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THE EFFECT OF GOLD NANOPARTICLES IN GROWTH AND BIOFILM FORMATION OF METHECILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* MRSA ISOLATED FROM VARIOUS CLINICAL CASES

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Abstract

A total number of 15 methicillin resistant *Staphylococcus aureus* MRSA were collected from wounds, burns, boils and urinary tract infections at Al-Ramadi Teaching Hospital. Twelve antibiotics were used to establish resistance patterns of these isolates. Results showed that these isolates were sensitive to amikacin, while they were resistant to augmentin, rifampicin, ceftriaxone, cephalothin, erythromycin, methecillin, cefoxitin, oxacillin. These isolates showed different resistant percentage to other antibiotics like vancomycin, tetracycline, trimethoprim / sulfamethoxazole. Mice injected with concentrations (75, 150, 300, 600 and 1200) µg/ml that showed there is no toxic effect of Au-NPs, and no change in the weights, behavior or death. Gold nanoparticles showed an inhibitory effect against all isolates at concentrations of 74.87, 56.15, 37.43, and 18.71 µg/ml with inhibition zones of (38, 26.7, 18.4, and 9.2) mm, respectively. Results showed that gold nanoparticles reduced the ability of MRSA to produce the biofilm by using sub – MIC of 72.2–95%.

Key words : Gold nanoparticles, *Staphylococcus aureus*, methicillin resistance, biofilm, TEM.

Introduction

Staphylococcus aureus strains were first discovered in Britain in 1961 after nearly a year of methicillin use in the treatment of bacteria. These bacteria have spread in poorer communities due to direct contact with patients and medical instruments contaminated with these bacteria, as well as through contaminated burns and wounds (Male, 2011). Since then there has been no hospital in the world free of similar cases, and the rates of infection vary from one country to another and from one hospital to another. The difference in rates is clear, sometimes reaching 1%, while in others it is 50%. MRSA have been responsible for severe epidemics and acute pathogens, particularly among hospital patients and nursing homes (Holden *et al*., 2013). *Staphylococcus aureus* was a medical hazard 100 years ago, causing epidemics and fatal deaths from pneumonia, brain abscesses, meninges, septicemia and other deadly diseases (Etinosa *et al*., 2016).

Nanomaterial's have the potential in the solution of many biological problems and the interference of nanotechnology with biology crystallizing the science of Nano-biotechnology (McQuillan, 2010), it has been observed in recent years the rapid growth and advancement of nanotechnology and most of its applications in medicine. This improves the quality of life and the elimination of many of the problems we face in our lives (Chatterjee *et al*., 2011).

Microorganisms in nature tend to aggregation and stabilization in a biofilm more than they survive in individual cells because the biofilm provides more survival opportunities for microorganisms than single-cell. Resistance in biofilm for antibiotics and host immunity is higher than resistance of free living bacteria at rate of 1000 times (Dadawala *et al*., 2010).

The development and spread of antimicrobial resistance, as well as the emergence of new strains of pathogens, is a major concern for researchers, doctors, society and public health, therefore in this present research gold nanoparticles synthesized from reducing (chloroauric acid; $HAuCl_4$) with citrate and investigated their effect on growth, biofilm formation of methicillin resistance *Staphylococcus aureus*.

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Materials and Methods

Chemicals and cultures

In the present study, the chemicals used are Gold chloride (chloroauric acid; $HAuCl_4.4H_2O$), tri sodium citrate, tryptone soya broth and mannitol salt agar, blood agar purchased from Oxoid Ltd., England and sterile deionized water was used in this experiment.

Collection of clinical specimens

Samples were collected from the patients in the Ramadi educational hospital. Samples were cultured on the blood and MacConkey agar and incubated at 37°C for 18-24 hours. After growth, the samples were kept at 4°C until use. Then conducted Biochemical tests: In order to diagnose bacterial isolates, a number of biochemical tests were carried out in the approved diagnostic sources (Monica, 2009; Brown, 2007).

Fabrication of gold nanoparticles solution

The method of Mohammed *et al.* (2014) was adopted to prepare the solution of the gold nanoparticles at a concentration of 74.87 µg/ml and the concentrations were obtained (56.15, 37.43, 18.71, 9.35) µg/ml as follow: Firstly, a $HAuCl_4.4H_2O$ solution with the concentration of 0.49 mol/L was prepared by dissolving 605 mg of $HAuCl_4.4H_2O$ into 3 ml of 10% HCl, then, a diluted 0.2 mM of $HAuCl_4.4H_2O$ solution was made by adding 40 µL (19.6 µmol) of $HAuCl_4.4H_2O$ solution into 100 ml of deionized water as to produce solution A. Secondly, 559 mg of Trisodium Citrate was added into 50 ml of deionized water to make solution B. The concentration of the solution was controlled at 38.8 mmol/L. Solution A was brought to a rolling boil at 150°C with stirring vigorously as to get a homogenous size of the gold nanoparticles GNPs solution. 10 mL of 38.8 mmol/L of sodium citrate was added rapidly into the vortex of the solution. The solution resulted in a color change from pale yellow to red. Boiling and stirring was continued for another 10 min. The heating was then removed and stirring was continued for an additional 15 min. When the solution cooled down to room temperature, it was filtered through a 0.8 µm membrane filter paper. The prepared solution was kept in the refrigerator with the temperature 4ºC and measured by using UV-Vis at the wavelength 400-800 nm, TEM and XRD, FT-IR spectrum.

Toxicity of gold nanoparticles (Au-NPs) *in vivo*

Selection of animal species: thirty Balb/c male mice, age between (8-10) weeks, weight (20-25) gm obtained from national center for drug control and research-Baghdad and kept in animal house in biotechnology research center of Al-Nahrain University.

Housing and feeding conditions: the temperature of animal room kept at (19-22^{0}C) and humidity at least between (15-30%). Lighting was synthetic 12 hours light; 12 hours dark, for feeding laboratory diets was use unlimited supply of drinking water. Animals were distributed into six group-caged by dose.

Preparation of animals: the mice are adapted to conditions of laboratory for at least seven days before start of study. Mice are randomly selected and cages marked to identify six groups as follow: G1= control (without treatment), G2, G3, G4, G5, G6 groups with 75, 150, 300, 600, and 1200 µg/ml Au-NPs, respectively. Administration: the test Au-NPs was given intraperitoneal at concentrations that mentioned. The results were interpreted in relation to mice survival and observable toxicity and it became possible to assign including mortality nature severity, duration, of effects and weight of mice (Chairman *et al*., 2013).

Antibiotic sensitivity test

Twelve antibiotics (Amikacin AK, Augmentin AMC, Rifampicin RA, Ceftriaxone CRO, Cephalothin CEP, Erythromycin E, Methicillin ME, Cefoxitin CX, Oxacillin OX, Vancomycin VA, Tetracycline TE, and Trimethoprim/ Sulfamethoxazole SXT manufactured by Bioanalyse Company Turkey origin) were tested for efficacy against Methicillin Resistance *Staphylococcus aureus* bacteria depend on disk diffusion method described by Bauer *et al*. (1966).

Antibacterial activity of GNPs against MRSA and determination of MIC

The antibacterial activity of synthesized GNPs was evaluated using disk diffusion method proposed by Jarosz and Lipa (1990). Pure cultures of selected bacteria were sub-cultured individually in tryptone soya broth for 18 hours at 37°C. A 20 ml volume of sterile Mueller Hinton Agar medium was poured into each petri-plate and each isolate was swabbed uniformly into plates using sterile cotton swabs. Paper Disks from Whatman No.3 prepared by piercer means of a 6 mm diameter paper, sterilized with autoclave and impregnated with a gold nanoparticle solution for one hour, then left to dry thoroughly. Disks placed onto each bacterium inoculated agar plate by using sterile plastic forceps. The bactericidal activity was determined by a clear inhibition zone around the sample loaded well after incubation of plates overnight at 37°C. The minimum inhibitory concentration of GNPs was determined by the method of NCCLS (1997) by preparing serial concentrations of GNPs (74.87, 56.15, 37.43, 18.71, and 9.35 µg/ml) and the lowest concentration inhibits the growth of bacteria considered MIC.

Effect of gold nanoparticles on Biofilm formation by using micro-titer plate method

The method described by Dheepa *et al*. (2011) was used to estimate biofilm formation as follows :

$$\% \text{ of inhibition of biofilm formation} = 1 - \frac{\text{O.D of treatment}}{\text{O.D of control}} \times 100$$

The bacterial suspension was prepared and compared with the standard McFarland No. 0.5, then 5 ml from tryptone soya broth inoculated with a loopful of test organism from overnight culture grown on nutrient agar, incubated at 37°C for 24 hours. Tryptone soya broth that contain Sub-MIC of gold nanoparticles was inoculated with bacterial suspension and incubated for 24 hours at 37°C. 200 µl of the culture was transferred into polystyrene micro titer plate in three replicates in the vertical rows of the plate for each isolate served as control, and 200 µl of culture that containing the sub-MIC concentration of the gold nanoparticles was added then incubated at a temperature 37°C for 24 hours. After the incubation period, all the wells were washed with normal saline to dispose of the non-adherent bacterial cells, and 200 microliters of methanol (concentration 99%) were added to fix the adherence bacterial cells. Alcohol was poured and the plate left to dry, then stained with 1% crystal violet for 5 minutes. Excess dye was removed and left to dry at room temperature. 160 µl of glacial acetic acid 33 % was added, and absorption was measured by by ELISA reader with a wavelength of 630 nm to determine the efficiency of the isolates production of biofilm and Comparing with bacterial cells treated with gold nanoparticles. Percentage of inhibition of bacterial adhesion by application of the equation described by Gudina *et al*. (2010).

Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and was analyzed using Two-way ANOVA at 5% level of significance using computer software SPSS version 22.

Results and Discussion

Isolation and identification of *Staphylococcus aureus* isolates

Among 83 clinical samples, 15 isolates were found to be Methicillin Resistance *Staphylococcus aureus*; that appeared circular white to creamy colored with smooth edges and slightly higher on the surface of Nutrient agar. They were characterized by a heavy growth in staphylococci No. 110 medium that contain 10% sodium chloride. The microscopic examination showed gram positive cocci arranged as clusters and characterized as positive for catalase, negative for oxidase test and fermentation of glucose and *Staphylococcus aureus* was distinguished from other species belonging to Staphylococcus genus depends on mannitol salt medium and its fermentation of mannitol sugar by converting the color of medium from red to yellow, as well as its ability to produce coagulase, DNase, hemolysin enzymes and positive to acetoin test (Collee *et al*., 1996; Kloos and Schleifer, 1986).

Antibiotic susceptibility testing

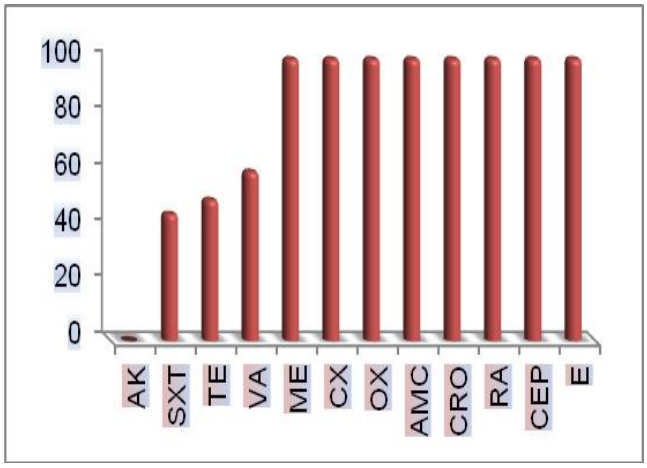
In this study, 12 antibiotics were used to establish resistant pattern of MRSA to several broad spectrum antibiotics that may associated with increased risk of MRSA infection. Results showed in graph (1) all isolates resistance for antibiotics (Methicillin, Cefoxitin, Oxacillin, Augmentin, Rifampicin, Ceftriaxone, Cephalothin, and Erythromycin). While they were resistance for Vancomycin, Tetracycline, Trimethoprim/ Sulfamethoxazole were (56.1, 48.8, 44%) respectively. As for Amikacin antibiotic, all isolates were 100% sensitive.

These isolates showed high resistance to the antibodies of the beta-lactam group, whether penicillins or cephalosporins, due to their production of the extended spectrum β-lactamase (ESBLs) (Shaikh *et al*., 2015). *Staphylococcus aureus* resistance to antibiotics may be due to mutations that lead to bacteria gaining resistance, loss of penicillin-binding proteins, lack of antibiotic permeability, or impact on DNA gyrase (Davies and Davies, 2010).

Synthesis of Gold Nanoparticles

Synthesis of gold nanoparticles was observed through a number of indicators as follows:

Color change : The results of present study showed



Graph 1 : Resistance of MRSA isolates to the antibodies.

that the gold nanoparticles (Au-NPs) formation by citrate reduction of the gold chloride solution, and appearance of red grape wine color (fig. 1). This chromatic change is caused by irritation of plasmon surface nanoparticles (Merza *et al*., 2012).

Absorption UV-light spectroscopy : The results of present study showed the production of Au-NPs by measuring the absorption spectra of UV-visible within the range (400-800) nm for gold chloride solution that used to prepare nanoparticles, which are important techniques for the detection of nanoparticles, the peak of absorption appeared at wavelength 520 nm (fig. 2), which represents the peak absorption of gold that agree with Haghshenas and Faraji (2016), Abalaka *et al*. (2014).

X-Ray Diffraction : Fig. 3 shows the X-ray diffraction spectra of the gold nanoparticles and observe the peaks of diffraction (111), (200), (220), (311) and (222) at angles (38.2°, 44.4°, 64.6 and 77.6 and 81.7, respectively). These angles were found to be close to the angles indicated with the JCPDS card and agree with Abdulghani and Mohuee (2015), Manivasagan and Junghwan (2015).

Transmission Electron Microscope (TEM) : Fig. 4 shows the shape of the gold nanoparticles in TEM with a magnification force of 46000 X, range of diameter between (17-25) nm. Particles are shown in clusters which confirm that nanoparticles are formed (Seoudi and Said, 2011).

Fourier Transform Infra-Red spectroscopy (FT-IR) : The infrared spectrum was recorded with wavelengths range between (500-4000 cm^{-1}). Fig. 5 that showing the absorption bands. A broad and heterogeneous band was observed at wave number (3261cm^{-1}) carboxylic hydroxyl group. The spectrum also exhibits two intense bands at (2853, 2920 cm^{-1}) for Au-NPs which is assigned to the symmetric and asymmetric stretching vibration of sp3 hybridized – CH^2 groups. The band around wave number 1733 cm^{-1}, a very strong and intense absorption band was observed due to the stretching vibration of the C = O group of ketones. At wave number (1531 cm^{-1}) showed a medium-intensity absorbance of C=C for aromatic group and a strong absorption band was observed at the wave number (1034 cm^{-1}) due to the stretching vibration of the C-O group.

Toxicity of gold nanoparticles (Au-NPs) *in vivo*

The result showed normal for the control and treated animals, and no significant changes observed in the body weight and no clinical signs change in (skin color, eyes, change in respiration and behavior) of toxicity observed

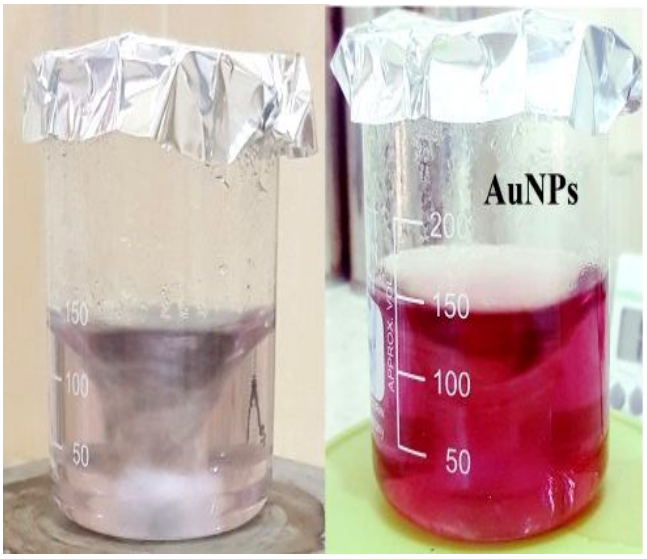


Fig. 1 : Color change of gold chloride solution and Au-NPs Synthesis.

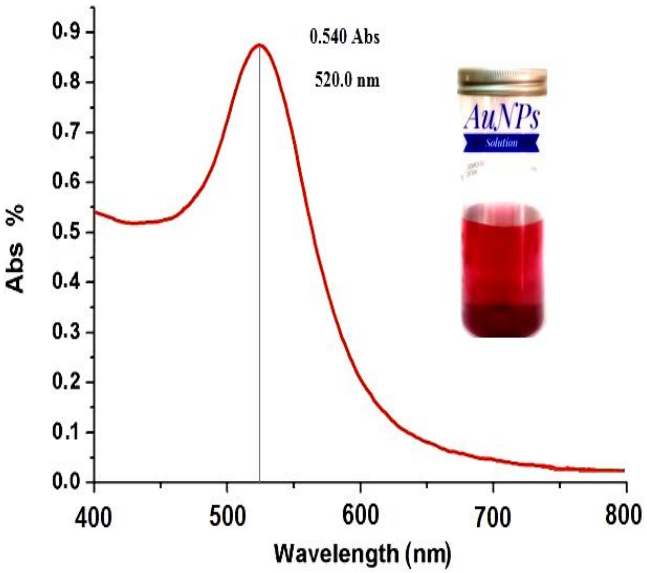


Fig. 2 : UV- visible light absorption of AuNPs solution.

for treated mice compared with control group, no mortality and toxic signs observed when different doses of Au-NPs were injected.

There are many probable ways to be exposed to Au-NPs including dermal contact, oral administration, inhalation, blood circulation, intraperitoneal injection etc. (Chen and Schluesener, 2008).

Typical acute effects are clinical signs of toxicity, abnormal body weight changes and/or pathological changes, in organs and tissues, which in some cases can result in death (Chairman *et al*., 2013). This result is agree with Jia *et al*. (2017) that show no any toxic signs after administration mice with difference concentrations of Au-NPs.

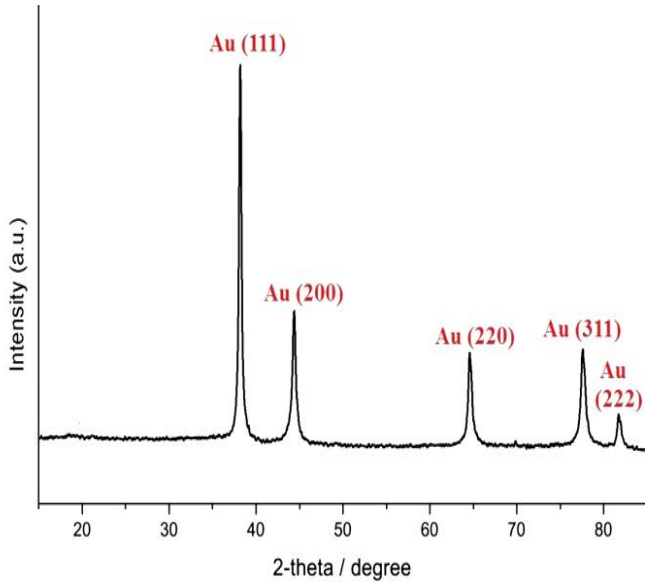


Fig. 3 : X-ray diffraction spectra of Au-NPs.

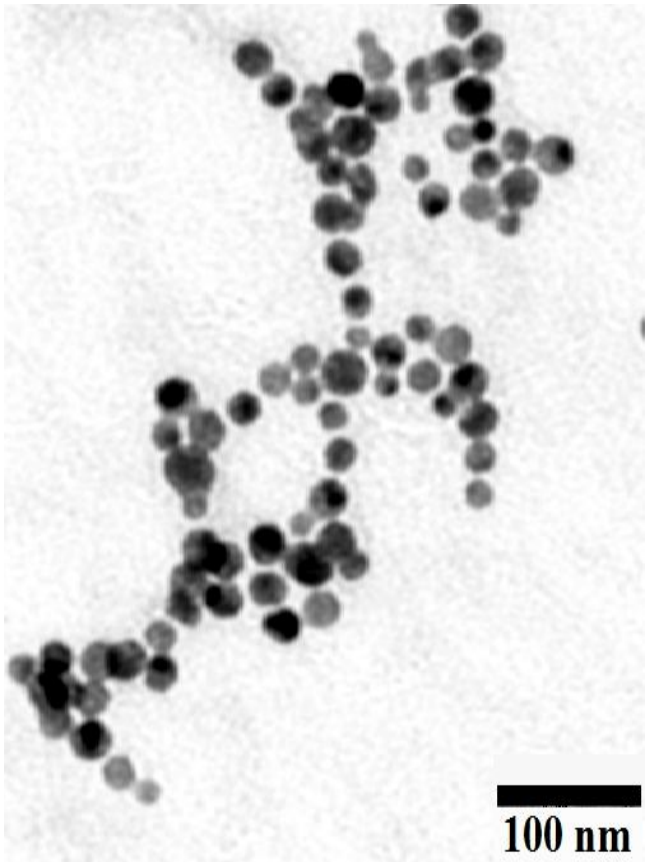


Fig. 4 : Shape of gold nanoparticles in TEM with 46000 X magnification force.

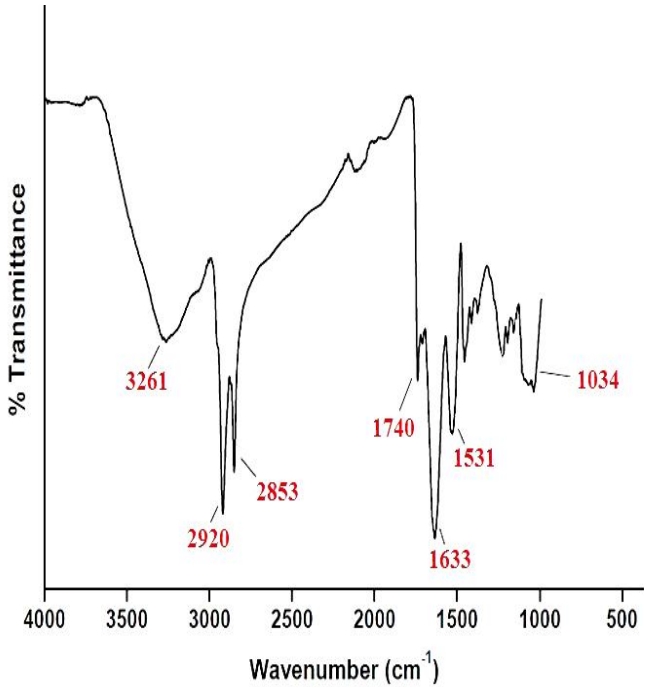


Fig. 5 : Fourier Transform Infra-Red spectroscopy (FT-IR) of Au-NPs solution.

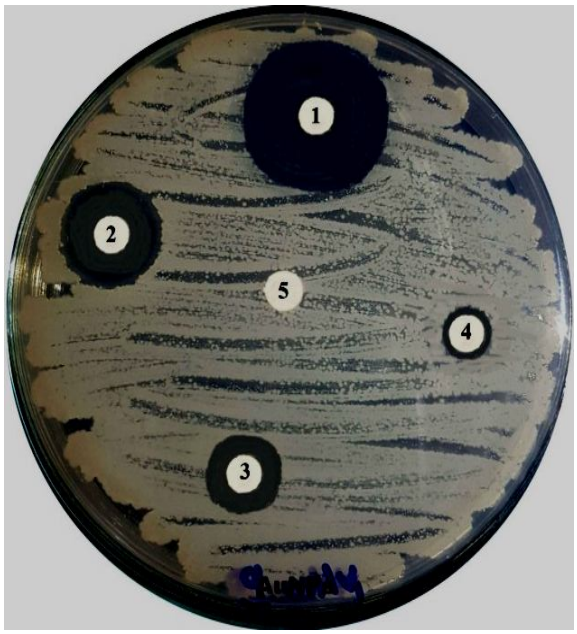


Fig. 6 : The inhibitory effect of gold nanoparticles with difference concentrations (74.87, 56.15, 37.43, 18.71, and 9.35) µg/ml against MRSA isolates

Antibacterial activity of Au-NPs against MRSA and their ability of biofilm formation

The results showed that gold nanoparticles were highly effective against MRSA as shown in table 1 and fig. 6 that show the diameter average of the inhibition zones (38, 26.7, 18.4, 9.2 and 0) mm at concentrations (74.87, 56.15, 37.43, 18.71, and 9.35 µg/ml), respectively.

The small size of the gold nanoparticles (Au-NPs) and their large surface area play important role in the toxicity. Whenever smaller size, accumulation is greater on the surface of the cells, which increases the toxicity against the bacteria through its effect on plasma membrane permeability leading to the death of bacterial

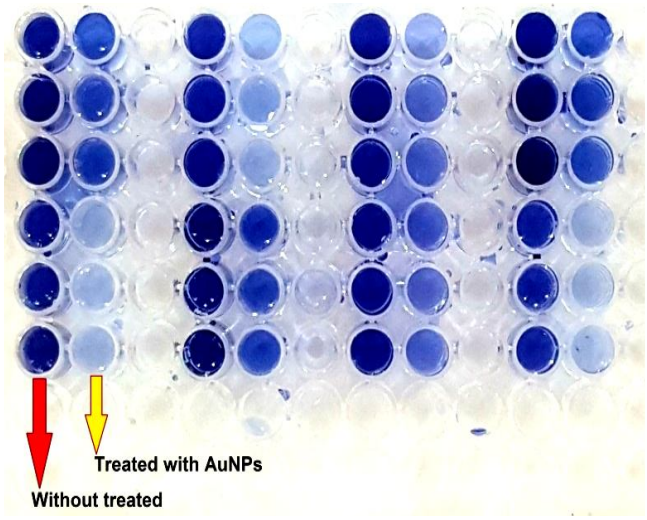


Fig. 7 : Anti-biofilm activities of Au-NPs against MRSA by Micro-titer plate (MTP) and crystal Violate staining method.

Table 1 : Effect of Au-NPs with different concentrations in MRSA growth.

Inhibition zone in mm (Mean ± SE)	Au-NPs concentrations (µg/ml)
38 ± 1.7	**74.87**
26.7 ± 1.3	**56.15**
18.4 ± 0.9	**37.43**
9.2 ± 0.6	**18.71**
0 ± 0.0	**9.35**

cell (Vimbela *et al*., 2017). The mechanism of nanoparticles action that interacts with bacterial cells, the bacterial cells have negative charges while the metal oxides have a positive charge, which creates electromagnetic attraction between the bacteria and particle surfaces. The particles release the ions that interact with thiol (-SH) group of transport proteins that emerge from the membrane of the bacterial cell and reduce the permeability of the membrane leading to bacterial cell death (Tauran *et al*., 2013). Gold nanoparticles inhibition mechanism of the ability of DNA multiplication and gene expression of proteins as well as various cellular proteins and enzymes, which are necessary in ATP production therefore become ineffective (Wang *et al*., 2017). Gold nanoparticles attack surface of the cell membrane, disrupt permeability and cellular respiration functions or interfere with system components of electron transport chain in bacteria (Dakal *et al*., 2016).

The results shown in table (2), figure (7) that Au-NPs have a high effectiveness in inhibiting the ability of MRSA biofilm formation, which was studied using Micro-titer plate assay method that consider the best and most reliable method of detecting biofilm production and the adhesion of bacteria.

Table 2 : Inhibition effect of Au-NPs on MRSA biofilm formation.

Isolates	Optical Density (O.D)		Inhibition of biofilm formation (%)
	Control without Au-NPs	Treated with sub-MIC concentration of Au-NPs	
S_1	0.158	0.0218	86.2
S_2	0.625	0.126	79.8
S_3	0.39	0.028	92.8
S_4	0.56	0.124	77.8
S_5	0.35	0.024	93.1
S_6	0.165	0.0271	83.5
S_7	0.273	0.0382	86
S_8	0.225	0.0367	83.6
S_9	0.713	0.153	78.5
S_{10}	0.864	0.24	72.2
S_{11}	0.285	0.014	95
S_{12}	0.227	0.019	91.6
S_{13}	0.318	0.02	93.7
S_{14}	0.166	0.021	87.3
S_{15}	0.280	0.025	91

Results shown differences in the inhibitory effect of Au-NPs on the bacterial isolates this is due to the difference in isolation site, environmental conditions that may cause appearance changes in the isolates, as well as the difference in the physiological activity of each isolate due to the difference in their genetic structure, which in turn reflects their different metabolic activities and enzyme activity. Au-NPs greatly attenuated the ability of MRSA to biofilm production. This is due to the ability of Au-NPs to penetrate the bacterial cell and the interaction with the proteins and enzymes responsible for adhesion and quorum sensing, leading to a reduction in the ability of bacteria to biofilm production (Haibo *et al*., 2016). On the other hand, Au-NPs inhibit biofilm production by preventing the formation of polysaccharides, and the small size of Au-NPs can penetrate biofilm matrix and communication between bacterial cells causing the inhibition of biofilm (Ansari *et al*., 2014).

Conclusion

The high level of antibiotic resistance among MRSA causing several diseases use of antimicrobial agents for treatment and also the spread of Multi Drug Resistance isolates is a threat for hospitalized patients. This study showed a significant relationship between biofilm

production of MRSA isolates and some antibiotic resistance. Thus, bacterial biofilms can play very an important role in resistance to antibiotics. In present study that gold nanoparticles no toxic effect against mice and have the high potential to inhibit growth of MRSA bacteria and reduce bacterial ability to biofilm production for 95%. Concentration plays a very important role, increasing concentration increases the inhibition zone.

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