

# Antibacterial and Antibiofilm Activity of Silver Nanoparticles Biosynthesized by *Mentha Spicata* Leaves Methanolic Extract Against *Staphylococcus Aureus* and *Acinetobacter Baumannii*

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## Abstract

Antibiotic resistance is a critical public health issue that necessitates more research and the development of alternate solutions. It is essential to identify novel antimicrobial agents (nanomaterials) capable of treating resistant isolates. Nanotechnology is predicted to pave the way for new ways to prevent and combat disease-causing microbes. Silver nanoparticles (AgNPs) were synthesized using an environmentally benign and straightforward method in which *Mentha spicata* leaf methanolic extract was used. UV-visible spectroscopy, an atomic spectrophotometer, and transmission electron microscopy were used to identify biosynthesized AgNPs (TEM). Silver nanoparticles showed one peak absorption at (450-500) nm in the UV-vis spectra of the solution. The silver nanoparticle concentration was found to be 240 g/ml using an atomic spectrophotometer. The AgNPs were predominantly spherical and of various sizes, according to TEM pictures. The size of the silver nanoparticles measured by TEM ranged from (9.8 nm – 51.42 nm), and the purity of the NPs was confirmed using a Fourier transform infrared spectrometer (FTIR). The findings of this study show that biosynthetic AgNPs exhibit antibacterial action against *S.aureus* and *A.baumannii* by measuring the diameter of the inhibitory zone around biosynthetic AgNP-containing wells. Against powerful biofilm producer isolates, AgNPs showed antibiofilm action. A total of 69 isolates of *S.aureus* & *A. baumannii* were tested to determine their ability to biofilm formation by microtiter plate method. The final score was 22. (53.6 %) 11 isolates (40%) produced a robust biofilm, 12 isolates (29.2%), and 8 isolates (28.3%) produced a moderate biofilm. 4 (9.7 %) *S.aureus* & *A. baumannii* concentration, 6 (21.4%) of isolates developed weak biofilm, and 3(7.3%) 3(10%) of isolates were nonbiofilm producers *S.aureus* & *A.baumannii*. The study concluded that the Utilization of secondary metabolites of *Mentha spicata* was used leaf extract as a reducing agent in the process of AgNPs synthesis. Biosynthesized AgNPs have antibacterial and antibiofilm activity.

**Keywords:** Green synthesis, Silver-nanoparticles, Anti-Bacterial, Antibiofilm, *S.aureus*, *A. baumannii*.

## 1. Introduction

Nanotechnology has sparked a lot of attention in recent years because of its potential impact on a range of fields, including medicine, agriculture, and industry. [1]. Nanotechnology has great potential for developing new nanomaterials that biomedical uses. [2]. Nanotechnology has great potential for developing new nanomaterials that biomedical uses. [3]. The name "Nano" comes from the Greek word "dwarf," and it refers to a size scale of one nanometer (9-10) nm of a meter [4]. Nanoparticles are atom clusters, that range from (1–100) nm in size. Nanomaterials have different physical and chemical properties than bigger bulk materials. [5]. Physical and chemical synthesis are inefficient because of their high cost, energy consumption, ecotoxicity, and lack of appropriateness for biomedical application [6],[7]. Biosynthesis technologies, on the other hand, may have more advantages, such as cheaper costs and

environmental benefits. Capping and stabilizing are also advantages, which can be accomplished with microbiological cultures or, nontoxic extracts. [8]. As a result, plant extracts, fruit, bark, fruit peels, root, and callus have all lately been discovered. added to the green production of, nanoparticles using phytochemicals as bio-reductants [9]. Mints are often considered to be one of the most important spices in the world. The goal of this work was to make silver nanoparticles out of *Mentha spicata* water leaves extract and test the antibacterial activity of the nanoparticles [10].

## 2. Material and Methods

### Isolation and identification of *S. aureus* & *A. baumannii*

Sixty nine *S. aureus* and *A.baumannii* isolates out of 200 of total isolates isolated from different sources samples for patients attending to Ramadi Teaching General Hospital and hospital Al-Ramadi for

maternity and Children, Specimens were cultured on MacConkey agar and blood agar and Mannitol salt agar in dishes at 37°C for 18-24 hr. Afterward, the grown colonies were identified by Vitek 2 compact system using GN and GP card depending on instructions of the manufacturer.

### Preparation of plant extract

The plant was taken, dried, and ground into powder. The plant extract was then made by dissolving 25 g of powder in 250 mL of solvent (methanolic) using Hot Plate for 2 hours. then suspension was shaking for 1 h. The extract is then filtered through medical gauze and using Whatman filter paper No.1 for the extract was filtered to obtain the methanolic plant extracts.

### Preparation of Silver Nitrate Solution

10 mM concentration AgNO<sub>3</sub>, The green, production of silver nanoparticles was performed, out by dissolving (0.169g) AgNO<sub>3</sub> in (100 ml) Deionized sterile distilled water [11].

### Synthesis of AgNPs by Mentha Spicata extract

For this experiment: 2 ml of Mentha Spicata 10 ml methanol extract with 2 ml of 1 M AgNO<sub>3</sub> aqueous solution heated at 60 °C for 15 minutes in a water bath with stirring. With the color change to brown, AgNPs were gradually produced. AgNPs were synthesized and centrifuged in centrifuges [12].

### Identification of bacterial Growth

Identification of bacterial isolates that grew after being incubated under aerobic and anaerobic conditions for an overnight period. The colony morphology and biochemical reactions are used to characterize possible colonies.

### Detection of biofilm by Microtitration Plate method

- The biofilm of bacteria infection isolates was studied using a 96-well flat-bottom microtiter plate according to [13]; all experiments were done in triplicate.
- Prepared bacterial suspensions, by inoculating tubes containing 5ml of normal saline with single colonies grown on agar for (18-24) hours with sterilized loops, then shaking the tubes for turbidity compared to Mcfarland standard (0.5-0.63).
- 20 µl of bacterial suspension overnight culture (equal to 0.5 Mcfarland standard) was used to inoculate microtiter wells with 180 µL of cerebral Heart Infusion Broth supplemented with 2% glucose, which were incubated at 37°C for 24 h.
- The plate was rinsed three times with normal saline after incubation to remove non-adherent cells.
- 200 µl of 99 % methanol per well was used to fix the adherent cells for 15 minutes. At room temperature, the plate was dried for 30 minutes.
- Then, for 15 minutes, 200 µl of 1 % crystal violet was added.
- After washing with sterile distilled water to

remove the dye solution, the attached dye was solubilized with 33 % glacial acetic acid, and the optical density was measured at 630 nm in a micro-titer plate reader.

Calculated the ability of biofilm by:

$$\text{Biofilm degree} = \text{OD}_{630} \text{ of tested bacterial} - \text{OD}_{630} \text{ control}$$

Cut-off value calculation	Biofilm production abilities
$\text{OD} > (4 \times \text{OD}_c)$	Strong
$(2 \times \text{OD}_c) < \text{OD} \leq (4 \times \text{OD}_c)$	Moderate
$(\text{OD}_c) < \text{OD} \leq (2 \times \text{OD}_c)$	Weak
$\text{OD} \leq (\text{OD}_c)$	None
OD: mean of sample absorption at 630 nm. OD <sub>c</sub> : mean of control absorption at 630 nm.	

### Serial Dilution

Stock solutions with concentrations of 120, 60 µg/mL, 240 µg/mL, were transferred to two sterile tubes in a rack. 2mL of normal saline is contained in each tube. After adding two ml of stock solution to the first tube and thoroughly mixing it, that 2ml was mixed and transferred from the first tube to the second tube. In this case, The first tube contains 120 µg/mL, whereas the second tube contains 60 µg/mL. to determine antimicrobial susceptibility

### Anti-bacterial efficacy of AgNPs by agar well method

Agar well-diffusion method was followed to determine the antimicrobial activity of biosynthesized AgNPs [14].

- Pure cultures of *S.aureus* and *A. baumannii* were subcultured in tryptone soya broth for 18 hours at 37°C.
- Up to 20 mL Mueller-Hinton Agar media was put into Petri dishes individually, and each isolate was uniformly swabbed on the dishes with a sterile cotton swab.
- Using a sterile cork borer, we bore 6mm diameter wells in each of these plates.
- Approximately 100 µl of varying concentrations of biosynthesized AgNPs were introduced to different wells using a sterile syringe. After overnight incubation at 37 °C, the clear zone of inhibition around the loaded well was used to determine bactericidal efficacy.

### Antibiofilm activity of silver nanoparticles by using micro-titer plate Method

[15] developed the following method for estimating biofilm formation

- A bacterial culture was modified overnight (in trypton soya broth) to be compatible with No. 0.5 McFarland standard.
- Inoculated with previously prepared bacterial suspension (step a), tryptone soya broth with sub-MIC of AgNPs was incubated at 37°C for 24 h.
- A total of 200 µl of the culture obtained in

step a was placed in triplicate into polystyrene microtiter plates in the vertical rows of the plate for each isolate as a control. ( Strong, Moderate and Weak).

d) A culture volume of 200  $\mu$ l that contains the sub-MIC, The AgNPs concentration (made in step b) was put into Three sub-MIC wells All plates were incubated for 24 h at 37°C.

e) Following that, biofilm production was assessed using the crystal violet assay (CV) assay, and optical density at 630 nm was obtained using a microtiter plate reader after dimethyl sulphoxide was used to dissolve the insoluble purple formazan (DMSO).

f) The percentage of bacterial adhesion inhibition achieved.

### 3. Results and Discussion

#### Isolation and Identification of *S.aureus* and *A. baumannii*

This study took nine months from the beginning of September 2021 and ended with the beginning of May 2022. Sixty-nine *S. aureus* & *A. baumannii* isolates were isolated from a total of 200 samples from different patient sources. Isolates from MacConkey Agar Mannitol salt agar were identified by the VITEK® 2 system.

#### Synthesis of AgNPs

##### UV-Vis Spectrophotometer analysis

In the nanoparticle preparation reaction, the hue changed to brown, indicating the development of silver nanoparticles. The existence silver nanoparticles with Plasmon resonance with a range between (450 to 500) nanometers was discovered using UV-Visible spectroscopy (Fig. 1). In the spectral pattern, there appears to be a strong band. because of the stimulation of localized surface plasmons, which causes substantial light dispersion when an electric field is applied at a certain angle wavelength at which resonance is place [16], [17]. Biosynthesis and Characterization of Silver Nanoparticle [18].

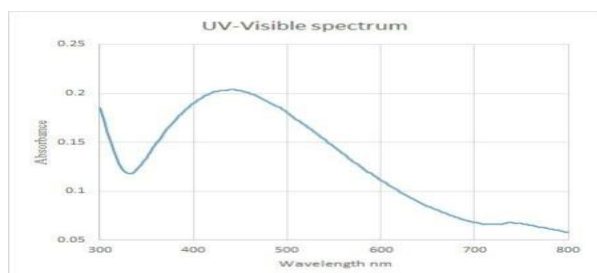


Fig. 1. UV-Visible spectroscopy of silver nanoparticles

#### Transmission Electron Microscope analysis (TEM)

Figure (2) shows a (TEM) image of silver nanoparticles (AgNPs) prepared from an alcoholic extract. The nanoscale particles were obtained in mostly spherical and spherical shapes and ranged between (9.8 nm – 51.42 nm) at a rate of the average

size of the nano-diameters is (25.76 nm), as shown in figure (3). These results are a greement with [19], [20].

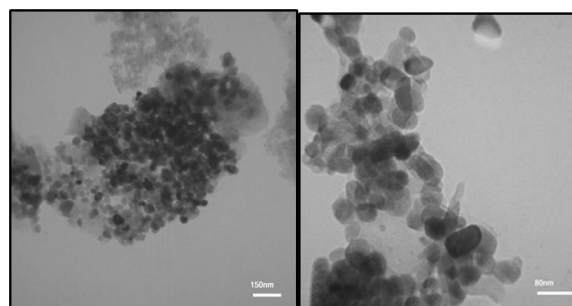


Fig. (2): Transmission electron image of for silver nanoparticles was synthesized by methanolic leaf extract of *M. spicata*

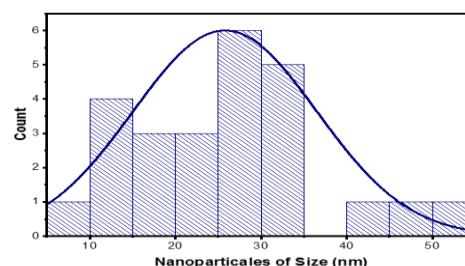


Fig. (3): Average diameters of silver nanoparticles(methanolic) by TEM

#### Fourier transform infrared spectrometer FTIR

FTIR spectroscopy shows that the Photochemical study of *M. spicata* methanolic leaf extract revealed significant absorbance bands at peaks 3332.99, 2121.70, 918.12, 540.07  $\text{cm}^{-1}$ .

Figure (4) shows a comparison of the FTIR spectra of methanolic leaf extract of *M. spicata* and the silver nanoparticles that were produced (5).

The stretching vibration of O-H of alcohols and phenols is ascribed to a shift in the band from 3332.99 to 3259.70  $\text{cm}^{-1}$ , whereas the stretching vibration of C-H aliphatic is related to a shift in the band from 2121.70 to 2113.98  $\text{cm}^{-1}$ . 1631.78 to 1631.78  $\text{cm}^{-1}$  band is attributed to Carbonyl group (C=O) stretching. Another shift (540.07 to 590.22)  $\text{cm}^{-1}$  was found, which corresponds to C-Cl stretching vibrations in alkyl halides. These functional groups could play an important role in the green synthesis of silver nanoparticles. Our findings were extremely similar to those of another study [21], [22].

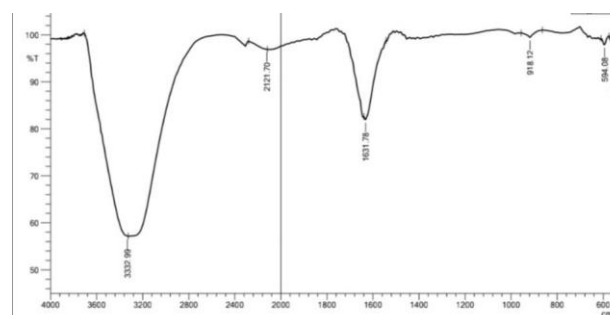


Fig. (4): FTIR analysis of methanolic leaf extract of *M. spicata*

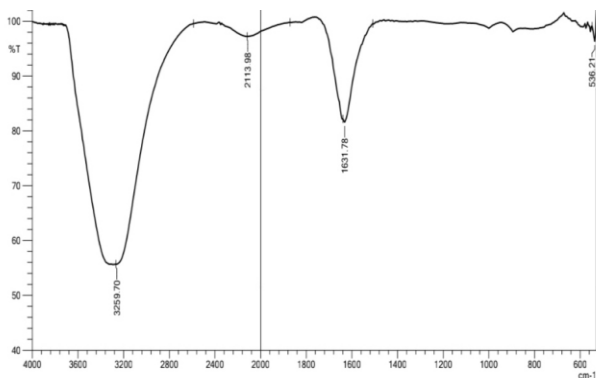


Fig. (5): FTIR analysis of biosynthesized silver nanoparticles by methanolic leaf extract of *M. spicata*

### Biofilm formation by Micro-titter plate Method

The results were 22 (53.6%) 11 (40%) of the isolates created a strong biofilm, 12 (29.2%),8 (28.3%) created a moderate biofilm, 4 (9.7%) 6 (21.4%) of the isolates formed a weak biofilm, and 3 (7.3%) 3 (10%) of the isolates were non-biofilm producers (*S. aureus* and *A. baumannii*) respectively. As shown in the table 1, figure (6) indicates the results of the statistical analysis for the presence of significant differences under the level of (1% and 5%). The study's results came close to researcher study [23]

**Table 1 :Distribution of *S.aureus* and *A. baumannii* isolate regarding their biofilm formation ability by microtiter plate**

Bacterial isolates	No	Strong	Moderate	Weak	None
<i>S. aureus</i>	41	22(53.6%)	12(29.2%)	4(9.7%)	3(7.3%)
<i>A. baumannii</i>	28	11(40%)	8(28.3%)	6(21.4%)	3(10%)
Total	69	No. (%) of isolates Specific biofilm formation			

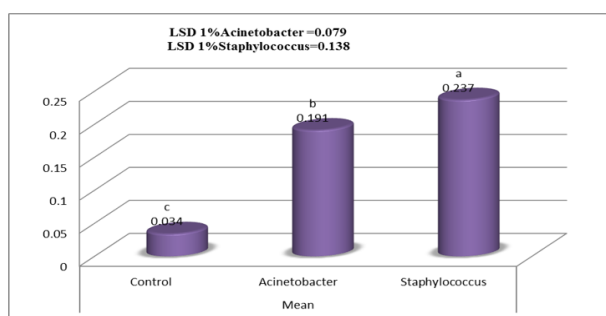


Fig. (6): Average of two types of bacteria *S. aureus* & *A. baumannii* compared to the control without bacteria for biofilm

### Antibiofilm activity of AgNPs against biofilm producing *S. aureus* & *A.baumanii*

**Table (2): Antibiofilm activity of AgNPs synthesized by methanolic leaf extract of *M. spicata***

NO.	Bacterial type	O.D. of untreated	O.D. of 0.5MIC	%Biofilm Inhibition
1	<i>S. aureus</i>	0.338	0.11	67.45%
2	<i>S. aureus</i>	0.778	0.077	90.10 %
3	<i>A.baumanii</i>	0.419	0.087	79.23 %
4	<i>A.baumanii</i>	0.675	0.126	81.33 %
5	<i>A.baumanii</i>	0.479	0.119	75.15 %

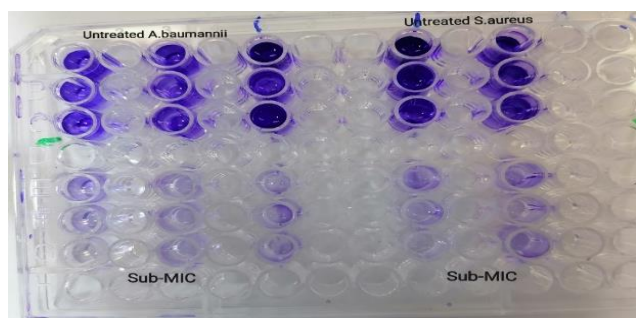


Fig. 6: Antibiofilm activity of AgNPs synthesized by methanolic leaf extract of *M. spicata*

### Anti-bacterial activity of silver nanoparticles against *S. aureus* and *A. baumannii* isolates

In this study,by agar well method. diffusion methods

### isolates

In this method, crystal violet and were used to determine the anti-biofilm activity of Silver nanoparticles by using AgNPs(SupMIC). The results of these tests showed the optical density of wells that contain AgNPs (SupMIC ) Approach to the optical density of the wells that do not contain bacterial isolates. This indicates that prevented the growth of bacteria and thus the absence of a biofilm in these wells. the effectiveness of biofilm inhibition of *S. aureus* and *A.baumannii* as shown in (table 2) and (figure 7). [24] where the Qader study was used the present finding agrees with the study of convolvulus and aloe vera that reduced biofilm formation ability

were performed to determine the effectiveness of Silver nanoparticles on *S.aureus* and *A. baumannii* isolates from Clinical cases, as these isolates were resistant to Ceftriaxone of cephalosporin.Silver nanoparticles showed anti-bacterial activity by measuring the inhibition zone around the wells a of AgNPs.

The results showed that the methanolic nano-extracts of *Mentha spicata* significantly inhibited the growth of *S.aureus* and *A. baumannii*. In this investigation when mixed with a solution of silver nitrate. The results showed that the methanolic nano-extracts of *Mentha spicata* had different activity in bacterial inhibition. figure (7 and 8). The highest inhibition results at 240 concentration were (32) for *S. aureus* and *A.baumannii* bacteria was (30), as

shown in Table 3 and figure (9) for damping diameters for concentrations. Included many significant studies on the antibacterial activity of silver nanoparticles have antibacterial activity against the tested pathogenic strains of *E. coli* and *S. aureus*, as well as antibacterial activity against other pathogenic bacteria [25].

No.	Con.	<i>S. aureus</i>	<i>A. baumannii</i>
1	240 µg/ml	32 mm	30 mm
2	120 µg/ml	26 mm	25 mm
3	60 µg/ml	23 mm	19 mm

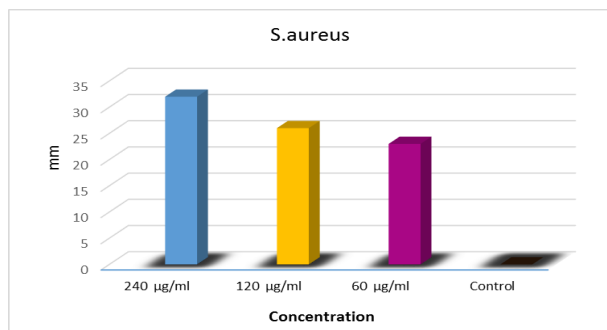


Fig. (7): Inhibitory activity of the nano methanol extract of *M. spicata* leaf against *S. aureus*

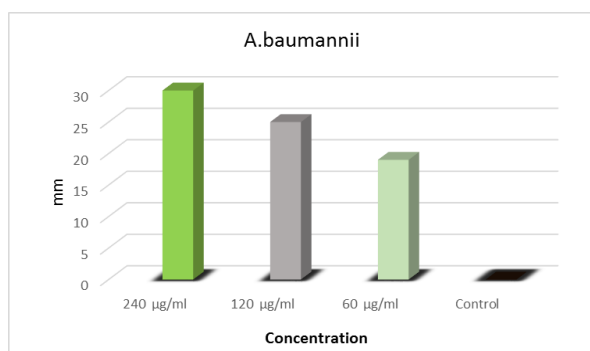


Fig. (8): Inhibitory activity of the nano methanol extract of *M. spicata* leaf against *A. baumannii*

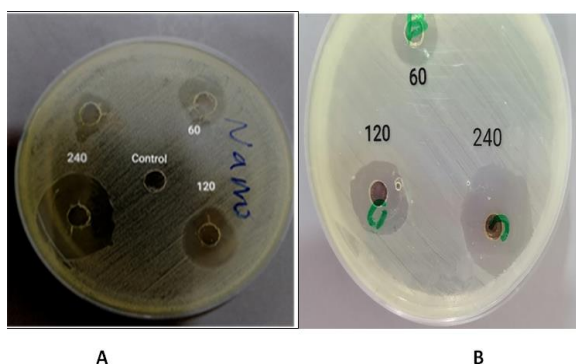


Fig. (9): Growth inhibition zones treated with silver Nano- methanol leaf extract of *M. spicata*: A - *S. aureus*, B- *A. baumannii*

## 4. Conclusion

Make use of the plant extracts of mint *spicata* leaves. As a reducing agent in the synthesis of green silver nanoparticles, it is a simple and environmentally friendly technology that can be used as an antibacterial and effective treatment in a promising future.

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