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Saprolegnia parasitica–mediated biosynthesis of silver nanoparticles and its antimicrobial activity

Majid Kadhim Al-Shibly¹, Syoof Khoman Alwan² and Rajaa Ali Habeeb³

¹ College of Education/Al-Qadisiya university/Diwaniya/Iraq
² college of Science/Al-Qadisiya university/Diwaniya/Iraq
³ college of Education/ Al-Qadisiya university/Diwaniya/Iraq

Email: syoofalwan@yahoo.com

Abstract. In this work, the effect of silver nanoparticles synthesized by Saprolegnia parasitica was investigated against Staphylococcus aureus, Proteus mirabilis, E.coli, Trichophyton rubrum and Candida albicans. The formation of the silver nanoparticles (AgNPs) was initially confirmed by the UV-Visible Spectroscopy. As well as using SEM, UV-Visible analysis showed a strong peak at 428 nm, the SEM images of the biosynthesized SNPs showed spherical shape with a particle size in the 37 nm range. The results of the study showed that the concentration of 150 microliters of SNPs solution was the most concentration for inhibition when it used as an antimicrobial against (E. coli, S.aureus, P.mirabilis, T.rubrum).

Keywords: Saprolegnia parasitica, Silver nanoparticles, Antimicrobial activity

Introduction

Nanotechnology has attracted the attention of scientists, especially nanoparticles because of their distinctive properties (optical, physical, biological). Scientists have begun producing nanoparticles using various sources such as plants, fungi, bacteria, metals; gold, silver, etc. (1). Fungi is one of the most important biological sources for producing nanoparticles because of its advantages such as its secretion of extracellular enzymes which works to reduce the metal ions and thus produce nanoparticles (2), their rapid growth, ease of culture and preservation in vitro (3). Therefore, their use in nanotechnology is somewhat inexpensive and nano production can be controlled by manipulating several conditions including temperature, pH, the concentration of metal ions, and reaction time (4, 5). Making them ideal for use in biosynthesis to produce nanoparticles. In addition, extracellular excretion of amounts of protein and secondary metabolites by fungi leads to the reduction of metal ions and the rapid production of nanoparticles. For these reasons, extracellular myco- synthesis is a high-yielding source of nanoparticles (6). Humans are surrounded by many pathogenic organisms (bacteria, fungi, viruses) that are resistant to many clinically agreed antibiotics, Which led to the emergence a medical problem that which requires the development of effective and inexpensive antimicrobials. (7). Recently, silver and gold nanoparticles (derived from biological sources) have been highlighted mainly as an effective solution to the problem of
microbial resistance, which has demonstrated its inhibitory capacity for many human pathogens that which resistant to many antibiotics. (8). Based on the scientific facts, the *Saprolegnia parasitica* was used in the current study of the synthesis of silver nanoparticles and its use as an antimicrobial agent.

**Materials and methods**

1- **Materials Collection:**

*Saprolegnia parasitica* was isolated from the water samples were collected from random sites from the river of AL-Diwaniya weekly, for this purpose, 500ml glass bottles use, they open under water and the depth 20-30 cm, after filling, they sealed tightly under the water and then transfer to the laboratory for tests. The samples need to placed inside a cooler box on days when the temperature was more than 25 C° and maintained on Sabroud dextrose agar (SDA) medium at 28C° The isolated fungus was identified according to the classification keys of fungi (9,10), Three bacterial isolates were obtained (two of which were Gram-negative bacteria) *P. mirabilis, E.coli,*. (one Gram-positive bacteria) *S. aureus*. and two isolates of fungi, one of which *Candida albicans*, the different clinical specimens taken from AL-Diwaniyah Teaching Hospital and the other *T.rubrum* (reference strain) obtained from mycology laboratory in the department of biology /college of education /University of AL-Qadisiyah for their susceptibility against silver nanoparticles.

2- **The extracellular production of silver nanoparticles**

For biosynthesis of silver nanoparticles from the fungus used the method was described by previous study (11) with modification

100 ml of sabroud dextrose broth was prepared inoculated with the *Saprolegnia parasitica* broth media in 250 ml Erlenmeyer flask and the chloramphenicol(250 mg/L) was add to the broth as an antibacterial agent and the pH was adjusted to 6.5 ,then flasks that containing media were incubated at 25 °C, 100 rpm for 7 days in shaking incubator After that, the culture solution was centrifuged at 13500 rpm for 2 min. Then the supernatant was taken into a clean 250 ml conical flask and 1 mM silver nitrate was added to 100 ml culture of the supernatant. Then the mixture was incubated in the shaking incubator for the synthesis of silver nanoparticles. The color change was observed visually and photographs were taken.

3- **Characterization of Silver Nanoparticles:**

- **UV-Vis spectroscopy analysis**
  At first, the brown color of the mixture was observed with the silver nitrate solution, which helps us to character the nano-silver particles. The spectra of the UV spectroscopy was recorded by taking a sample of the reaction solution at different time periods and conditions (12).
- **Scanning electron microscope (SEM)**
  The colloidal solution was centrifuged for 15 min at 4000 rpm the supernatant again centrifuged (25 and 900 rpm) for 30 min the pellets were dissolved in 0.1 ml of distilled water then placed on cover slip and dried by air and the sample coated with gold the images of silver nanoparticles obtained using scanning electron microscope(13) this test was done at Nanotechnology Center at Babylon University
4. The antimicrobial activity of SNPs

A) Antibacterial Susceptibility Test

The method of disks diffusion was used according to the CLSI guidelines (CLSI, 2017) for a pharmacological sensitivity test on Muller Hinton Agar Amikacin (30 g) Gentamicin (10 g), Erythromycin (10 g) Ciprofloxacin (5 g) acquired from Bioanalyse / Turkey.

- Few colonies from overnight culture of E. coli, Staphylococcus, Proteus were transferred to 2 ml of normal saline to prepare the bacterial suspension and adjusted to 0.5 McFarland turbidity.
- The bacterial suspension was inoculated into Muller Hinton agar plates using a sterile cotton swab. The plates were left to dry for 5 min.
- The tablets of antibiotic are placed in the plates with bacterial isolates in advance by sterile cotton swabs. Then incubating the plates overnight at 37 °C,
- The bacterial inhibition zone was calculated and taken as a measure of sensitivity. At the same time, nine mm in diameter wells have been done on Muller Hinton Agar medium and swabbed with bacteria using cotton swabs. a hundred micro liters of SNPs were distributed into one of the well and the other scattered Comparative, distal water is placed in the other well and the third end of the petri dish in which placed a loaded nano silver particle was done by depositing every 10 tablets of filter paper in 5 ml of SNPs for two-days as reported by (14) and incubated at the same degree and measured different levels of inhibition zones

B) Antifungal Sensitivity:

Preparation of fungal suspension

The fungal suspension was prepared according to (15) by taking fungal colonies and cultured them on the SDA medium after activation by using a sterile needle and placing it in a vial tube containing 5 ml of normal saline and Solution by vortex. The inhibitory ability of some antifungal agents that used as a treatment for skin lesions was measured. Four antifungal agents were clotrimazole 50mg, ketoconazole 10mg, fluconazole 25mg, and 10mg miconazole.

The test was as follows:

1. SDA was prepared and incubated for 24 hours at 28 C
2. A fungal suspension was prepared from a culture of T. rubrum as well as to the candida albicans yeast.
3. The plates were inoculated by fungal suspension using a sterile cotton swab. Antifungal disks were placed immediately after culture.
4. The plates were incubated for 7 days (T. rubrum) at 28 C, and two days at 37 C for Candida albicans, after which the diameter of the inhibition zone was measured around the antimicrobial disk (16)

Preparation of soaking filter paper of 150 μl of SNPs:
1-cut the filter paper into small circle about 2 cm.
2-Place the filter paper in a small container and Add 2 mL of 1mM of silver nanoparticles over them. Let the filter paper soak for about 10 minutes
Results and discussion

Visual Analysis of silver nanoparticles:

*S. parasitica* showed ability to biosynthesis of SNPs after their growth on SDA and treated the fungal culture supernatant (FCS) with 1mM of AgNO₃ through primary examination with visual observation by bio reduction that causes the color changes in mixture of fungal supernatant with 1mM AgNO₃ after 24hr. of incubation at darkness condition, the fungal culture supernatant showed that color changing from Light yellow to dark brown color at first( 4 ), then the color becomes darker by increasing the incubation period ,this refers to the bio-reduction of silver ions and the formation of nanoparticles in the fungal culture supernatant, this is a result of Plasmon Resonance Surface of nanoparticles while no change in color was observed in the control container that containing fungal supernatant without AgNO₃ when incubated in the same case as shown in Figure (1).

![Containers containing (A) (FCS) (B) Mixture of (FCS) with 1mM AgNO₃.](image.jpg)

**Figure (1):** Containers containing (A) (FCS) (B) Mixture of (FCS) with 1mM AgNO₃.

UV-Vis Spectrophotometer Analysis:

The results of UV-visible absorption spectrometry after 24 hours showed different absorption peaks at limited wavelengths and the absorption density gradually increased to the highest absorption indicating a continuous decrease in AgNO₃ and thus increasing the concentration of SNPs and the formation of SNPs in the reaction mixture were monitored using visible ultraviolet radiation in the range of 200-1100 nm (Figure2). A characteristic peak was seen at 428 nm. This was caused by the phenomenon of surface plasmon resonance. The range within this area was reported to correlate with the surface plasmon of silver nanoparticles(17,18) while fungal supernatant that untreated with silver nitrate and used as a control agent(blank) showed no absorption peaks at the wavelengths mentioned.
Figure (2): UV-Vis spectrophotometer analysis of myco-synthesized silver nanoparticles

**Scanning Electron Microscope**

The images of scanning electron microscopy gave the shapes and sizes of biosynthesized SNPs and using different magnification forces. The results showed that different sizes and variable shapes of particles were found but the spherically shaped particles were predominantly with dimensions ranging from (32-44) nm and distributed uniformly (mono-dispersion) with no large conglomerate (Figure 3).

Figure (3): Image of electron microscopy of the AgNPs particles produced from the *Saprolegnia parasitica*. Power magnification 79,959 x.

**The susceptibility test of antibiotics**

The results of the sensitivity test showed that the various antibiotics which were used against different bacterial and fungal isolates, these isolates have appeared different sensitivity against the antibiotics used in this study, as seen in Table (1) and (2).

The susceptibility test of antibiotic was performed for bacterial isolates (*E.coli*, *Proteus mirabilis*, *Staphylococcus aureus*) for the following antibiotics (Amikacin (30μg), Ciprofloxacins (5μg), Erythromycin (10μg), Gentamicin (10μg), Ampicillin-sulbactam (10μg), Tetracycline (30μg), Augmastin...
(10μg) by disk diffusion method then measuring the diameter of the inhibition zone around the disk and compare results with standard tables(19)

the table (2) show E.coli was resistance for (Gentamicin (10μg), Erythromycin (10μg), while was sensitive for Ciprofloxacins (5μg), Amikacin (30μg) , S.aureus was sensitive to (Ampicillin-sulbactam (10μg), Amikacin (30μg) , but shows resistance to (Tetracycline (30μg), while the least resistance was towards Augmastin (10μg) whereas P.mirabilis show resistance to (Ampicillin-sulbactam (10μg), Gentamicin (10μg), Tetracycline (30μg) but susceptible to Amikacin (30μg).

The cause of bacterial resistance to these antibiotics may be due to changes that occur in the genes found on the chromosome or as a result of a mutation leading to the loss of penicillin binding proteins or the loss of antibiotic activation or the change of Gyra , which is one of the basic units of DNA enzyme or for its production of special enzymes to resist the antibiotic group of betalactam(20,21).

| Table(1): Zone of inhibition(mm) of antibiotics and SNPs that used against some bacterial species. |
|---------------------------------------------------------------|
| **Type of Antibiotics** | **Zone of inhibition against bacteria species** |
| | **E.coli** | **S.aureus** | **P.mirabilis** |
| AK | 25.33±0.33A | 30.33±0.33A | 22.66±0.66A |
| CIP | 23±0B | ----- | ----- |
| SAM | ----- | 20.66±0.66B | 11.66±0.66B |
| ER | 10±0C | 17.66±0.66C | 10±0B |
| AUG | ----- | 17±0C | ----- |
| GM | 10.66±0.33C | 0±0D | 0±0C |
| TE | ----- | 0±0D | 0±0C |
| SNPs | 30.66±0.66B | 26±0.57E | 29.66±0.66D |
| LSD | 1.689 | 1.668 | 1.782 |

- The result represent means of three isolates ± standard error
- The similar letters represent to statistical non-significant differences while the different letters represent to significant differences at (p<0.05)

| Table(2): Zone of inhibition(mm) of antibiotics and SNPs that used against fungal species. |
|---------------------------------------------------------------|
| **Antibiotics** | **Zone of inhibition against fungal species** |
| | **T.rubrum** | **C.albicans** |
| KET | 18.66±0.33A | 15±0.57A |
| MIC | 10.33±0.33B | 20.66±0.33B |
| FLC | 0±0D | 0±0C |
| CLO | ----- | 10.66±0.33B |
| SNPs | 20±0D | 0±0C |
| Laden disk of SNPs | 0±0C | 0±0C |
| LSD_{0.05} | 1.307 | 1.194 |
The result represent means of three isolates ± standard error
The similar letters refers to statistical non-significant differences while the different letters refers to significant differences at (p<0.05)

The susceptibility test of the standard antifungal, although the Fluconazole strength was up to 25 micrograms per tablet, but no inhibition zone was observed around the disks when tested against *T. rubrum*. Several studies have suggested that fluconazole was less effective against dermatophytes (22,23). Our results were consistent with those reports as well as gave the same result when tested against *Candida albicans* indicating that this strain was resistant to this common antifungal drugs. Azoles such as fluconazole are often prescribed for treatment Candidiasis infection and the prolonged use of this azole resulted in the emergence of resistant isolates and caused therapeutic failure. Unfortunately, the prescription of this drug by doctors for the prevention and treatment of fungal infections led to the development of resistance (24).

This is maybe due to the possibility of the patient's use of antifungal drugs previously, which was reflected in the results of this study or this is due to the fact that fluconazole is triazole, and Sabroud dextrose agar components interfere with the test. In addition, the results of the use of fluconazole against skin dermatophytes are variable due to the use of different methods and media(25,26).

This study was consistent with other studies on the use of fluconazole as it was less active against skin fungus (27,22), the inhibition zone of miconazole against *T. rubrum* was about 10 mm, Ketoconazole was the best activity against isolates. Ketoconazole is one of the oldest anti-fungal drugs designed as a treatment against dermatophytes, the inhibition zone of it was about 20 mm, and its effect is due to its ability to inhibit the action of the cytochrome P450 dependence C-14x demethylase (cyp), which is necessary for the conversion of Lanosterol to Ergosterol which leads to weakness in the construction of the plasma membrane and create gaps in it and then imbalance of the cell and the exit of substances out of them as these antibiotics have a deadly effect of fungus

The results of our study showed that miconazole unlike fluconazole, was the best effect against *c.albicans*. The inhibition zone of it was about 20 mm, while *c.albicans* was resisted to ketoconazole and clotrimazole, it works on the accumulation of ROS strongly in the cells of the fungi (24)

**Effect of SNPs against tested Bacteria and fungi:**

The indiscriminate use of drugs has led to the emergence of strains of drug-resistant bacteria carrying genes that make them resistant strains(28) that pay attention to how the consumption of antibiotics, Thus, the search for alternatives to antibiotics has become an urgent need to solve the resistance problem that arises from many pathogenic microorganisms against most commonly used antibiotics (Cassir et al., 2014; Dos Santos et al., 2014). At present, efforts and studies on the use of nanoparticles are intensifying to become a viable alternative to antibiotics, with distinct properties that make them an optimal choice to solve the problem of bacterial resistance to multiple drugs.

AgNPs have become the focus of many researchers in the scientific field. In the past silver were a disinfectant and antimicrobial agent of Gram-positive bacteria (29) because of its low cellular toxicity. In recent years, AgNPs have been considered an alternative to producing a new class of antimicrobials (30) and their mechanism has not yet been fully defined. In fact, antibacterial activity appears to be linked to a multifaceted mechanism in which nanoparticles interact with microbes. In addition, its special structure makes it interact with the bacterial surfaces and may provide a unique antibacterial mechanism and controlled for exploitation.
Three concentrations (50, 100, 150) micro liters of SNPs solution were used against the bacterial and fungal species studied to show the best concentration for example as shown in *E. coli* figure (4). The concentration of 150 l was the optimal concentration as an antimicrobial.

In addition, filter paper was soaked in silver nanoparticles at 150 l concentration.

![Image](image.jpg)

**Figure (4):** Figure showing the use of different concentrations (50 l, 100 l, 150 l) of SNPs solution against bacteria *E. coli*.

The results of our study indicate that the nanoparticles produced by *Saprolegnia parasitica* have an inhibitory effect against the tested pathogenic bacteria (*E. coli*, *Proteus mirabilis*, *S. aureus*), these are highly effective against *E. coli* as shown in Table (1) figure (5,6,7). The diameter of the inhibition zone of *E. coli* was 29 mm and was also effective against *Proteus mirabilis*, as shown in the same table, the inhibition zone was about 25 mm and the inhibition diameter was 30 mm against bacteria *S. aureus*.
**Figure (5)**: Antibacterial activity of SNPs against *E.coli* (A) SNPs at 150 μl, (B) Laden disk of SNPs, (C) D.W Control.

**Figure (6)**: Antibacterial activity of SNPs against *P.mirabilis* (A) SNPs at 150 μl, (B) Laden disk of SNPs, (C) D.W Control.
Figure (7) : Antibacterial activity of SNPs against *S.aureus*  
(A) SNPs at 150 μl , (B) Laden disk of SNPs, (C) D.W Control.

The small size of the nanoparticles and the large surface area impact on the permeability of the plasma membrane of the bacteria and therefore the death of cells as small size collected in larger numbers on the surface of the cell and this led to increased toxicity to microorganisms . (31,32)

As was evident from the (table 2) that the Gram-negative bacteria (*E. coli* and *P. mirabilis*) have shown higher inhibitory values when compared with *S. aureus* bacteria this result was compatible with (33) who synthesized the silver nanoparticle by banana peel extract and used it as an antimicrobial against *E. coli* and *P. aeruginosa*. *S.aureus* then he was noticed that the inhibition zones were 13 nm, 15 mm, 16mm respectively , explanation of this case is that the nature of the composition of the bacterial cell wall, Gram-positive bacteria are characterized by the thickness of the peptidoglycan layer, which forms the cell wall and is made up of polysaccharide chains. This leads to a more rigid structure and thus difficult to penetrate by silver nanoparticles, As opposed to gram-negative bacteria, whose cellular wall is characterized by a thinner peptidoglycan layer (34).

The mechanism in which nanoparticles interact with bacterial cells is that microorganisms carry a negative charge while nanoparticles carry a positive charge, creating an electromagnetic attraction between bacteria and the surface of nanoparticles, the nanoparticles release the ions that interact with the total of thiol, which represent the proteins that transport the food that protrudes from the bacterial cell membrane, leading to reduced permeability of the membrane and thus cell death (35).

Mechanically, the inhibition of nanoparticles of DNA susceptibility to the replication and gene expression of proteins as well as the various cellular proteins and enzymes necessary in ATP production leading to inhibit microbes (36).

Nanoparticles attacking the surface of the cell membrane and disrupting the permeability and respiratory functions of the cell or interfering with Components of the electron transport system for bacteria as well as lead to the creation of gaps in the outer membrane of bacteria which affects on membrane permeability (37).
AgNPs also release ROS, which reduces the activity of dehydrogenase (LDH), which is important in cellular respiration (31).

This results agreed with the results of a previous study (38), who reported that the biosynthesized silver nanoparticles that prepared by *Streptomyces hygroscopicus* and *Streptomyces parvulus* significantly inhibited the growth of medically important pathogenic of bacteria *Escherichia coli* and *Proteus mirabilis*, there were significant differences between the effects of nanoparticles compared with the antibodies used in the study as shown in the Figure (1).

The ability of silver nanoparticles during this study was shown to inhibit the growth of some fungal species, where nanoparticles showed an inhibitory effect against *T. rubrum* and the inhibition zone was about (20 mm) as shown in the figure (8) and the results showed significant differences between silver nanoparticles compared with the antimicrobial (P <0.05) which were used in this study as shown in the table (2).

**Figure (8)**: Antifungal activity of SNPs against *T. rubrum*

(A) SNPs at 150 μl, (B) Laden disk of SNPs, (C) D.W Control.

while no inhibitory activity against *Candida albicans* this unexpected finding was observed in the case of using the SNPs effect against *Candida albicans*, As we have not seen inhibition zones when the silver nanoparticles or loaded disk of SNPs were used against *Candida albicans*, as shown in figure (9) using 150 μl of SNPs at a concentration of 1 mM by agar well diffusion method, of SNPs at a concentration of 1 mM by agar well diffusion method, this result is close to (33) who shown the effect of silver nanoparticles against *C. albicans* was low and the inhibition diameter was too small It seemed like there was no inhibition zone.
Figure (9) : Antifungal activity of SNPs against *C. albicans*

(A) SNPs at 150 μl, (B) Laden disk of SNPs, (C) D.W Control.

The Silver nanoparticles have an effective antimicrobial effect and have been successfully demonstrated by many researchers, although fungal resistance to antifungal agents has been widely discussed in the literature, resistance to silver nanoparticles has not been fully explored. In our study, *Candida albicans* can be resistant to silver nanoparticles after exposure to these particles. This resistance may be arises from the production of the adhesion protein, which leads to the aggregation of nanoparticles. The emergence of this resistance has nothing to do with any genetic changes. There is only a need to change the phenotype to reduce the stability of colloidal nanoparticles thus eliminating their antifungal activity. The mechanism of resistance cannot be overcome by additional fixation of silver nanoparticles using surfactants or polymers, but this resistance can be overcome with the inhibitor of the adherent protein produced by the fungus.

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