ASPERGILLUS NIGER AS A BIO-LAB FOR EXTRACELLULAR SYNTHESIS OF SILVER NANOPARTICLES AND ITS ANTIBACTERIAL ACTIVITY

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Antibiotic resistance is one of the world’s maximum urgent public healthcare problems. Silver nanoparticles (AgNPs) are appealing alternative due to the fact they may be non-poisonous to the human frame at low concentrations and feature antibacterial actions. The present study aimed to biosynthesis of silver nanoparticles (AgNPs) extracellularly using Aspergillus niger extracts and to evaluate their antibacterial activity. The biosynthesized AgNPs were characterized by UV-Vis, FT-IR, SEM, and EDX. Furthermore, antibacterial activity was evaluated against bacterial strains of Staphylococcus aureus (ATCC 6538, Gram-positive) and Escherichia coli (ATCC 8739, Gram-negative) through the well diffusion method. The results confirmed that the synthesized AgNPs were spherical and their dimensions were less than 100 nm. AgNPs revealed a good antibacterial activity (for 20 μg/ml) against E. coli and exhibited an excellent synergistic effect against S. aureus when combined with vancomycin. The current research had concluded that AgNPs have the potential to be an alternative or partner to antibiotics to control microbial infections caused by multidrug-resistant (MDR) pathogens.

Keywords: Biosynthesis, Silver nanoparticle, Aspergillus niger, Antibacterial activity.

INTRODUCTION

Nanotechnology is a branch of science that achieved formidable progress during recent decades due to the synthesis and varied applications of metal nanoparticles (MtNPs) in different areas, such as biology, medicine, agriculture, engineering, electronics, and biomedical devices. The field offers a hopeful way to improve the properties of metals by transforming them into nanoparticles within a size range of 1-100 nm. The nanoparticles (NPs) have reached a position of great importance due to their certain physicochemical characteristics and significant biotechnological applications.¹²

The nanoparticles synthesized can have an organic structure such as carbon or inorganic (metallic) structures such as silver, gold, and copper. Various methods have been done to synthesize silver nanoparticles (AgNPs) consist of physical, chemical, and biological methods which are also known as green synthesis. In general, silver nanoparticles syntheses are classified as "bottom-up" and "top-down" methods. The bottom-up methods consist of sono-decomposition, chemical reduction, and electrochemical methods while the top-down method is the physical method consisting of mechanically grinding the silver bulks.⁴

Green synthesis is non or minimally toxic, environmentally friendly, and cost-effective which based on the utilization of plants extracts or microbial cells or their metabolites for NPs synthesis. This method has become popular over the years as an alternative to the classical chemical approach.⁶ Fungi are among the most important organisms utilized in the biosynthesis of nanoparticles (NPs) because the NPs synthesized present good dimensions, stability, and polydispersity. Furthermore, Fungi are distinguished and superior to other
living organisms as biofactors of nanoparticles due to their diversity, ease of isolation, and their ability to secrete large amounts of extracellular enzymes, which facilitates the biosynthesis of NPs.

Nanobiotechnology has emerged as a promising technology to develop new therapeutically active nanomaterials. The synthesis of nanosized drugs with qualitative requirements in terms of size, shape, and physical and chemical features is of massive interest in the formulation of new pharmaceutical preparations.

So, the aim of this study was the biosynthesis of AgNPs using *Aspergillus niger* and evaluated its antibacterial activity.

## MATERIALS AND METHODS

### Aspergillus niger isolate culture

Aspergillus niger isolate was obtained from the Microbiology lab at Pharmacy College, Al-Baath University, Syria. The isolate was refreshed on sterile medium of Sabouraud Dextrose Agar (SDA). Potatoes Glucose Broth (PGB) medium was utilized to grow the fungi. 750 ml of the PGB medium was prepared and evenly distributed into 4 flasks of 250 mL and autoclaved at 121°C for 20 min. 6 mm discs of Aspergillus niger were taken using a sterile cork auger. 2 discs were added to each flask containing PGB medium and left in the microbiological incubator at 25 °C for 10 days.

### Biomass Preparation

The grown fungi on the PGB medium were filtered by sterile gauze in a sterile atmosphere within the isolation room. The raw filtrate was saved for other utilizes while the collected fungi were taken over the gauze and washed thrice with sterile deionized water. 10 g of wet fungi mass were added to a flask containing 100 ml sterile distilled water and placed on an electric vibrator at 120 rpm for 4 days, at 25 °C in the dark then the solution was filtered using several layers of sterile gauze and Whatman filter papers No1. The filtrate was centrifuged at 5000 rpm for 10 mins. The supernatant in the centrifugation tubes was transferred to a 100 mL flask.

### Synthesis of silver nanoparticles

17 mg of silver nitrate (AgNO₃) was added to the flask containing the supernatant to obtain a concentration of 0.001 M then the flask was placed in the dark for 4 days at 25 °C.

### Characterization of silver nanoparticles

The biogenic AgNPs were characterized using standard techniques; namely, Ultraviolet-Visible (UV-Vis) spectrum, Fourier Transform Infrared (FT-IR) spectrum, Scanning Electron Microscopy (SEM), and Energy Dispersive X-Ray (EDX).

### UV–Vis spectroscopy

To obtain the UV–Vis absorption spectrum, a stock solution of the AgNPs was diluted with distilled water (1:1 ratio) and the spectrum was recorded in the range of 350–700 nm to ensure the presence of specific Surface Plasmon Resonance (SPR) peak of AgNPs. The crude fungal filtrate with freshly added silver nitrate was utilized as a blank.

### FT-IR spectroscopy

The solution of AgNPs with acetone in (1:5) was subjected to centrifugation at 4000 rpm for 15 min after continuous shaking (4 times). Later, supernatant was discarded then 1-2 ml acetone was added into the pellet. After shaking thoroughly, it was poured into the watch glass. Acetone was allowed to evaporate to obtain the powder of NPs. AgNPs were characterized by FTIR in the range of 4000 – 400 cm⁻¹. The dried sample was mixed with spectral grade KBr (ratio 1:1) and pressed into discs under hydraulic.

### SEM

SEM analysis are closely related techniques that utilize an electron beam to image a sample to determine the size and surface morphology of the silver nanoparticles synthesized using *Aspergillus niger* extracts.

### EDX

The EDX analysis conducted with scanning electron microscope to confirm the presence of silver in the solution.

### Antibacterial assay

The well diffusion method was utilized to evaluate the efficacy of AgNPs against
Staphylococcus aureus (ATCC 6538) and Escherichia coli (ATCC 8739)\textsuperscript{21,22}. A bacterial suspension was prepared from both S. aureus and E. coli with turbidity equivalent to 0.5 McFarland standard [approximately 1.5 × 10\(^8\) colony-forming unit (CFU)/mL]\textsuperscript{23}. Mueller Hinton Agar (MHA) medium was prepared and autoclaved at 121\(^\circ\)C for 20 mins. Six Petri plates containing MHA medium were cultured with 100 μL of S. aureus bacterial suspension while the other six plates were cultured with 100 μL of E. coli bacterial suspension. To prepare a solution of AgNPs at a concentration of 20 μg/ml, the solution of the fungal extract added to silver nitrate was centrifuged; the precipitate was weighed and dissolved in dimethyl sulfoxide (DMSO), which is an inactive solvent against bacteria. Each Petri plate containing MHA medium cultured with S. aureus. Four wells were made in each plate, one for silver nanoparticles solution (for 20 μg/ml), the second for DMSO, the third for (silver nanoparticles solution with a concentration of 20 μg/ml + vancomycin), and the fourth well for the fungal extract solution was placed as a control, vancomycin was placed at the tip of the plate. The same procedure was repeated in the plates cultured with E. coli, but vancomycin was replaced with ciprofloxacin. Later, all plates were incubated in the microbiological incubator at 37 \(^\circ\)C for 24 hrs. The antibacterial activity of AgNPs were evaluated by measuring inhibition zone.

RESULTS AND DISCUSSION

Biosynthesis and Characterization of silver nanoparticles

UV-Vis Spectrum

Silver ions were bioreduced to silver nanoparticles when added Aspergillus niger extracts. The formation of AgNPs was confirmed by UV-Vis spectrophotometer studies with a range of 350-700 nm. As shown in Fig. 1, the specific SPR peak of AgNPs after 24 hrs was found to be centered at 400 nm in the spectrum indicating the presence of AgNPs.

Compared to similar studies, our results according to\textsuperscript{17} while differing from other studies. For instance, Sagar and Ashok (2012) showed the peak of absorption appeared at 440 nm\textsuperscript{24}, the difference of absorption peak due to many differences between the synthesized AgNPs such as size, shape and particle interaction with the medium like agglomeration. In another study, Lotfy et al (2021) had used Aspergillus terreus extracts for the synthesis of AgNPs, the peak of absorption appeared at 420 nm\textsuperscript{19}, the difference due to using another specie of Aspergillus.

FT-IR Spectrum

The FT-IR analysis of silver nanoparticles (Fig. 2-A) showed intensive peaks at 3426.89, 2927.45, 2857.92, 1536.3, 1630.52, 1387.53, 1389.46, 1322.93, 1120.44, and 1041.37 cm\(^{-1}\). Those peaks were, respectively, corresponding to Normal polymeric O-H stretch, Methylene-CH, unknown, Organic nitrate, Aromatic nitro compounds, Aromatic nitro compounds, Aliphatic fluoro compounds, Aliphatic fluoro compounds while the other peaks that appeared were unknown, it is according to\textsuperscript{25}.  

Fig. 1: UV-Vis spectra showing a strong broad peak at 400 nm of AgNPs synthesized by A. niger extracts.
SEM

The SEM micrographs of the AgNPs obtained in the filtrate showed that AgNPs were spherical shaped and well distributed in the solution without aggregation. As shown in Fig. 3, the average measured particle size was 57.4 nm.

Compared to similar studies, our results relatively correspond to many studies while differing from other studies. For instance, Li et al. (2021) who using Aspergillus japonicas for the biosynthesis of AgNPs, the diameters of AgNPs were 3.8 ± 1.1 and 9.1 ± 2.9 nm\(^2\), the difference due to the use of another species of the Aspergillus genus. In another study, Heflish et al. (2021) who using Acalypha wilkesiana for the biosynthesis of AgNPs, the diameters of AgNPs were in the range of 10–30 nm\(^2\), the difference due to the biosynthesis being done using plants instead of microorganisms.

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**Fig. 2:** (A) FT-IR spectra of *Aspergillus niger* extracts with silver nitrate. 
(B) FT-IR spectra of *Aspergillus niger* extracts (control).

**Fig. 3:** (A & B) SEM images of AgNPs. 
(C) SEM images of *Aspergillus niger* extracts (control).
EDX

The EDX analysis showed a good silver signal together with remarkably stronger peaks confirming that the pellets were AgNPs. The other elements are thought to be originated from the fungal extracts which are depicted in Fig. 4 along with typical silver peaks, thus confirming the formation of AgNPs.

Antibacterial assay

After 24 hrs of incubating the dishes in the microbiological incubator, the antibacterial activity of the 20 μg/ml silver nanoparticle solution was evaluated and compared with an antibiotic known for its tested antibacterial activity (Fig. 5). The antibacterial activity of silver nanoparticle solution was compared with vancomycin against strains of *S. aureus* while the activity of silver nanoparticles was compared with ciprofloxacin against strains of *E. coli*. Silver nanoparticles showed weak activity against *S. aureus* strains, the diameter of growth inhibition was 12 mm, while vancomycin did not show activity against *S. aureus* but the combination of AgNPs solution and vancomycin showed a synergistic effect where the average diameter of inhibition reached 26 mm which confirms that the combination of an antibiotic such as vancomycin with a solution of silver nanoparticles promises a very good efficacy in the eradication of resistant strains of *S. aureus*, it is according to\(^{28}\). In another study, Gouyau *et al* (2021) found that AgNPs have no activity on *S. aureus*\(^{29}\) while other researchers found that *S. aureus* has good sensitivity for AgNPs, this may be due to the use of another strain of *S. aureus*. For example, Gade *et al* (2008) used *S. aureus* (ATCC 25923)\(^{30}\). In contrast, silver nanoparticles showed good activity against *E. coli* strains, it is according to many studies. The average diameter of growth inhibition was 16 mm, while ciprofloxacin showed slightly higher activity against *E. coli*, the average inhibition diameter was 19 mm, but better activity appeared against *E. coli* when the solution of AgNPs and ciprofloxacin was shared, where the average diameter of the inhibition was 20 mm. Thus, it can be confirmed that the strains of *E. coli* are well sensitive to the combination of ciprofloxacin and silver nanoparticle solution. On the other hand, neither DMSO nor the fungal extracts showed any antibacterial effect (Table 1).
Fig. 5: Average diameters of inhibition zones (mm) of a solution of AgNPs against the tested bacterial strains. (A) *Staphylococcus aureus*. (B) *Escherichia coli*.

1. AgNPs. 2. DMSO. 3. Combination between AgNPs and Antibiotic. 4. Fungal extract. 5. Antibiotic is Vancomycin in dish (A), Ciprofloxacin in dish (B).

| Tested bacteria | AgNPs | Antibiotic | Combination of (AgNPs & Antibiotic) | Fungal extracts | DMSO |
|-----------------|-------|------------|------------------------------------|----------------|------|
| *S. aureus*     | 12    | 0          | 26                                 | 0              | 0    |
| *E. coli*       | 16    | 19         | 20                                 | 0              | 0    |

* Values were expressed as the means of six replicates.

As a result, the AgNPs have an excellent antibacterial activity against *S. aureus* and *E. coli*. It is due to AgNPs can permanently release Ag\(^0\), which may be considered a means of killing bacteria where the adherence of Ag\(^+\) to the cell wall and cytoplasmic membrane occurs due to the electrostatic affinity of Ag\(^0\) towards sulphur proteins. This causes the disruption of the bacterial envelope by enhancing the permeability of the cytoplasmic membrane. The uptake of silver ions into the cells results in the deactivation of respiratory enzymes, the formation of Reactive Oxygen Species (ROS), and interruption of Adenosine Triphosphate (ATP) production. ROS can play a major role in the processes of cell membrane disruption and Deoxyribonucleic Acid (DNA) modification. The interaction of Ag\(^0\) with the phosphorus and sulphur components of DNA results in DNA modification. Furthermore, Ag\(^0\) can inhibit the formulation of proteins by denaturing ribosomes in the cytoplasm\(^31\).

**Conclusion**

The current study revealed that silver nanoparticles can be prepared using biological methods based on fungi. *Aspergillus niger* is considered one of the most important and best fungi that can be adopted in biomanufacturing. Analytical techniques such as UV-Vis and IR spectroscopy are also considered among the most important techniques that are used to characterize silver nanoparticles. SEM analysis showed that the sizes of the formed AgNPs ranged from 39 to 85 nm. Silver nanoparticles showed good antibacterial activity, especially when combined with an antibiotic.

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فطور الرشاشيات السوداء كمُعامل حيوي للاصطناع خارج خلوي لجسيمات الفضية النانوية وتأثيرها المضاد للجراحيات
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تعد مقاومة المضادات الحيوية إحدى مشكلات الرعاية الصحية العامة الأكثر اكتئابًا في العالم. تعتبر جزيئات الفضية النانوية (AgNPs) بديلًا جذابًا عن المضادات الحيوية نظرًا لحقيقة أنها قد تكون غير سامة للبشر بتراكيز منخفضة وتمييز تأثير مضاذا للجراحيات. هدفت الدراسة الحالية إلى التصنيع الحيوي لجسيمات الفضية النانوية (AgNPs) باستخدام مختلطات خلويات فطر الرشاشيات المصنعة حيوية AgNPs السوداء وتقديم نشاطها المضاد للجراحيات. تم تصنيف جسيمات الفضية النانوية المصنوعة حيوية AgNPs وتحديد نشاطها المضاد للجراحيات. تم تقييم فعاليتها المضادة للجراحيات أولاً، EDX و SEM و FT-IR و UV-Vis بواسطة وسائل جرثومية من العنقوديات المذهبة (ATCC 6538) والإشريكية القولونوية ATCC 8739، ونجمة السرطان ATCC 8739. أظهرت النتائج أن جسيمات الفضية AgNPs النانوية المصنوعة حيوية وقعت كروية وأبعادها كانت أقل من 100 نانومتر. أظهرت جيدة مضادة للجراحيات بتركيز 20 ميكروغرام / مل ضد الإشريكية القولونوية وأظهرت تأثيرًا تأريثًا ممتازًا ضد العنقوديات المذهبة عند المشاركة مع فانكومياسين. خلص البحث الحالي إلى أن جسيمات الفضية AgNPs الجريثومية التي تسبّبت في العوامل المسببة للأمراض المقاومة للأدوية المتعددة (MDR) يمكنها أن تكون ميدلًا أو مشاركًا للمضادات الحيوية للسيطرة على الإنتانات.