

DETECTION OF THE ANTIBACTERIAL ACTIVITY OF AGNPS BIOSYNTHESIZED BY *PSEUDOMONAS AERUGINOSA*¹ N. N. Hussein² A. H. Muslim

Assist. Prof.

Researcher

Departments of Applied Sciences, Biotechnology, University of Technology, Iraq

nehiahussein@yahoo.com

ameensoso77@gmail.com

ABSTRACT

Both bacterial isolates were obtained from Al-Elwiya Teaching Hospital for Children, Baghdad, Iraq. Clinical specimens were isolated from wounds and identified by Gram staining and by Vitek 2. Silver nanoparticles (AgNPs) were synthesized by *Pseudomonas aeruginosa*, and Characterized by UV-Vis spectroscopy (UV), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction(XRD). Antibacterial activity toward *S. aureus* was detected for the Silver nanoparticles that created by bacteria, Also the synergistic effect that will be detected between the antibiotics and nanoparticles(AgNPs). The highest effect was recorded in the case of the combination of (Doxycycline antibiotic with AgNPs) by a diameter of inhibition zone reached to 45.33 mm ,followed by 41.16 mm for the combination of (Clindamycin and AgNPs).Antagonistic effect also observed in the case of the combination of (Erythromycin with AgNPs) by inhibition zone reached to 16 mm. Minimum inhibitory concentration(MIC) of the biosynthesized AgNP detection for *S. aureus* was carried out with the following concentrations (0.005, 0.010, 0.015, and 0.020 mg/mL), the MIC of the biosynthesized AgNPs was 0.020mg/mL. About the detection of kill time for the biosynthesized AgNPs, the result showed that AgNPs required 120 min with 0.020mg/mL to kill the pathogenic bacteria *Staphylococcus aureus*.

Keywords:., *Staphylococcus aureus*, Silver nanoparticles, antibiotic disc, synergistic effect.

حسن ومسلم

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الكشف عن الفعالية المضادة للبكتريا لدقائق الفضة النانوية المخلفة حيويًا بواسطة *Pseudomonas aeruginosa*

أمين هادي مسلم

نهية نعمة حسين

باحث

أستاذ مساعد

فرع التقنيات الاحيائية، قسم العلوم التطبيقية، الجامعة التكنولوجية

المستخلص

تم الحصول على بكتريا الزئانفة الزنجارية *Pseudomonas aeruginosa* والمكورات العنقودية الذهبية *Staphylococcus aureus* معزولة من الجروح من مستشفى العلوية التعليمي للأطفال، بغداد، العراق. شخصت العينات السريرية وتم تحديدها بواسطة ملون غرام والاختبارات الكيموحيوية القياسية وفحص فائتك2. صنعت دقائق الفضة النانوية بواسطة بكتريا الزئانفة الزنجارية *Pseudomonas aeruginosa* ، وصفت من خلال التحليل الطيفي للأشعة فوق البنفسجية UV، التحليل الطيفي للأشعة تحت الحمراء FTIR وحيود الأشعة السينية XRD. تم الكشف عن فعالية دقائق الفضة النانوية المخلفة حيويًا المضادة للبكتريا تجاه بكتريا المكورات العنقودية الذهبية *Staphylococcus aureus*. استخدم مزيج دقائق الفضة النانوية مع اقراص المضادات الحيوية، في مزج المضادات الحيوية مع دقائق الفضة النانوية لوحظ أن هناك تأثير تآزري في معظم الحالات. تم تسجيل أعلى تأثير في حالة مزج المضاد الحيوي Doxycycline مع دقائق الفضة النانوية بقطر من منطقة التثبيط وصل إلى 45.33 ملليمتر ، يليه 41.16 ملليمتر لمزيج المضاد الحيوي Clindamycin ودقائق الفضة النانوية. ظهر تأثير تثبيطي لمزيج المضاد الحيوي Erythromycin مع دقائق الفضة النانوية حيث بلغ قطر منطقة التثبيط 16 ملليمتر. فيما يخص تحديد أقل تركيز مثبط للبكتريا باستخدام عدة تراكيز (0.005, 0.010, 0.015, 0.020 مايكروغرام/مل) فقد وجد بان أقل تركيز مثبط لنمو بكتريا *Staphylococcus aureus* هو 0.020 مايكروغرام /ملليمتر. تم ايضا تحديد وقت القتل بالنسبة الى دقائق الفضة النانوية المصنعة بواسطة البكتريا *Pseudomonas aeruginosa* وقد وجد بأن الدقائق النانوية تحتاج الى 120 دقيقة لغرض القضاء على البكتريا المرضية *Staphylococcus aureus*.

كلمات مفتاحية: اختبارات كيموحيوية، المكورات العنقودية، التحليل الطيفي.

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INTRODUCTION

Nanotechnology including nanomaterials synthesis and applications as developing field of nanoscience with important applications in biology and medicine due to their unique particle size and shape depending biological, chemical and physical properties (21). Silver is broadly used in nanotechnology applications since it has elevated toxicity toward pathogenic microbes (10). To overcome problem using toxic chemicals and high energy required physical procedures, the nanoparticles have been synthesized by biological materials which have been used for the synthesis of various metal and oxide nanoparticles. Hence, the biogenic approach, the usage of natural organisms or materials in particular, has offered a reliable, simple, nontoxic and eco-friendly method (11). In addition to different types of plants, many microorganisms, such as algae, fungi and bacteria have been used in nanoparticles synthesis (1). Biosynthesis of metal nanoparticles can take place either intracellularly or extracellularly (17). Extracellular biosynthesis is cheap and it requires a simpler downstream processing than the intracellular biosynthesis which requires additional steps such as ultrasound treatment or reactions with suitable detergents to release the synthesized nanoparticles (15). This favors large-scale production of silver nanoparticles to explore its potential applications. Because of this, many studies focused on extracellular methods for the synthesis of metal nanoparticles (7). With an increased surface area and a high proportion of surface atoms of AgNPs, the effectiveness of their antimicrobial activity increases compared with that of bulk silver metal (34). Among the metal nanoparticles, silver nanoparticles (AgNPs) have received much attention in different fields, such as therapeutics (23), antimicrobial activity (2), bio-molecular detection (31) and silver nanocoated medical devices (9). *Staphylococcus aureus* causes pyogenic disease, boils, carbuncles, impetigo, and surgical or accidental wounds and burns (13). *Staphylococcus aureus* has demonstrated a unique ability to rapidly respond to each new antibiotic with different resistance mechanisms (25). Therefore, antibacterial agents should be

synthesized from natural and inorganic substances (4), such as silver, which has been used since ancient times (27). Our work focused on AgNP synthesis by *P.aeruginosa* and evaluate their antibacterial activity against *S.aureus*.

MATERIALS AND METHODS

Isolation of bacteria: Bacterial isolates from contaminated wounds were obtained from Al-Elwiya Teaching Hospital for Children in Baghdad City. The isolates were diagnosed by gram stain and biochemical tests. These identified cultures were transferred to nutrient agar slants for preservation and then store in the refrigerator at 4°C (30).

Antibiotic efficacy assay for *S. aureus*: This was performed using Muller–Hinton agar (MHA, Difco USA.), inhibition zones were compared with standards to determine the susceptibility or resistance of organism to each antibiotic (33).

Synthesis of AgNPs by *P.aeruginosa*: Biosynthesized AgNPs were produced through the inoculation of *P. aeruginosa* on nutrient broth, incubation for 3 days, with shaking at 120 rpm at laboratory temperature, and centrifugation at 8,000 rpm for 10 min. The bacterial supernatant was used for the synthesis of AgNPs by adding it to 100 mL of 1mM aqueous silver nitrate with stirring at 200 rpm for 8 h at room temperature (19).

Characterization of biosynthesized AgNPs Characterization was performed using UV-visible spectroscopy, X-ray diffraction (XRD) and Fourier Transform Infrared (FTIR) spectroscopy (14).

Evaluation of the antibacterial activity of AgNPs: Nutrient broth was inoculated with *S. aureus* for 3 h at 37 °C, and the turbidity was compared with 0.5 McFarland standard. The culture (100 µL) was poured on the MHA and spread using a spreader and left for 10 min to settle down. Wells with 8 mm diameter were made using gel puncture. Nanoparticle solution (50 µL) was poured onto each well and incubated for 24 h at 37 °C. The inhibition zone diameter was calculated and recorded as mean ± SE of the triplicate experiment (19).

Estimation of the increase in fold region: The enlargement in fold region was assessed by determination of the inhibition zone produced by an antibiotic alone and in

combination with silver nanoparticles. Then four to five antibiotic discs were transferred to each plate of MHA and incubated at 37°C for 24 h with each plate prepared in triplicates. The increasing fold region was calculated using the following equation:

$$\text{Fold increase (\%)} = (b - a)/a \times 100$$

where *b* and *a* refers to the inhibition zones for the antibiotic with and without AgNPs, respectively (35).

Estimation of minimum inhibitory concentration: MIC for *S. aureus* by serial dilution method was determined according to (5,22). Sterilized brain heart infusion broth (0.8 mL) (Difco USA) was distributed in test tubes, inoculated with 0.1 mL of *S. aureus* culture and compared with 0.5 McFarland standard. Then 0.1 mL of the different AgNP concentrations (0.005, 0.010, 0.015, and 0.020 mg/mL) were added into all tubes, except the control tube. Normal saline (0.1 mL) was added instead of AgNPs. All tubes were incubated at 37 °C for 24 h. The result was recorded depending on the turbidity.

Kill time assay: Nutrient broth (10 mL) (Difco USA) added to test tubes and sterilized using an autoclave; then 0.1 mL of prepared AgNPs and 0.1 mL of fresh culture of *S. aureus* were added into the tubes, incubated in shaking incubator (150 rpm) at 37°C. Viable bacterial cells were counted at 0, 30, 60, 90,

and 120 min by spreading 0.1 mL of each tube on MHA and incubated at 37 °C for 24 h to detect the growth (6).

RESULTES AND DISCUSSION

Isolation of *P. aeruginosa* Bacteria: Gram negative *P. aeruginosa* rods with actively motile. Bacterial isolates were examined morphologically by Gram staining and subjected to biochemical tests for further confirmation. Vitek 2 (Bio MÉRIEUX, France) was used for diagnosis (24,28).

Antibiotic Susceptibility Assay For *S. aureus*: The antibiotic susceptibility test showed in (Fig.1) was used to detected the *S. aureus* sensitivity or resistance to antibiotics and were compared with the standard tables mentioned in (32). While *S. aureus* resistance to Carbencillin 100%. Doxycycline, Ampicillin, Ciprofloxacin had an inhibition rate of 29.83 mm, 26.83 mm and 26.16 mm respectively. Whereas Clindamycin, Chloramphenicol, Azithromycin, Amikacin Tobramycin, and Erythromycin showed inhibition zones 25.16 mm, 24.5 mm, 22 mm, 20.66 mm, 20 mm and 19.66 mm respectively, and were consistent with (3). Where Ciprofloxacin inhibits the work of the enzyme topoisomerase and thus prevents the construction of DNA, Amikacin, Tobramycin, Chloramphenicol, Erythromycin, Azithromycin and Doxycycline all are inhibiting the protein formation (16).

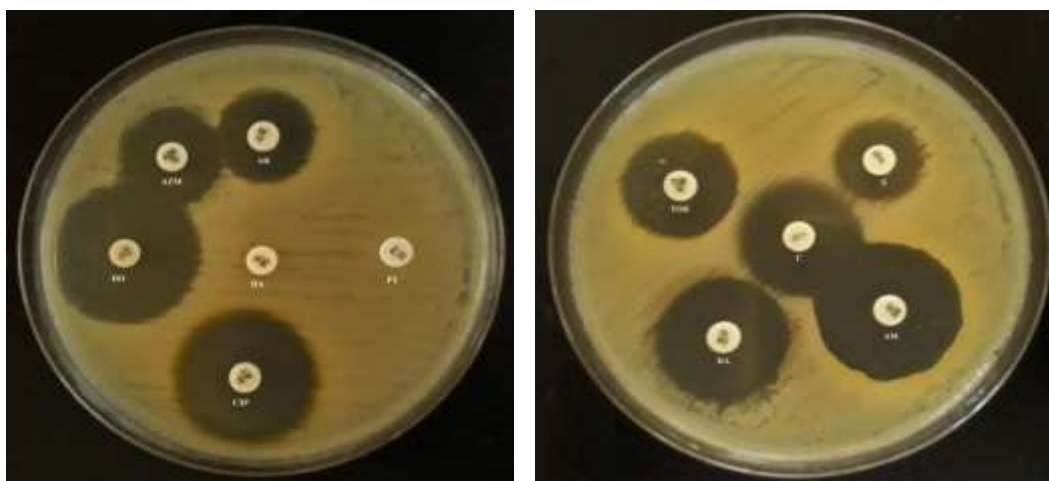


Fig. 1. Susceptibility test of antibiotics against *S. aureus*; CIP=Ciprofloxacin, PY=Carbencillin, AK=Amikacin,AM=Ampicillin,AZM=Azithromycin,E=Erythromycin,C=Chloramphenicol, TOB=Tobramycin, DO=Doxycycline and DA=Clindamycin.

Biosynthesis of AgNPs: The synthesis of nanoparticles in the medium was characterized by the changes in color from light yellow to

light brown (Fig.2). The color density increases with increase in incubation period due to the reduction of Ag⁰. In addition, the

synthesis of AgNPs depends on the incubation period of the culture as stated in previous studies (29).

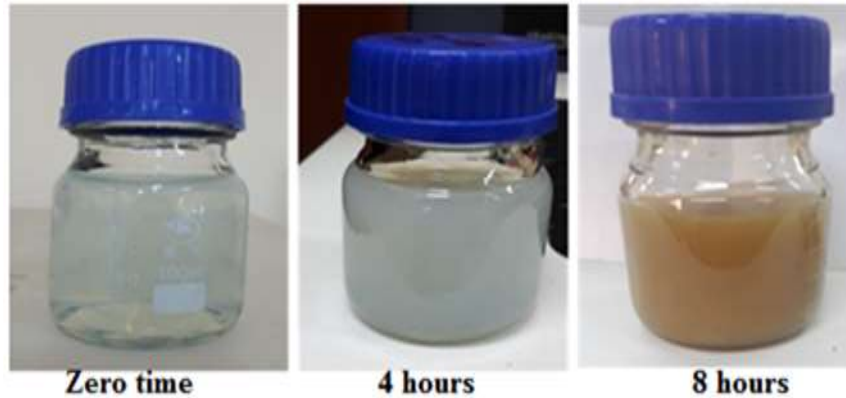


Fig. 2. Stages of AgNPs biosynthesis

Characterization of the Biosynthesized AgNPs UV-Visible spectra analysis
Reduction of silver ions into silver nanoparticles using bacterial cultures was evidenced by the visual change of color from yellow to brown due to excitation of surface plasmon vibrations in silver nanoparticles. The synthesized silver nanoparticles was then

characterized by UV spectrophotometer. The absorbance spectra of reaction mixture containing aqueous solution of 1mM silver nitrate and the pellet of *P.aeruginosa* after incubation. The band corresponding to the surface plasmon resonance at 410 to 430 nm. The strong peak at 420 nm (Fig.3) (14).

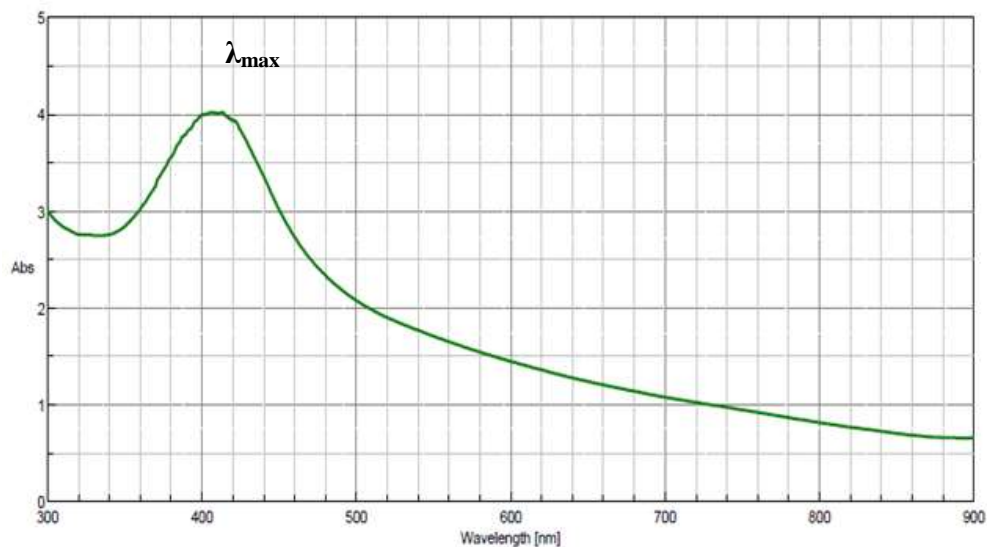


Fig. 3. UV-Visible spectra of biosynthesized AgNPs

X-ray diffraction analysis: XRD pattern shows three intense peaks in the total spectrum of 2θ values ranging from 20–60 (Fig.4). The AgNPs formed in this study were in the form of nanocrystals, as obvious from the peaks at 2θ standards of, 39.31, 44.73, and 56.93, respectively for silver. The particle size was 32.8nm, calculated by using Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos \theta}$$

where D refers to the crystallite size; K refers to the shape factor of crystallite, in which a good approximation is 0.9; λ is the X-ray wavelength; and B is the full width at half the maximum of XRD peak in radians; and θ is the Bragg's angle (14).

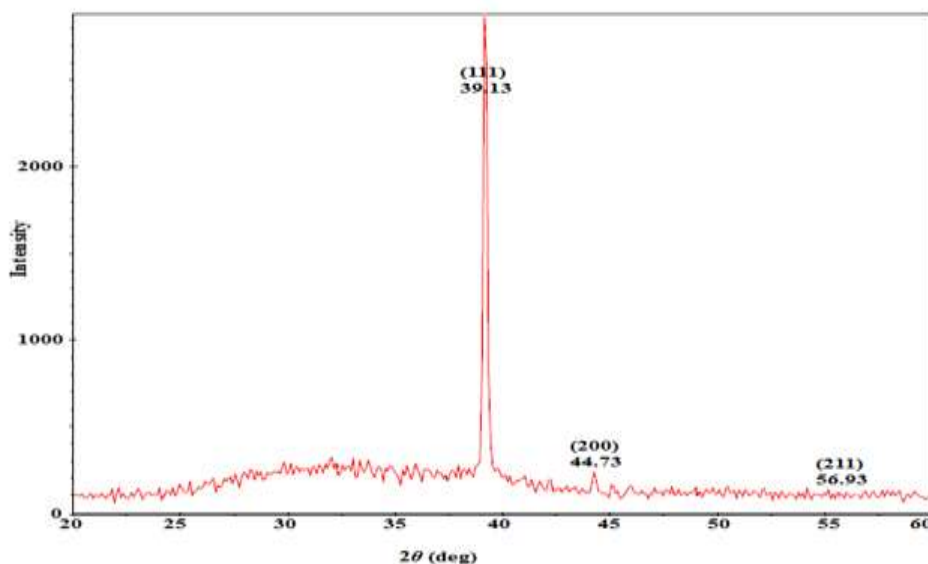


Fig. 4. XRD of biosynthesized AgNPs

Fourier transform infrared analysis: FTIR measurements were used to determine the possible interaction among silver salts and protein molecules, which can detect the reduction of silver ions and stabilization of AgNPs (Fig.5). The amide bonds among amino acid residues in proteins increased the well-known signatures in the infrared area of the electromagnetic range. The bands were observed at 3363, 2943, 2885, 1897, 1712,

1577, 1500, 1408, 1165, 1029, 75, 933, 802, 732, 705, 624, 601, 578, 543, 509, 486, and 455 cm^{-1} . The peaks at 3363.86, 2943.37, 1897.95, 1577.77 and 1408.04 cm^{-1} correspond to free O–H, C–H, C=O, C=N and C–N bonds, respectively. Obviously, protein was present in AgNPs. This protein may be attached with free amine groups or cysteine residues (35), which results in the stability of the nanoparticles.

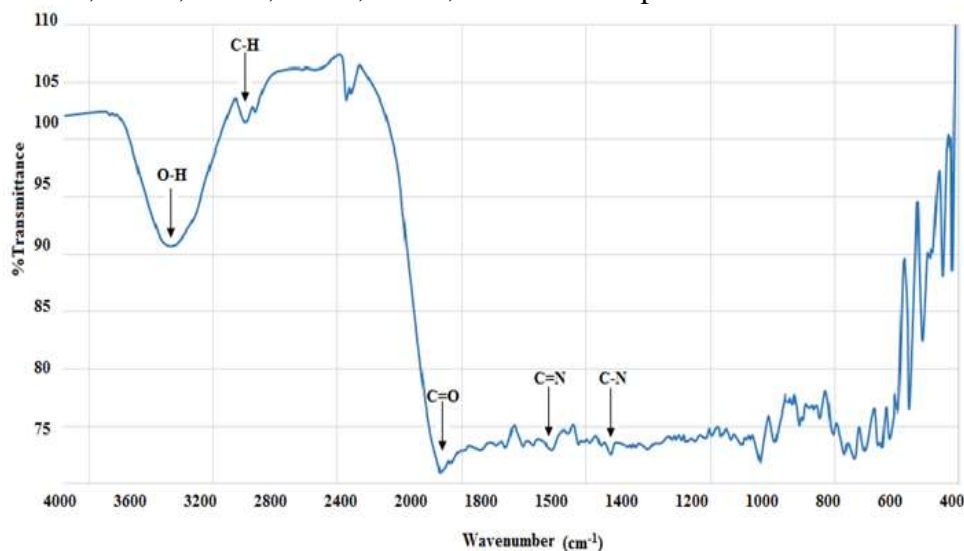


Fig. 5. FTIR spectra of biosynthesized AgNPs

Antibacterial Activity of biosynthesized AgNPs: The antibacterial activity of biosynthesized silver nanoparticles was examined towards *S.aureus* bacteria (Fig.6). The biosynthesized AgNPs effected on the growth of pathogenic bacteria by zone of

inhibition reached to (14mm).The antibacterial effect mechanism of AgNPs is poorly understood but they supposed that bacterial DNA replication capability loses and cellular proteins become inactivated after silver ion treated (18).

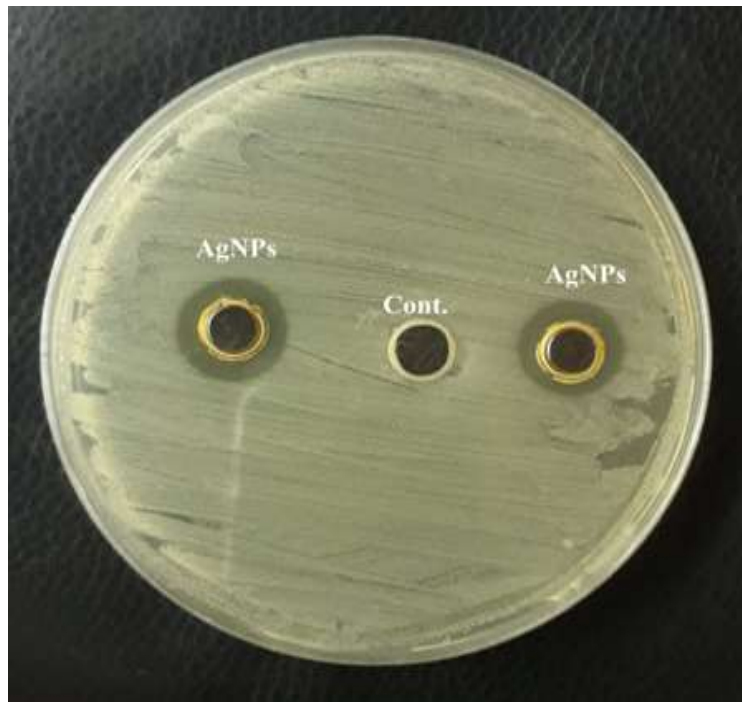


Fig. 6. Effect of AgNPs on *S. aureus* Cont = control D.W

Estimation of antibiotic resistance with and without combination of AgNPs: The antibacterial activities of the combined formulation of AgNPs through different antibiotic discs, including ciprofloxacin, carbencillin, amikacin, ampicillin, azithromycin, chloramphenicol, tobramycin, doxycycline, clindamycin, and erythromycin (Fig. 7). The results showed that resistance to antibiotics increased after treatment with AgNPs in the case of carbenicillin 100%. The results showed an increase in the effect of antibiotics with nanoparticles as in the case of

doxycycline, clindamycin, chloramphenicol, ciprofloxacin and ampicillin (45.33 mm, 41.16 mm, 37.83 mm, 34.33 mm and 34 mm, respectively) after the zone of inhibition prior to treatment with nanoparticles (29.83 mm, 25.16 mm, 24.5 mm, 26.16 mm and 26.83 mm, respectively). This is an evidence of a synergistic effect of the combination of antibiotics and nanoparticles. The increases in the fold area was detected by measuring the inhibition zone produced by an antibiotic before and after treatment with AgNPs (Fig.7 and Table 1) (8).

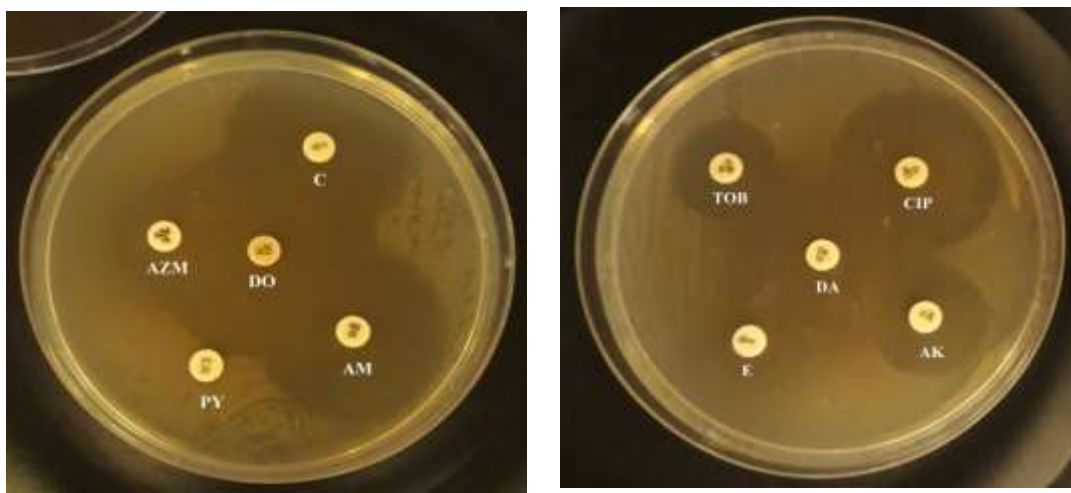


Fig. 7. Effect of antibiotics and biosynthesized AgNPs combination toward *S. aureus*.

Table 1. Inhibition rate of *S. aureus* by antibiotics alone, antibiotics with AgNPs, and increased fold

Antibiotics	Antibiotics effect (A)	Antibiotics with AgNPs effect (B)	Increase in fold area †
Carbenicillin	6 [‡]	6 [‡]	0
Erythromycin	19.66	16.00	-18.61
Amikacin	20.66	24.33	17.76
Tobramycin	20.00	21.66	8.3
Azithromycin	22.00	22.50	2.27
Chloramphenicol	24.5	37.83	54.4
Ciprofloxacin	26.16	34.33	31.23
Ampicillin	26.83	34.00	26.72
Clindamycin	25.16	41.16	63.59
Doxycycline	29.83	45.33	51.96

† Mean surface area of the inhibition zone was calculated for each tested antibiotic from the mean diameter. Fold increases for different antibiotics were calculated as $((b-a)/a)*100$, where *a* and *b* are the inhibition zones for A and B, respectively

‡ = In the absence of bacterial growth inhibition zones, the disc diameter (6 mm) were used to calculate the fold increase fold

In this study, the effects of AgNPs with many antibiotic combination were studied. Antibiotics, such as ciprofloxacin, carbencillin, amikacin, ampicillin, azithromycin, chloramphenicol, tobramycin, doxycycline, clindamycin, and erythromycin against *S. aureus* bacteria. The exact mode of action of AgNPs is still under investigation (10). AgNPs as other NPs may disrupt the bacterial cell wall formation and cause damage to the cellular proteins and nitrogenous bases in the bacterial components (12). The antibacterial effect of antibiotics in combination with AgNPs is enhanced due to the fact that antibiotics–AgNPs complex is formed, in which an AgNP is surrounded by antibiotics molecules (20). As a result, the effective number of molecules of antibiotics present at the site of action increased.

Determination of minimum inhibitory concentration: The MIC of the biosynthesized AgNP detection for *S. aureus*

was carried out with the following concentrations (0.005, 0.010, 0.015, and 0.020 mg/mL). After the incubation period, the result showed that the MIC of the biosynthesized AgNPs for *S. aureus* was 0.020mg/ML.

Kill time test: The kill time test of AgNPs was performed to estimate its bacteriocidal effect on the studied bacteria. The results showed that the time of killing of *S. aureus* by AgNPs was 120 min (Fig. 8). The result of this study showed that *S. aureus* bacteria required 120 min to be Killed with (0.020mg/mL) of biosynthesized AgNPs. The mechanism of the nanoparticles action on bacteria is not well understood, and may be due to the interaction between these particles and the groups of sulfur and phosphorus found in the bacterial cell membrane because the proteins of cell membrane are the preferred sites for the work of these particles, which lead to the destruction of the cell and death (12,26).

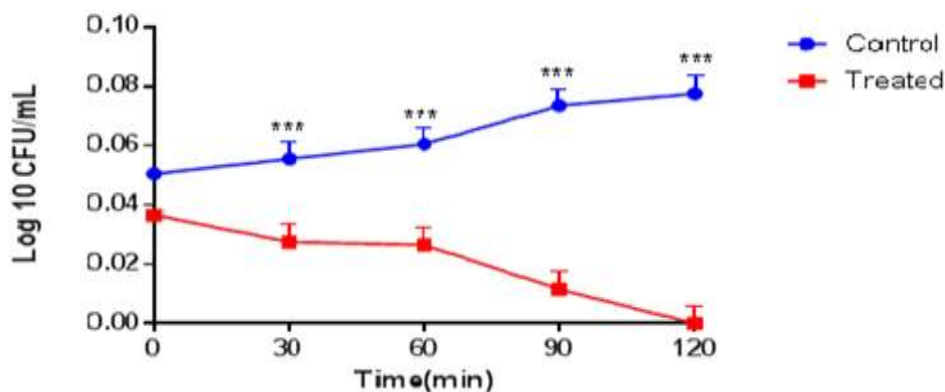


Fig. 8. Kill time of *S. aureus* by AgNPs (0.020mg/mL) Control=*S. aureus* without treatment; Treated=*S. aureus* treated with AgNPs. The values represents the Mean \pm SE, ***P< 0.001

The present investigation support the use of biosynthesized AgNPs by *P. aeruginosa* elicited a strong antibacterial activity. Thus, the biological approach could be a economical alternative to conventional chemical and physical methods of AgNP synthesis and would be suitable for the development of a biological method for commercial large-scale production. AgNPs have wide applications in various fields, such as antibacterials. Therefore, the improvement of their synthesis for nanoparticle production is the main objective of nanotechnology.

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