Antibacterial, antivirulence and antifungal activity of silver nanoparticles synthesized using alkhal mother shae

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Abstract. Silver nanoparticles were biosynthesized using alkhal mother shae. Nanoparticles were characterized using four methods. The results of the fourth methods proved that silver nanoparticles are spherical shape with a size ranging between 30-40 nm. AMS-AgNPs has antibacterial and antibiofilm activity against gram negative and gram positive tested bacteria, and it has antifungal and antivirulence activity against five types of fungi. Results declare the effect of AMS-AgNPs on the fungus, it can disrupt the integrity fungal cell wall, promoting the permeability and the leakage of the cell constituents, and eventually induce cell death, which is reached from the Congo red dye absorption test and the formation of swelling in the hyphae of the fungal. This study revealed that AMS-AgNPs can be used as an alternative medicin for pathogenic bacteria and fungi.

Keywords. Alkhal Mother Shae, Silver nanoparticles, antibacterial, antifungal, antivirulence.

1. Introduction

Alkhal Mother is a substance composed of yeast and acetic acid bacteria especially, Acetobacter xylinum which forms a cellulose pellicle on shae broth. It is produced by fermenting shae using a "symbiotic 'colony' of bacteria and yeast" (SCOBY). Actual contributing microbial populations in SCOBY cultures vary, but the yeast component generally includes Saccharomyces and other species, and the bacterial component almost includes A. xylinus to oxidize yeast-produced alcohols to acetic and other acids [1]. Tea is the oldest and cheapest health beverage in the world next to water [2]. Today, tea is produced in over 20 countries in tropical, sub-tropical and temperate regions. It is the most widely consumed beverage after water, due to its health, sensory, stimulant, relaxing and cultural properties [3]. The beneficial effects of tea are owing to its polyphenolic compounds. Among the shae polyphenols, flavonoids, especially catechins, are the leading functional components, which accounts for 30% of the dry weight of green tea leaves. Fresh tea leaves are very rich in catechins, which include mainly epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), and gallatechin (GC). EGCG is the most abundant catechin in green tea which accounts for at least 65% of the total catechin [4]. Black tea which grows in many parts of world, is used as an herbal tea for Drinking. Camellia sinensis leaves (Black tea) have been reported to contain considerable amounts of tannin products [5]. In Iraq, mats of personally are

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individually circulated among those people seeking for health remedy. So, this mat is called “Alkhal mother” as an acquired local traditional Iraqi name, however, it has little scientific studies. The goal of this study is to examine the antibacterial and antifungal activities of silver nanoparticles synthesized using Alkhal Mother Shae (AMS).

2. Materials and Methods

2.1. Collection of bacteria

The bacteria were isolated from patients with infection wounds and burns attending to Al-Yarmouk Teaching Hospital for the period from October to December, 2019, and used as an antimicrobial.

2.2. Collection of fungi

Five types of fungi isolates from Mycotoxins laboratory. Faculty of Sciences, University of Baghdad.

2.3. Alkhal Mother SCOBY

Alkhal Mother SCOBY was obtained from (Dr. Al-Kalifawi, Esam J.), as described by [6].

2.4. Cultivation of Alkhal Mother SCOBY

Alkhal Mother cultured as described by [7].

2.5. Extracellular synthesis of silver nanoparticles

AMS-AgNPs synthesized as described by [8].

2.6. Properties of AgNPs

2.6.1. UV-Vis analysis

The silver nanoparticle colloidal was examined for the absorption peak, which falls within the range 350-450 nm

2.6.2. FT-IR Spectroscopy

The active biological groups present in Alkhal Mother Shae, which serve to reducing and capping the resulting silver nanoparticles, have been identified as described by [8].

2.6.3. XRD analysis

The peaks of the AgNPs were determined using the XRD device as described by [9].

2.6.4. SEM Analysis

The silver nanoparticles were scanned to find out the shape and clustering of the particles.

2.6.5. Antibacterial activity determination
The effect of AMS-AgNPs was determined as described by [10].

2.6.6. Antibiofilm activity determination

Detection of antibiofilm activity of AMS-AgNPs against *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates using Microtiter plate method as mentioned in [11].

2.6.7. Antifungal activity determination

The antifungal activity of AMS-AgNPs was tested by the radial growth of tested fungi, after the addition of different concentration of silver nanoparticles to potato dextrose agar medium. The media inoculated with the tested fungi and incubated at 25±2°C [12].

2.6.8. The effect of AMS-AgNPs on cell wall function

The effect of AMS-AgNPs on cell wall efficacy was studied by phenotypic changes and congo-red dye test [13].

3. Results and Discussion

The fresh suspension of (AMS) was bright brown but it turned reddish brown after adding it to the silver nitrate solution. This color change indicates the completion of the reaction and the formation of silver nanoparticles Figure 1.

![Figure 1. Synthesis of AMS-AgNPs: A- Solution of silver nitrate. B- (AMS), and C- AMS-AgNPs colloidal.](image)

3.1. UV-Vis Spectrophotometry

UV-Vis absorption spectrum of AMS-AgNPs is shown in figure 2. It’s at 430 nm.
3.2. Fourier Transform Infra-Red Spectroscopy

Figure 3 shows the spectra for Alkhal Mother Shae were obtained using an FTIR spectrophotometer. Several peaks were observed indicating the Alkhal Mother Shae is composed of various functional groups. The broad band at about 3448.72 cm$^{-1}$ can be attributed to bond –OH groups. The band at about 2356.89 cm$^{-1}$ can be attributed to C=N bond Amine I groups are also observed at 1635.64 cm$^{-1}$. The peak at 1543.05 cm$^{-1}$ is attributed to secondary amine groups. The peaks at 1458.18 and 1396.46 cm$^{-1}$ are both related to the symmetric bending of CH3. While the peak at about 1107.14 cm$^{-1}$ can be attributed to C=O stretching vibrations groups. The peak at 470.63 cm$^{-1}$ correspond to stretching vibration of amine groups.
3.3. XRD pattern of AMS-AgNPs

Figure 4 shows the main peaks obtained at 111, 200, 220, and 311 correspond to reflections with 2θ values of the Bragg angles 38.12°, 44.31°, 64.46°, and 76.98°, respectively. These results confirm that the material tested are AgNPs and are of high purity. The average crystallite size of AMS-AgNPs in range (30-40 nm).

![Figure 4. X-ray diffraction of AMS-AgNPs.](image)

3.4. SEM analysis of AMS-AgNPs

The scanning showed that the AMS-AgNPs are spherical in shape and are aggregated in clusters Figure 5.

![Figure 5. SEM micrographs of AMS-AgNPs.](image)
The results in Table (1) and Figures (6 and 7) shows the inhibition zone was 18 mm for *P. aeruginosa* No.1, 3, 4, 7, 9, 10 and *S. aureus* No.3, 5, 6, 9. 16 mm for *P. aeruginosa* No.2, 8 and *S. aureus* No.4, 10, 14 mm for *P. aeruginosa* No. 5, 6 and *S. aureus* No.1, 2, 7. The IZ was 10 mm for *P. aeruginosa* No.1, 4, 6, 8, 9, 10 and *S. aureus* No.2, 3, 7, 8, 9. 11 mm for *P. aeruginosa* No.2, 5 and *S. aureus* No.1, 5, 10, 12 mm for *P. aeruginosa* No.3, 7 and *S. aureus* No.4, 6.

**Table 1.** Activity of the AMS-AgNPs against the tested bacteria.

| Isolated bacteria | Zone of Inhibition (mm) |  |
|-------------------|-------------------------|--|
|                   | Silver nitrate solution | Alkhal mother tea | Silver nanoparticles colloidal |
| *P. aeruginosa* No.1 | 0 | 10 | 18 |
| *P. aeruginosa* No.2 | 0 | 11 | 16 |
| *P. aeruginosa* No.3 | 0 | 12 | 18 |
| *P. aeruginosa* No.4 | 0 | 10 | 18 |
| *P. aeruginosa* No.5 | 0 | 11 | 14 |
| *P. aeruginosa* No.6 | 0 | 10 | 14 |
| *P. aeruginosa* No.7 | 0 | 12 | 18 |
| *P. aeruginosa* No.8 | 0 | 10 | 16 |
| *P. aeruginosa* No.9 | 0 | 10 | 18 |
| *P. aeruginosa* No.10 | 0 | 10 | 18 |
| *S. aureus* No.1 | 0 | 11 | 14 |
| *S. aureus* No.2 | 0 | 10 | 14 |
| *S. aureus* No.3 | 0 | 10 | 18 |
| *S. aureus* No.4 | 0 | 12 | 16 |
| *S. aureus* No.5 | 0 | 11 | 18 |
| *S. aureus* No.6 | 0 | 12 | 18 |
| *S. aureus* No.7 | 0 | 10 | 14 |
| *S. aureus* No.8 | 0 | 10 | 14 |
| *S. aureus* No.9 | 0 | 10 | 18 |
| *S. aureus* No.10 | 0 | 11 | 16 |

**Figure 6**: Antibacterial of AMS-AgNPs using the test bacterium Pseudomonas aeruginosa No.4. A - Solution of silver nitrate, B - Alkhal Mother Shae, C - AgNPs colloidal.
The results showed the inhibition of biofilm formation for *P. aeruginosa* and *S. aureus* was 70% and 60%, respectively when using AMS-AgNPs at 0.1 concentration. While the inhibition of biofilm formation for *P. aeruginosa* and *S. aureus* was 80% and 70%, respectively when using AMS-AgNPs at 0.5 concentration table 2 and figures 8 and 9.

**Table 2.** Inhibition of biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* using AMS-AgNPs.

| Isolate number | The value of biofilm | The value of biofilm after addition 0.1 mg/ml AgNPs | The value of biofilm after addition 0.5 mg/ml AgNPs |
|----------------|---------------------|-----------------------------------------------|-----------------------------------------------|
| *P. aeruginosa* No.1 | 0.176 | 0.90 | 0.80 |
| *P. aeruginosa* No.2 | 0.169 | 0.75 | 0.62 |
| *P. aeruginosa* No.3 | 0.188 | 0.95 | 0.86 |
| *P. aeruginosa* No.4 | 0.179 | 0.88 | 0.75 |
| *P. aeruginosa* No.5 | 0.295 | 0.280 | 0.200 |
| *P. aeruginosa* No.6 | 0.289 | 0.222 | 0.210 |
| *P. aeruginosa* No.7 | 0.170 | 0.89 | 0.78 |
| *P. aeruginosa* No.8 | 0.187 | 0.170 | 0.91 |
| *P. aeruginosa* No.9 | 0.200 | 0.93 | 0.85 |
| *P. aeruginosa* No.10 | 0.199 | 0.87 | 0.70 |
| *S. aureus* No.1 | 0.274 | 0.200 | 0.192 |
| *S. aureus* No.2 | 0.217 | 0.195 | 0.187 |
| *S. aureus* No.3 | 0.223 | 0.102 | 0.89 |
| *S. aureus* No.4 | 0.224 | 0.188 | 0.171 |
| *S. aureus* No.5 | 0.193 | 0.88 | 0.76 |
| *S. aureus* No.6 | 0.184 | 0.90 | 0.83 |
| *S. aureus* No.7 | 0.234 | 0.210 | 0.200 |
| *S. aureus* No.8 | 0.310 | 0.304 | 0.298 |
| *S. aureus* No.9 | 0.198 | 0.84 | 0.71 |
| *S. aureus* No.10 | 0.186 | 0.160 | 0.90 |
Inhibition of biofilm formation of Pseudomonas aeruginosa using AMS-AgNPs.

Inhibition of biofilm formation of Staphylococcus aureus using AMS-AgNPs.

The results in Table (3) and Figure (10, 11) shows that AMS-AgNPs has effective antifungal activities on the Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Fusarium oxysporum and Fusarium verticillioides as indicated by the diameter of their radial growth. The radial growth of Aspergillus flavus, Aspergillus parasiticus and Aspergillus niger reduce from 9 cm for control to 2 cm at 15% concentration of silver nanoparticles in the culture media. Whereas the radial growth of Fusarium oxysporum and Fusarium verticillioides reduce from 7 cm for control to 1 cm at 15% concentration of silver nanoparticles in the culture media.

Table 3. The antifungal effect of the AMS-AgNPs.

| Isolated microbes       | control | 5%  | 10% | 15% |
|------------------------|---------|-----|-----|-----|
| Aspergillus flavus      | 9       | 7   | 4   | 2   |
| Aspergillus parasiticus | 9       | 7   | 4   | 2   |
| Aspergillus niger       | 9       | 7   | 3   | 2   |
| Fusarium oxysporum      | 7       | 2   | 1.5 | 1   |
| Fusarium verticillioides| 7       | 3   | 2   | 1   |

* Radial growth of fungi (cm).
Figure 10. Antifungal activity of AMS-AgNPs against *Aspergillus flavus*.

Figure 11. Antifungal activity of AMS-AgNPs against *Fusarium oxysporum*.

The effect of silver nanoparticles on the fungus was also studied and it was found that AMS-AgNPs lead to swelling in the fungal hyphae Figure 12. These swelling effect on permeability of hyphae cell and thus kill it, which is reached from the Congo red dye absorption test Figure 13.
Figure 12. Show the effect of AMS-AgNPs on the *Fusarium verticillioides* hyphae. A- Control. B- treated with AMS-AgNPs. Power of magnification 100X.

Figure 13. Show the Congo red dye absorption test. A- Normal hypha of *Fusarium verticillioides*. B- treated with AMS-AgNPs. Power of magnification 100X.

4. Discussion

In the present study, AgNPs was biosynthesized using alkhal mother shae. Production of AgNPs was confirmed by the change in colour and formation of gray aggregates of AgNPs. This result is in agreement with many studies [16, 17, 18] which found that the formation of gray aggregates is
evidence of completeness of reaction and formation of AgNPs. The crystalline AgNPs were confirmed by UV absorption spectrum at 430 nm. This result is accordance with several studies [19, 20, 21] which reported that the peak absorption of AgNPs is around 300-500 nm. The functional groups associated with the process of reducing and stabilizing AgNPs were analyzed using Fourier Transform Infrared Spectroscopy. Several peaks were observed indicating that the alkhal mother shae is composed of various functional groups are attributed to secondary amine groups. These finding are acceptable with many studies [22, 23, 24] which found these groups are responsible for reducing and capping of AgNPs. The results of X-ray Diffraction spectrum with 2θ values was at Bragg angles 38.12, 44.31, 64.46 and 76.98, respectively. These results are consistent with several studies in which biosynthesis of nanoparticles of silver molecules using tea leaf extract and kombucha tea [25, 26, 27]. Particle size causes the broadening of peaks in the XRD patterns and by using Debye-Scherrer’s equation, the average particles size were arrange 30-40 nm. Results of the present study show that AMS-AgNPs have antibacterial activity against Gram positive and Gram negative bacteria which tested. The antimicrobial activity of alkhal mother shae has less effect against tested isolates. These finding are in agreement with several studies [28, 29, 30, 31]. Who’s found that the silver nanoparticles synthesized by leaf tea extract had antibacterial activity against Gram-negative and Gram-positive bacteria. The inhibition of biofilm formation for P. aeruginosa and S. aureus was 70% and 60%, respectively when using AMS-AgNPs at 0.1 concentration. While the inhibition of biofilm formation for P. aeruginosa and S. aureus was 80% and 70% respectively when using AMS-AgNPs at 0.5 concentration. These finding are in agreement with several studies [32, 33, 34] which found that the antibiofilm activity of AgNPs was between 50 to 90 in Gram negative bacteria and about 50 to 80 in Gram positive bacteria. AMS-AgNPs has antifungal activity on the Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Fusarium oxysporum and Fusarium verticillioides as indicated by the diameter of their radial growth. Whereas the radial growth of Aspergillus flavus, Aspergillus parasiticus and Aspergillus niger reduce from 9 cm for control to 2cm at 15% concentration of silver nanoparticles in the culture media. These finding are in agreement with several studies which found the effect of silver nanoparticles against various plant pathogenic fungi [35, 36, 37]. The two species have been selected because they are producers of the mycotoxins. The Aspergillus, especially A. flavus and A. parasiticus, which produce aflatoxins and Fusarium, which produces Trichothecenes. Aflatoxins is the leading cause of cancer. Trichothecenes is used in chemical warfare [38, 39]. For these reasons, the eradication of these fungi protects plants, from the infection of and animals, humans from their toxins [40, 41]. The effect of silver nanoparticles on the fungus was also studied and it was found that silver nanoparticles lead to swelling in the fungal hyphae and thus kill it, which is reached from the Congo red dye absorption test [42]. These finding are in agreement with several studies [43, 44, 45] in which found AMS-AgNPs disrupt the integrity fungal cell wall, promoting the permeability and the leakage of the cell constituents, and eventually induce cell death.

5. Conclusions

We concluded that the AMS-AgNPs can be used as an alternative medicin for pathogenic bacteria and fungi.

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