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Detect the Antibacterial and Antitumor of synthesized Silver Nanoparticles Using *Microbacterium sp*

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Abstract: Metal nanoparticles are widely utilized in biotechnology and biomedicine for various applications, including medication delivery, imaging, and bacterial growth control. Silver nanoparticles (AgNPs) were synthesized by bacteria, fungi, algae, and plants. The Study aimed to synthesize nanomaterial with a cost-effective, environmentally friendly, and the uses of AgNPs as antibacterial (against pathogenic bacteria) and anticancer (on MCF7 cell line). In this Study, bacteria were collected from different soil samples. Isolated, purified by selective media, identification genotypically by 16rRNA sequencing analysis, then compared with NCBI, GenBank as *Microbacterium sp.* Biosynthesis of silver nanoparticles using *Microbacterium* for extracellular synthesis by reducing silver ions to silver nanoparticles. The color change to brown and reddish-brown was the first indication of the AgNPs formation; physical characterization using UV-Visible spectroscopy showed a wavelength in 489 nm, while X-ray diffraction (XRD) revealed that the silver nanoparticles were crystalline; transmission electron microscope (TEM) image showed that AgNPs spherical in shape with an average diameter of around 50 nm, in SEM (Scanning electron microscope) AgNPs formed with a diameter of 41-44 nm, spherical and uniform, while Energy-dispersive X-ray show very high silver peaks. Bioactivity of AgNPs by antimicrobial on pathogenic bacteria, which collected from Al- Sadr hospital in Misan (identified by using VITEK). This experiment showed that the inhibition zone was rung from (6- 38mm) on pathogenic bacteria; it was tremendous compared with antibiotics (Gentamycin in this project ranged from(7-28.5mm). Antitumor activity of extracellular biosynthesized AgNPs was determined using the MTT test against breast cancer cells (MCF7 cell line), which showed very high results. AgNPs inhibition breast cancer cell line by about 81% at 100ug/ml, indicating that the rate is outstanding. Finally, different biomedical approaches can benefit from AgNPs as antibacterial agents and anticancer agents with many results.

Key words: Silver Nanoparticles, biosynthesis, antibacterial, and antitumor.

Introduction

Recently, the immunological compatibility of humans has greatly enhanced the emergence of microbial diseases. As a result, many novel antibiotics and therapeutic pharmaceutical substances with a wide range of applications have been made available on the market to protect humans from various diseases[1], but they can potentially affect the environment, especially in developing nations[25]. Metals containing nanoparticles have the potential to be used in the control of several types of infection, but little is known about their antibacterial capabilities18. Due to a growing desire to generate environmentally friendly products, the synthesis of nanoparticles has become a hot topic at the junction of nanotechnology and biotechnology[2,26,27]. Using bacteria, fungus, algae, actinomycetes, plants, and other organisms, biogenic synthesis of metal nanoparticles has been demonstrated[3], Actinobacteria such as *Streptomyces sp.*[4], *Nocardia sp.*[5], and *Rhodococcus sp.*[6], have been reported to synthesize and characterize silver and gold nanoparticles. Metals such as zinc, silver, titanium, and copper have antibacterial properties that have been recognized for decades, allowing them to be used in various current medical applications to manage microbial infection disorders[33]. According to one idea, free metal ion toxicity arising from nanoparticle surfaces may play an essential role in infection prevention[34]. The catalytic, electrical, and optical characteristics of AgNPs are well-known[7,8]. A new generation of dressings comprising antimicrobial compounds like silver was created to prevent or minimize infection[9]. Hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules have recently been proven to have efficient antibacterial surface coating[10]. Because of their widespread use, silver nanoparticles are in high demand. Silver nanoparticles have attracted much attention among noble metal nanomaterials because of their appealing physicochemical feature[11]. Individual silver nanoparticles are great candidates for molecular labeling because of their surface Plasmon resonance and large effective scattering cross section[12,13]. Even Antifungal candidates could be AgNPs[19]. Silver ions have a well-known bactericidal action on microorganisms; however, the bactericidal process is only partially understood. It's been proposed that ionic silver interacts significantly with the thiol groups of essential enzymes, rendering them inactive[29].

Experiments show that when bacteria are exposed to silver ions, their DNA loses its replication capacity. Other research has found indications of structural alterations in cell membranes and the creation of tiny electron-dense granules from silver and sulfur[30,31]. Chemotherapy is one of the

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most common treatments for cancer, and a large number of antitumor compounds are found in nature as a whole or as derivatives, mostly formed and produced by microorganisms, particularly Actinomycetes, which produce a large number of natural products with various biological and bioactive properties, as well as antitumor properties, they work by inducing apoptosis through one of the suitable mechanisms. Topoisomerase, I or II inhibition, causes such DNA cleavage. Inhibition of essential enzymes affects signal transduction, such as proteases, mitochondrial permeabilization, cellular metabolism, and tumor-induced angiogenesis in some situations[32]. The Study's goal was to isolate and identify *Microbacterium* from the soil. Biosynthesis of silver nanoparticles, which were characterized using physicochemical methods such as UV-spectroscopy, XRD, SEM, TEM, EDX, and Study of the antibacterial activity of the AgNPs synthesized biologically against pathogenic bacteria; the last goal was evaluation the activity of AgNPs that synthesized in the lab as antitumor on MCF7 cell line (breast cancer cells).

Materials and methods

Soil Samples Collecting

Tn sterile polythene bags, soil samples were collected from sugar cane fields and gardens in Misan at 11cm below the surface. The samples were named with numbers. Closely tightened, and were taken to the laboratory[14].

Isolation of Actinomycetes

The soil samples were dried in the oven at 60oC for three hours to reduce the number of bacteria other than Actinomycetes in the soil. Actinomycetes form spores and then grow in the media. Serial dilution was done for each sample. The isolation media SCN Agar (Starch-casein-nitrite agar)[28] contained 1 ml of Cycloheximide (100ùg/ml) as antifungal agents; the samples were incubated for 5 days 30oC. The isolation bacteria were Isolated in pure culture on transfer medium YEG agar (Yeast Extract Glucose agar)[28], with one colony on each plate.

16S rRNA Gene Sequencing of Isolated Bacteria

Several methods for determining DNA sequence have been used using a universal primer (Macrogen/Korea), as shown in table1.

Genomic DNA was extracted from isolates using DNA Kit (Presto' Mini g DNA Bacteria Kit, Geneaid, Taiwan), PCR reaction mixture with a final volume of 20µl consisting of 2µl for each 27F and1492R primers (10 picomoles), 9µl De-ionized water, and 7µl of the DNA of the isolate, were added into the Maxime TM PCR Premix i-Taq (Intronbio/Korea), then amplified by polymerase chain reaction (PCR) technique under the following conditions: An initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min. Annealing at 58°C for 30 sec. and 72°C for 1 min, with a final extension at 72C° for 7 min. The PCR product that amplified was then Electrophoresis on Agarose Gels[34]. The isolation strain was identified genotypically by 16S rRNA sequencing analysis and then compared with (NCBI) GenBank[14].

Biosynthesis of AgNPs Laboratory

Colonies transfer into a conical flask containing 200 ml of MGYP (Malt extract glucose yeast extract peptone broth)[7] at PH 7.0 and put in a shaker incubator (rpm 150) at room temperature for 7 days. After that colony developed on the medium, filtrated through Whatman filter paper No.1 (Sigma/USA). The supernatant was added to 2mM of AgNO3(V/V) and incubated in an orbital shaker (rpm 150) for 7days at room temperature; after that, the color changed into dark color (reddish-brown dark) indicating the formation of AgNPs in the culture solution[7].

Characterization of Biosynthesis AgNPs

The AgNPs were Characterized physically by using:

UV-Visible spectroscopy

UV-Vis analyzed silver nanoparticles that were biosynthesized laboratory. Spectroscopy (Elettrofor/Italy) to determine the absorption spectrum, The sample of bio- AgNPs collected in a quartz cuvette (1cm path length) contains 2ml of the solution to fill past the instrument light path. At room temperature, the untreated supernatant was set as reference control while treated supernatants were used to monitor their UV-Visible absorbance Spectra between 300-800 nm wave length[36].

X-ray diffraction (XRD) analysis

X-ray diffraction (Broker/Germany) is one of the most widely used techniques for characterizing NPs. XRD usually provides information regarding crystal structure, phase nature, lattice dimensions, and crystal sizes[20].

Transmission Electron Microscopy (TEM) examination

The formation type (shape) and size of the generated silver nanoparticles were determined by Transmission Electron Microscopy examination (Broker/Germany), according to magnification TEM micrographs[37].

Scanning Electron Microscopy (SEM)

SEM (Buker/Germany) was used to examine the AgNPs in the sample. Thin films of the sample were made on carbon-coated copper grids by dropping an amount of the filtrate on the grid and blotting away the excess solution using blotting paper, then allowing the films to dry overnight at room temperature under sterilized conditions. The silver nanoparticles were imaged using a scanning electron microscope equipped with EDX attachment[38,39].

Primer	Sequence (5´- 3´)	Length	Amplicon size	Reference
27F	AGAGTTTGATCCTGGCTCAG	20bp	1500bp	36
1492R	GGTTACCTTGTTACGACTT	19bp		

Table 1. This is a table of the sequence of Universal primer kite[36] used in this experiment.

Antibacterial Activity

The antibacterial activity of synthesized AgNPs was tested using the disc diffusion method[15] against some human pathogens from both gram-negative and gram-positive bacteria collected from Al-Sadr hospital in Misan/Iraq, as shown in table 2.

NO.	Pathogenic bacteria
1	*Staphylococcus* aureus
2	*Staphylococcus haemolytic*
3	*Staphylococcus hominis*
4	*Escherichia coli*
5	*Pseudomonas aeruginosa*
6	*Klebsiella pneumonia*
7	*Salmonella typhi*
8	*Enterobacter cloacae*
9	*Staphylococcus lentus*
10	*Proteus mirabilis*

Table 2. This is a table of pathogenic bacteria that were collected from Al-Sadr hospital in Misan and used in our experiment.

Using sterile cotton swabs, each strain was swabbed uniformly into the individual Muller Hinton agar plates. 30ul of synthesized AgNPs were placed onto a plate using a sterile micropipette. It was then applied to a sterile paper disc (0.6 mm) and left to dry. After putting the AgNPs disc on the plates then incubation for 24 hours at 37°C, inhibitory zones appeared around the filter paper disc, showing the bioactivity of produced AgNPs[40]. The clear zone diameters were measured and compared to Gentamycin (30ul).

Antitumor activity

Mcf7 (breast cancer cells) were obtained from the IRAQ Biotech Cell Bank Unit in Basrah and cultured in RPMI 1640 (Gibco/USA) with 10% Fetal bovine serum (Sigma/USA), 100 units/mL penicillin, and 100 g/mL streptomycin. Cells were passaged twice a week with Trypsin-EDTA, reseeded at 50% confluence, and incubated at 37°C with 5% CO2[41]. The cytotoxicity test (measured by MTT assay) performed the MTT cell viability assay on 96 well plates to detect the cytotoxic effect. The mcf7 cell line was planted at 1 $\times 10^4$ cells per well. Cells were treated with the tested substance at a final concentration of 1000ug/ml after 24 hours or when a confluent monolayer was attained. After 72 hours of treatment, cell viability was determined by removing the medium, adding 28 liters of a 2mg/ml MTT solution (Gibco/USA) and incubating the cells for 2 hours at 37°C. Following removal of the MTT solution, the crystals in the wells were solubilized by adding 100ul of DMSO (Dimethyl Sulfoxide) and incubating at 37°C for15minutes with shaking[42]. The absorbency was measured using a microplate reader at 620 nm (test wavelength), and the assay was done three time[16,17].

Results

After incubation period the bacteria was grown on culture media, then purified on transfer media. The isolates were identified genotipically[14].

Identification of the Isolated Strains genotypically

After DNA extraction from isolated bacteria, the DNA must be amplified by polymerase chain reaction (PCR) technique, then the nucleotide sequences of the 16S rRNA gene were compared to the nucleotide sequences of reference strains retrieved from the GenBank database. One of the isolated was a new strain, so it was registered in my name on GenBank the other one was already registered on GenBank; the first isolation (the new one) was:

1. *Microbacterium paraoxydans* strain shahooda, 16S rRNA gene, partial sequence100% identical, Sequence ID: MZ701742.1, Length: 1388bp. The second isolation:
2. *Microbacterum lacticum*, strainSTM54,16S rRNA sequence gave 949 base pair, an NCBI, BLAST search revealed that the sequence was 100% identical to the sequence of *Microbacterium lacticum*. strain STM54 16S rRNA gene, partial sequence, Sequence ID: KY393059.1, Length: 949bp.

Producing Nanoparticles

This Study was focused on the extracellular synthesis by supernatant to form AgNPs using *Microbacterium sp*, after incubation period the color change from white to reddish brown was the first indicating of silver nanoparticles formation[7], as shown in figure 1.

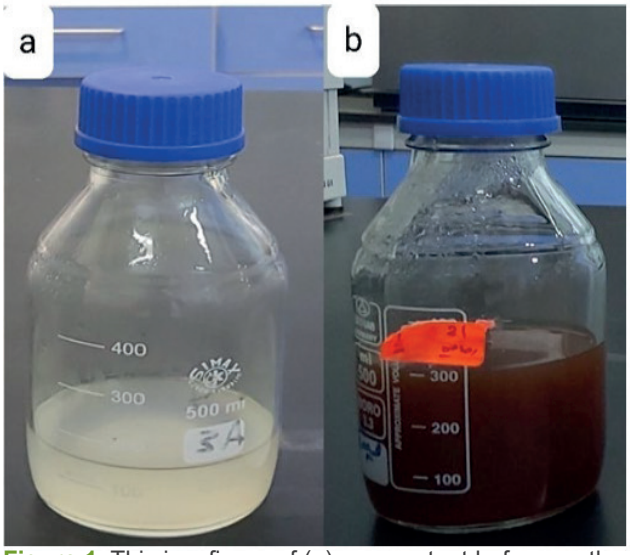


Figure 1. This is a figure of (a) supernatant before synthesis. (b) supernatant after the formation of AgNPs.

Characterization of Ag Nanoparticles

UV-Vis spectroscopy

Metallic bio-nano particles have a distinct optical absorption spectrum in the UV visible region (300-800)[36], and the optical absorption spectrum in this Study was at 489nm wavelength, as shown in figure 2.

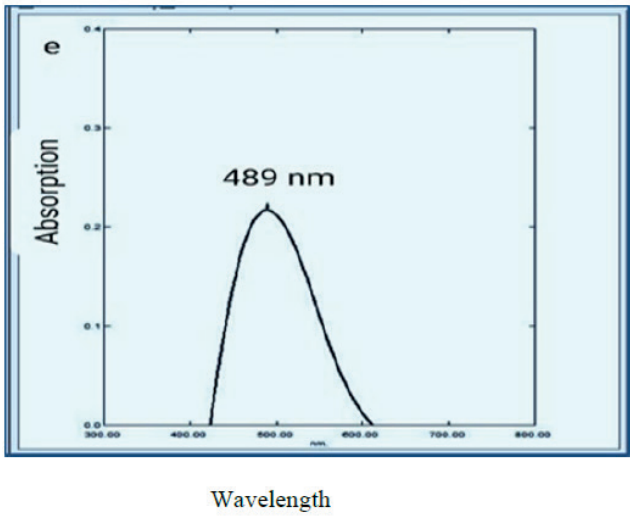


Figure 2. This figure of UV-Vis spectroscopy of AgNPs showed a peak at 489nm wavelength.

As the process progressed, the spectra indicated a rise in silver solution intensity with time, indicating the creation of more silver nanoparticles in the solution. Shows that after 72 hours, there is no discernible difference in the UV-Vis spectra of the reaction product, indicating that the process has reached equilibrium.

XRD analysis

Further studies were carried out on silver nanoparticles using X-ray Diffraction. Depicts the evaluation of the XRD phase and crystal structure analysis of green produced AgNPs. In the XRD investigation, 2□degrees = 32.5, 28.3, and 48.1 values were used to determine the reflections (122), (111) and (200), respectively, diffraction Standards (JCPDS) silver file No. (04–0783)[23], as shown in figure 3.

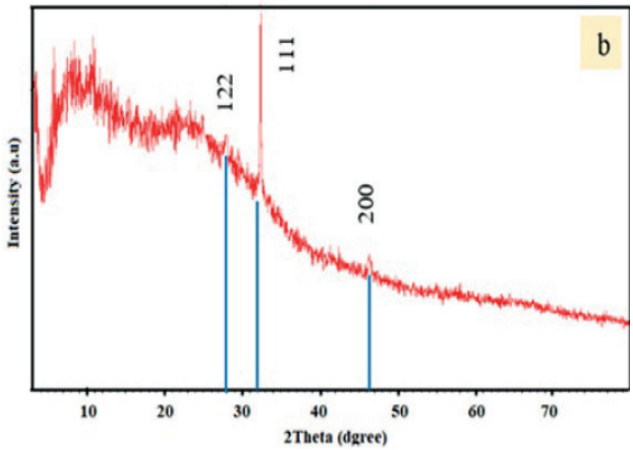


Figure 3. This figure of X-ray diffraction of synthesized AgNPs analysis, diffraction standards (JCPDS), silver file No. (04–0783).

AgNPs have an elemental (Ag^0) and spherical and generated crystalline, indicating that they are face-centered and cubic. Similar results have been presented in another research[46-48].

TEM analysis

TEM was used to determine the size and shape of particles. Silver nanoparticles which examine with TEM were of an average diameter of around 50 nm as shown in figure 4.

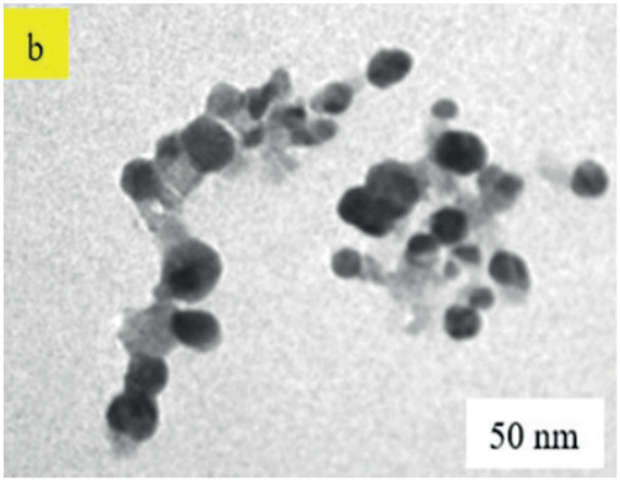


Figure 4. This figure of Transmission Electron Microscopy image showed the size of silver nanoparticle with an average diameter of around 50 nm.

SEM analysis

As showed in the previous studies on biosynthesized silver nanoparticles on SEM image found to be generally spherical and uniform[49,50], and the size was between 41-44 nm, as shown in figure 5.

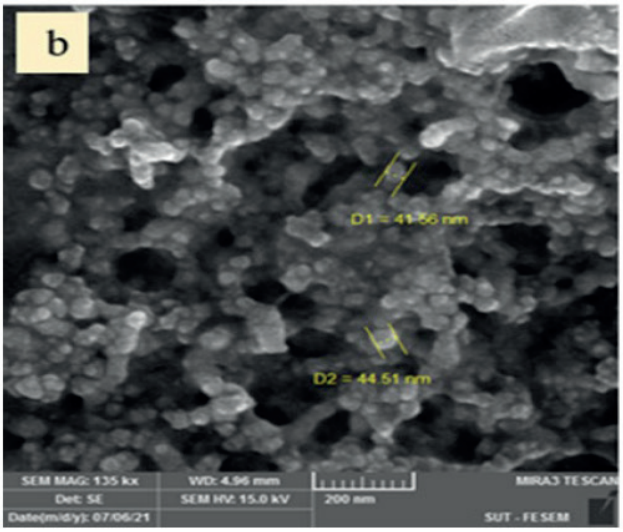


Figure 5. This is a figure of a Scanning Electron Microscopy image of AgNPs fabricated by *Microbacterium sp.* with a 41-44 nm diameter.

Biological Characterization of Synthesized Silver Nanoparticles

Antibacterial Activity

The data from energy dispersive spectroscopy X-ray (EDX) show very high silver peaks, indicating that the reduction of silver ions to elemental silver may have come

from molecules connected to the AgNPs' surface. The silver's dense peak was a clear indicator of the formation of silver nanoparticles from silver ions[20]. As shown in figure 6.

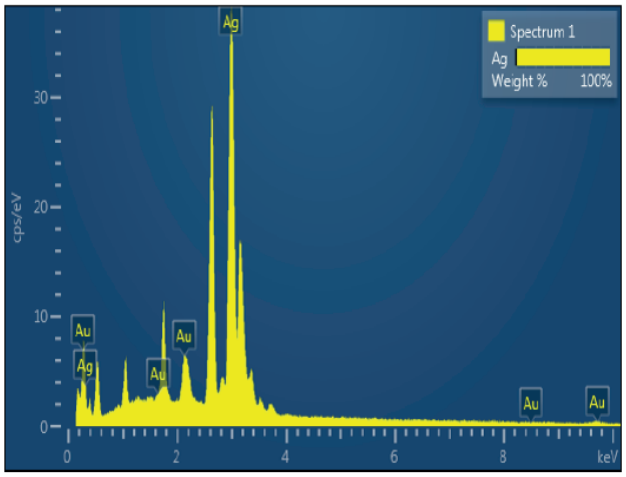


Figure 6. This figure of EDX analysis showed a strong peak of silver at 3kev.

Antibacterial testing revealed that the Nanoparticle is effective against bacterial pathogens[21,22]. Both Gram-negative and Gram-positive bacteria alike (identified by using-VITEK-2)[23] and compared with Gentamycin antibiotic, as shown in figure 7 and figure 8.

Microbacterium's silver nanoparticles displayed the most significant inhibition zones (38mm) against *S.aureas* bacteria; it was enormous compared with antibiotics (Gentamycin) in this project; the lowest inhibition zone was 7 mm on pseudomonas aeruginosa bacteria, as shown in table 3.

Antitumor activity of AgNPs biosynthesized laboratory

Silver nanoparticles play important role as antitumor[24]. At low concentrations demonstrated very great activity on MCF-7 cell line, AgNPs inhibition breast cancer cells by about 81.732 % at 100ug/ml, and only18.268 % of MCF7 were able to form formazan product and remained as alive cells (viability, after 72hr of incubation period. There was significant change in cancer cell that inhibition with synthesized silver nanoparticles at a different concentration (10,30,60,80,100ug/ml) of AgNPs, by about (9.263%,65.252%,81.565%, 80.447%,81.732%), as shown in) figure 9.

The median inhibitory dose (IC50) value of 24.93ug/ml. This means that biosynthesis AgNPs have excellent antitumor activity on breast cancer cells. These results agreed with those described by (24).

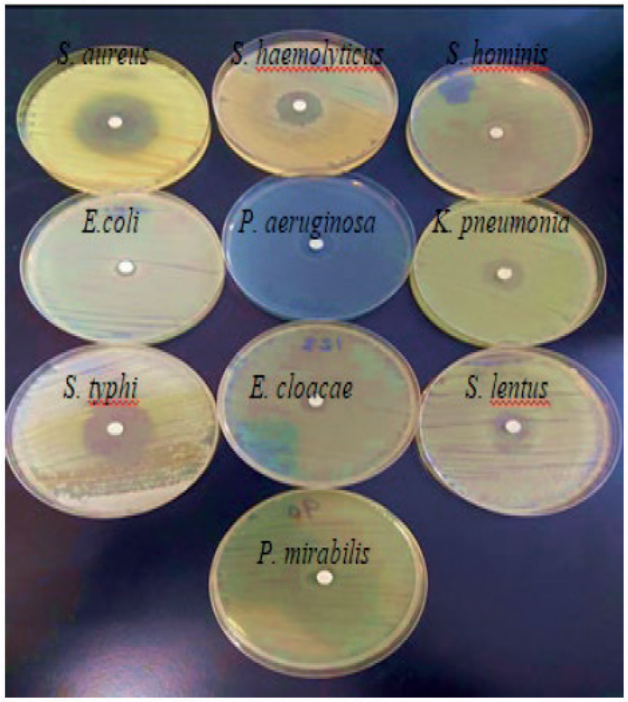


Figure 7. This figure of the Antibacterial activity of synthesized AgNPs against ten pathogenic bacteria.

Discussion

Compared to other bio-reductants, the manufacture of metallic nanoparticles using microorganisms is more fruitful in terms of ease[51]. Phytochemicals are well known for converting Ag^{1+} to Ag^{0} and capping these nanoparticles, making them highly stable[52]. In this Study, *Microbacterium* was used after being isolated from soil to green synthesis of AgNPs, and This indicates that this isolated bacteria had the enzyme that reduced Ag^{+} to Ag^{0} by Nitrate reductase, then the aggregation of silver atoms was formed AgNPs. Then was tested of the synthesized AgNPs were for bactericidal and cytotoxic activity. Spectroscopy in the ultraviolet and visible ranges was used to determine metallic nanoparticles' formation and exploit their optical properties. Plasmon bands were recorded at various points during the bioreduction process. The AgNPs were prepared principally by the emergence of a reddish-brown color after 72h. The reaction was finished and validated by the Plasmon absorption peak, which reached a constant value. The SPR (surface Plasmon resonance) band of the spherical AgNPs at 489 nm is visible

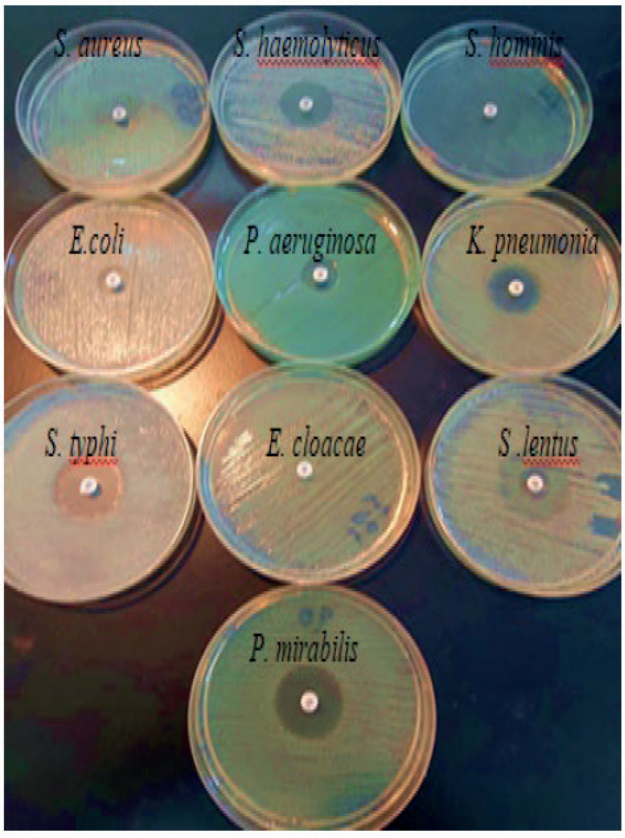
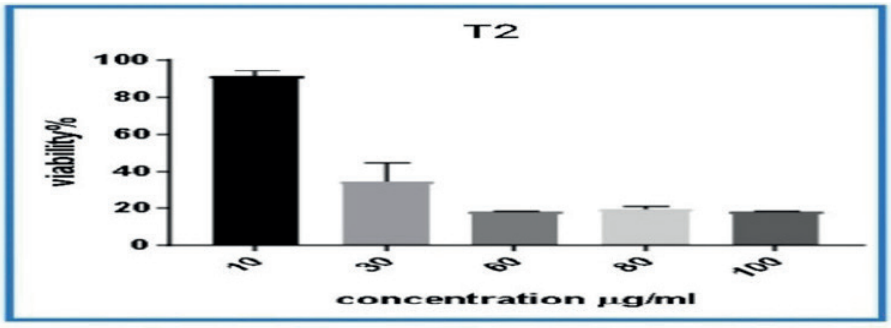


Figure 8. This figure of the Antibacterial activity of antibiotic (Gentamycin) against the same pathogenic bacteria.

Pathogenic bacteria	Inhibition zone of Gentamycin (antibacterial agent) (mm)	Inhibition zone (mm) of synthesized AgNPs
Staphylococcus aureus	9 mm	38 mm
Staphylococcus haemolytic	24.5 mm	22.5 mm
Staphylococcus hominis	7 mm	13 mm
Escherichia coli	14 mm	9.5 mm
Pseudomonas aeruginosa	13.5 mm	7 mm
Klebsiella pneumoniae	16.5 mm	18 mm
Salmonella typhi	24.5 mm	33.5 mm
Enterobacter cloacae	8 mm	12 mm
Staphylococcus lentus	28.5 mm	21 mm
Proteus merabilis	24.5 mm	10 mm

Table 3. This table of Inhibition zone on ten pathogenic bacteria by synthesized AgNPs and Gentamicin.



Compound µg/ml	10	30	60	80	100
Viability%	90.837	34.748	18.435	19.553	18.268

Figure 9. This figure of viability percent on the mcf7 cell line in different concentrations of synthesized AgNPs (10, 30, 60, 80, 100) ug/ml.

in the spectra, showing that the synthesis of AgNPs in the reaction mixture is consistent with the AgNPs synthesized before[53]. After one month under ambient circumstances, the stability of the nanoparticles was assessed throughout time (30 days), and no change in absorption peak value was detected, indicating that the nanoparticles are highly stable.

The particle size and shape of the produced AgNPs were studied using TEM and SEM. The synthesized AgNPs were monodispersed, spherical in shape, and ranged in size from 41 to 44 nanometers. The interaction of hydrogen bonds and electrostatic interactions between the bioorganic capping molecules linked to the AgNPs resulted in silver nanoparticles. Even within the aggregates, the nanoparticles were not in direct contact, indicating that the nanoparticles had been stabilized by a capping agent and crystalline in nature[45], and well dispersed, primarily spherical, which is agreed with other reports[44,46].

Correlated to the (111), (200), and (220), and indicating that AgNPs have been prepared. Several Bragg reflections with 20 values of 32.5°, 28.3°, and 48.1° are achieved

and related with the diverse set of (111), (200), and (220), which can be recorded as the band for face-centered cubic (JCPDS file no. 89-3722). As a result, XRD indicates that the samples are pure AgNPs that are highly crystalline. The silver particle size histograms revealed that the particles varied in size. Planes appear in the selected area Electron Diffraction Pattern (EDX), indicating that the produced AgNPs are crystalline. The dots are aligned with a face-centered cubic structure. The AgNPs are crystalline, according to the EDX pattern. The elemental detection of AgNPs was also done with the EDX. The presence of a prominent peak of silver at 3 KeV can be seen in the EDX pattern; the produced AgNPs have outstanding antibacterial effectiveness. When clinical bacterial pathogens are exposed to AgNPs, the membrane permeability is altered, resulting in cellular leakage, restricting cell growth and replication. Some bacterial macromolecules can be affected by AgNPs, resulting in disintegration and cell death[54]. Compared to chemically synthesized AgNPs, green produced AgNPs are more biocompatible and have a stronger antibacterial impact[55]. The AgNPs first bind the cell membrane at numerous points before quickly penetrating it, causing structural changes and, as a result, perforations that allow compounds from intracellular storage to flow out[56]. When AgNPs reach the interior, silver ions are released, resulting in the production of reactive oxygen species (ROS), which can affect membrane proteins, causing the electron transport chain to be disrupted[57].

The MTT assay findings show that AgNPs have high cytotoxicity against MCF7. The MTT assay was carried out in triplicate. AgNPs have higher toxicity at lower concentrations as they were incubated for 24 hours. At doses of 10,30,60,80, and 100ug/ml of AgNPs at a different concentration, respectively, toxicity if there were (9.163%,65.252% ,81.565%,80.447%,81.732%) found after 72 hours of incubation respectively this indicates that as the concentration of AgNPs rises so does its toxicity. The results showed that AgNPs strongly suppressed MCF-7 cell proliferation. For AgNPs-treated MCF7, IC50 values were calculated over a wide concentration range and incubation duration. Several studies have previously concentrated on using cell culture methods to perform AgNPs cytotoxicity tests[58,59]. The cytotoxicity of any natural or synthetic substance on an established cell line must be determined before moving to in vivo experiments[60]. When tested against breast cancer cells, our biogenic AgNPs have high cytotoxicity.

Conclusions

The soil was shown to be a rich supply of various bacteria in the current study, and even a novel strain was discovered (*M. paraoxydans* strain shahooda). As a result of this research, it can be concluded that it has been employed effectively for AgNPs extracellular synthesis using soil microbes. The presence of elemental silver and its crystalline structure and size were confirmed by using UV-Vis. Spectroscopy, EDX, SEM, TEM, XRD. Both Gram-negative and Gram-positive pathogenic bacteria are susceptible to AgNPs. The anticancer effects of extracellular AgNPs on MCF7 (breast cancer cells) yielded the best results, with AgNPs significantly suppressing MCF7 cell multiplication. Finally, compared to commercially available antimicrobial drugs, the current Study provides an environmentally friendly and cost-effective approach for synthesizing powerful antibacterial silver nanoparticles (biologically) against pathogenic bacteria and the capacity of silver nanoparticles as antitumors on breast cancer cells line.

Authors contributions

Conceptualization, TMD and RRH; methodology, TMD; software, TMD; validation, TMD and RRH; formal analysis, TMD; investigation, TMD; resources, TMD; data curation, TMD; writing—original draft preparation, TMD; writing review and editing, TMD; visualization, TMD; supervision, RRH; project administration, RRH. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board

Al- Sadr hospital in Misan had been informed about the aims of the Study before collecting samples and declared their agreement to give samples (175; 1/6/2021). The Study follows the rules of scientific research at Misan University, Iraq.

Informed Consent

Informed consent was obtained from all subjects involved in the Study. The patient's consent was oral.

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Conflicts of interest

The authors declare no conflict of interest.

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