THE SYNERGISTIC EFFECT OF GOLD NANOPARTICLE LOADED WITH CEFTAZIDIUM ANTIBIOTIC AGAINST MULTIDRUG RESISTANCE PSEUDOMONAS AERUGINOSA

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ABSTRACT
This study was aimed to evaluate the antimicrobial activity of gold nanoparticles that was synthesized by biological method using Aloe Vera extract. The Surface morphology of the synthesized gold nanoparticles was confirmed by Atomic force microscope (AFM) while the nature of functional groups present in gold nanoparticles was determined by FT-IR analysis. The antibacterial activity of gold nanoparticle was tested against multidrug resistance (MDR) pseudomonas aeruginosa, the results showed a significant effect against MDR isolates. Gold nanoparticle was loaded with ceftazidium antibiotic in order to improve the antibacterial activity and drug delivery efficiency. The synergistic effects of biosynthesize gold loaded with ceftazidium antibiotic at different concentration against MDR bacteria were also investigate. The result showed that ceftazidium-loaded nanoparticles have superior effectiveness compared to native ceftazidium against pseudomonas aeruginosa.

Keywords: Nanoparticles, Antimicrobial effect, biological method.

المستخلص
ركزت هذه الدراسة على تقييم الفعالية ال ضد بكتريا ceftazidime لدقائق الذهب النانوية مع المضادات الحيوية MDR pseudomonas aeruginosa. حيث أن النتائج تأثيراً كبيراً على هذه العزلات. ثم تم تح🏻يم جزيئات الذهب بالمضاد الحيوي ceftazidium في الدهس. أظهرت النتائج أن المضاد الحيوي المحمل بواسطة الدقائق النانوية لديه تأثير كبير مقارنة مع المضاد وحده ضد بكتريا pseudomonas aeruginosa.

الكلمات المفتاحية: الدقائق النانوية، الفعل الضد ميكروبي، الطرق النباتية
INTRODUCTION
A wound (burn wounds) infections are considered one of the most common causes of serious problems worldwide. (10) it can be defined as a damage of the protective skin barrier that normally prevents bacterial invasion making burn wound the most frequent origin of sepsis. (26) (3). One of the most common pathogens that are colonized burns wounds are *Pseudomonas aeruginosa*, they are found everywhere in water, soil and moist environment and have the ability to adapt to different environmental conditions. (22). The ability of *Pseudomonas aeruginosa* to cause severe wound infections are related to presence of multitude of pathogenicity factors during infections and resistance to antibiotics. (9). Some of these virulence factors are structural constituents and others are secreted or directly injected into host cells. Among structural constituents, *P. aeruginosa* flagellum and pili are responsible for motility and bacterial adhesion to host cells. In addition, the outer membrane of *P. aeruginosa* contain lipopolysaccharide (LPS), a complex glycolipid, and lectins (LecAand LecB) that are also contributed to its pathogenicity Nanotechnology is increasingly being utilized for clinical applications, especially as a new technology for infectious diseases treatment. (14) (4) (13). Antimicrobial agent as a gold nanoparticles, has been reported to possess antimicrobial activities. (11). GNPsw with antibacterial have also emerged as an alternativeways to high-dose administration of antibiotics and proven their effectiveness against infectious diseases including antibiotic-resistant ones. (16). This study aimed to synthetize gold nanoparticles by biological method and evaluate their antimicrobial activity against multi-drug resistance bacteria which were isolated from burn wounds. As well as study the synergistic effect of gold nanoparticle loaded with ceftazidium antibiotic with different concentrations against multidrug resistance *pseudomonas aeruginosaisolates.*

MATERIALS AND METHODS
Samples collection
One hundred and fifty samples were collected randomly from burn wounds. During the period of sep 8th to feb 2nd from different hospitals (Al- swairah hospital, Medical City, Alzahraa hospital, Al yarmook hospital and Al-Karama Hospital). Prevalence of bacteria was found to be in 122 isolates from 150 samples.

Isolation and Identification of bacterial isolates
The identification of *pseudomonas aeruginosa* isolates were confirmed by culturing in selective cultural media and incubated at 37 °C for 24 hrs. under aerobic conditions, and biochemical tests(Catalase test, Oxidase test, Indole test, Urease production test, Citrate utilization test and Kligler’s Iron agar). Other Biochemical tests using the API 20 E were employed to confirmed the diagnosis for *pseudomonas aeruginosa*.

Antibiotic susceptibility testing
The susceptibility of pseudomonas aeruginosa isolates were carried out by Kirby-Bauer’sdisk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines. (5) one to three colonies from *pseudomonas aeruginosa* isolates were grown over night on Müller-Hinton agar at optimal incubation temperature 37°C for 24 h. Cultures of *P. aeruginosa* were adjusted to 0.5 McFarland standards and streaking method was used to plated the bacterial suspension on Müller-Hinton agar by sterile swab. The outcomes were communicated as sensitive (S) or resistant (R) as indicated by the criteria prescribed by the (CLSI) 2018. The following antibiotics were tested: Ciprofloxacin, azithromycin, cefoxitin, clindamycin, Gentamicin, Rifampin, Tetracycline and chloromphenicol. All Antibiotics were used in this study were purchased from Himedia, India.

Preparation of gold nanoparticles by using aloe vera extract
Gold nanoparticles was prepared in two-step based on biological method already mentioned in (23) with a little modification according to ideal preparation. Twenty five gram of aloevera were collected and cut into small pieces, then dispensed in 10 ml of sterile distilled water and boiled for 10 minutes at 70-80°C, then filtering and centrifuging the extract and stored at 4°C - 0.2 ml of 1.5 mM Aqueous chlorauric acid (HAuCl4) solution was added to 10 ml of extract at ratio 1:5 and put the solution in the stirrer for 2 hr. Within a
particular time (overnight), the color of the solution changed from yellow to red color which depends upon the extracts of plants and species. The gold nanoparticles so prepared were stabilized in dark place.

Characterization of prepared nanoparticle
3D surface topography is provided by Atomic Force Microscopy (AFM) which measures relies on Van der Waals or other attractive and repulsive forces (12). 5 drops of gold nanoparticle has been added at glass slide and leave it until drying and precipitated on it. On the other hand FTIR is another characterization tool for obtaining the nature of functional groups present in gold nanoparticles.

Minimal inhibitory concentration
Inoculum preparation: The bacterial inoculum was prepared according to CLSI instruction: A loop full of single bacteria isolate was inoculated in 10 ml tube of Muller Hinton broth and incubated overnight for activation, after 24 hours the bacterial suspension compared with McFarland tube to obtain culture with 1.5*10^8 CFU/ml, which was confirmed with a spectrophotometer at absorbance of (600 nm), and absorbance between (0.08-0.1) was acceptable.

Antibacterial activity of gold nanoparticles
The antibacterial activity of GNPs was evaluated by using Different concentrations (250, 125,62.5,31,2,15.6, and 7.8) µg/ml and plate count method(17). one mL from the previously prepared culture medium was added into each tubes, then 1 ml of Au NPs were added in each tubes in the following concentration(250, 125,62.5,31,2,15.6, and 7.8)µg/ml for control negative 1 ml of normal saline was added to 1mof bacterial inoculum in another tube. finally 1ml of inoculum bacteria added to each tube. The tubes were incubated in a shaking incubator at 37C for 24 h. After incubation, 100 µl from each tube was spread onto MHA and incubated at 37C for 24 h; the numbers of colonies growing on agar were estimated.

MIC of ceftazidium
The Minimum Inhibitory Concentration Assay was performed by preparing of Serial dilutions of the antibiotic (representing different concentrations of the antibiotic16, 32, 64, 128) µg/ml according to (CLSI). that were added to a growth medium(prepared as mentioned above) in separate test tubes. These tubes are then inoculated with the pseudomonas aeruginosa then incubated at 37 c for 24h

Preparation of gold NPs loaded with ceftazidium antibiotic (synergistic effect)
The ceftazidium antibiotic prepared at different concentration (16, 32, 64, 128) µg/ml according to (CLSI). Then, all concentrations of ceftazidium added to each concentration of gold nanoparticles (250, 125, 62.5,31.2,15.6 and 7.8) µg/ml respectively.

RESULTS AND DISCUSSION
Isolation and Identification of bacterial isolate
The results of bacteriological examination for 150 samples (from wounds and burns), cleared that 122 samples gave a positive bacterial growth ,and out of 122 bacterial isolates, 56 isolates showed a green-blue color colonies with a sweet grape-like odor of Pseudomonas aeruginosa. To confirm the diagnosis of Pseudomonas aeruginosa isolates after the work of classical diagnosis on culture media and biochemical tests, the Api 20E system was used.

Antibiotic susceptibility testing
The results of antibiotic susceptibility test showed that all isolates were appeared high resistance to B-lactam group ceftazidium(76%), azetroinem(71.4%) and imipenem (59%) while the isolates showed low resistance to piperacillin(21%) . Our study is consistent with(25) who mentioned that P. aeruginosa isolates were mostly resistant against aztreonam (86.7%) but inconsistent with him in piperacillin rate (93.3%), Also, (19) reported that many isolates of P. were resistant to used antibiotics, aztreonam (80.2%), and ceftazidime (74.8%). Other studies recorded variable rates of piperacillin resistance 69.9% and 75%(15)(1). The resistance to Fluoroquinolones including ciprofloxacin was (75%). In Saudi Arabia, resistance to ciprofloxacin was 50.9%, (2). Comparable rates were also reported from Iran (58%), (7). In addition, the isolates showed high resistance to colistin in percentage 83.9%. While many other studies recorded high sensitivity to colistin in 96%-100%(27) (18), However, increasing
administration of colistin for treat the infections caused by MDR organisms may lead to the emergence of colistin-resistant strains in some countries.

**Fig1. Percentage of Antibiotic susceptibility patterns of Pseudomonas aeruginosa**

**Characterization of prepared gold nanoparticle**

**Atomic force microscope (AFM):** Atomic force microscope (AFM) was used to know the surface morphology and to determine topography in addition it was chosen as imaging method which provides nanometer resolution and three-dimensional surface imaging, requires minimal sample preparation and allows imaging in ambient and liquid conditions. The (AFM) gives a two and three-dimensional image of the surface of nanoparticles at an atomic level.

**Figure2. Atomic Force Microscopy of gold nanoparticle illustrate 2D and 3D topological of gold nanoparticles**

**FT-IR analysis**

FTIR measurements were performed to identify the potential biomolecules in Aloe Vera responsible for the reduction capping and efficient stabilization of the bio-reduced gold nanoparticle. FT-IR spectrum of Aloe Vera aqueous extract shows different bands positioned at 3402.20, 2929.67, 1741.60, 1568.02, 1423.37, 1041.49 and 460.96 cm⁻¹ bands. The absorption band at 3402.20 cm⁻¹ is related to the symmetrical and asymmetrical hydroxyl functional group in alcohols and...
phenolic compounds. And 2929.67 cm\(^{-1}\) band is also characteristic of the presence of aliphatic (-CH) groups in these compounds. The band at 1741.60 cm\(^{-1}\) is characteristic of C=O stretching indicates the presence of Carbonyl groups that present in ketones, aldehydes, and carboxylic acids (24). And, the 1568.02 cm\(^{-1}\) band is attributed to amine groups, while 1423.37 cm\(^{-1}\) related to the symmetric bending of CH\(_3\), 1041.49 is corresponded to –coc group. The peak at 470.63 cm\(^{-1}\) correspond to stretching vibration of amine groups. The peaks of gold nanoparticle were 3444.63-3460.06, 2923.88, 737.74, 1639.38, 1546.80, 1460.01, 1141.78, and 491.81 cm\(^{-1}\). FTIR analysis showed shifting in carboxyl group from 3402.20 cm\(^{-1}\) to 3444.63-3460.06 cm\(^{-1}\), carbonyl group at 1741.60 cm\(^{-1}\) to 1737.74 cm\(^{-1}\) that is attributed to binding of aldehydes/ketones with the gold surface, also shifting the peak of amine group from 1568.02 to 1546.80 cm\(^{-1}\), also CH\(_3\) group 1423.37 to 1460.01 cm\(^{-1}\). These shifting or replacement and deleting of some peaks mean that aloe Vera made up as a capping agent for synthesis Au NPs and confirmation the formation of Au NPs. This observation is similar to (21) who report that on gold NPs synthesis using aloe vera extract, phenolic and carbonyl groups were found to play an important role in the stabilization and capping of the gold NPs.

Figure 2. The Fourier transforms infrared (FT-IR) spectroscopy measurement of aloe vera

Figure 3. The Fourier transforms infrared (FT-IR) spectroscopy measurement of gold nanoparticle
The antibacterial activity of gold nanoparticles: The antimicrobial activity of gold nanoparticle was examined against pathogenic *Pseudomonas aeruginosa* isolate which were selected depending on the susceptibility testing because it showed a highly resistance rate to many antibiotics. The results showed that the high concentration (250 µg/ml) gave the highest antibacterial activity by killing of about 77.1% of *Pseudomonas aeruginosa* isolates. While the lowest concentration (7.8 µg/ml) gave also a good antibacterial activity by killing about the half of bacterial growth. In addition, other NPs concentrations gave antibacterial activity ranged between (68.2% for 125 µg/ml NPs), (65.5% for 62.5 µl NPs), (53.41% for 31.2 µg/ml NPs), and (49.9% for 15.6 µg/ml NPs).

**Table 1. Effect of different concentration of gold nanoparticle and percentage death for each one for *Pseudomonas aeruginosa***

| NPs µg/ml | % of dead bacteria |
|-----------|--------------------|
| 250       | 77.1%              |
| 125       | 68.2%              |
| 62.5      | 65.5%              |
| 31.2      | 53.41%             |
| 15.6      | 49.9%              |
| 7.8       | 47%                |
| control   | Growth             |

**Figure 4. Antibacterial activity of gold nanoparticle at different concentration**

Among these different concentration, 250 µg/ml NPs concentration was considered the best antibacterial concentration. NPs are considered as next-generation antibiotics and have been shown to exhibit activity against gram-positive and gram-negative bacteria. AuNPs did not directly interact with the bacterial cell membrane but permeate across cell membrane and induced membrane potential disturbance. However, mechanism of antibacterial activity of GNPs are various, such as disturbance of membrane structure and function, inhibition of DNA replication, and inhibition of protein synthesis and energy metabolism. Gold nanoparticles (AuNPs) represent a revolution in drug delivery, and are considered safe and non-toxic antimicrobial agents.

**Figure 5. The antibacterial activity of gold nanoparticle against *Pseudomonas aeruginosa***

A: negative control with high concentration gold nanoparticle (250 µg/ml)

B: negative control with low concentration gold nanoparticle (7.8 µg/ml)
The synergistic effect of gold nanoparticle loaded with ceftazidime antibiotic: The results of synergistic effect to determine the potency of the combination of AuNPs and antibiotics showed that the bacterial cells are completely inactivated with no growth, this mean the efficiency of gold nanoparticle when combined with antibiotic.

Table 2. synergistic effect of different concentration of gold nanoparticle with different concentration of ceftazidium for Pseudomonas aeruginosa

| Gold conc µg/ml | Conc 1 of antibiotic µg/ml | Conc 2 of antibiotic µg/ml | Conc 3 of antibiotic µg/ml | Conc 4 of antibiotic µg/ml | Percentage of death |
|-----------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------|
| 250             | 16                          | 32                          | 64                          | 128                         | 100%                 |
| 125             | 16                          | 32                          | 64                          | 128                         | 100%                 |
| 62.5            | 16                          | 32                          | 64                          | 128                         | 100%                 |
| 31.2            | 16                          | 32                          | 64                          | 128                         | 100%                 |
| 15.6            | 16                          | 32                          | 64                          | 128                         | 100%                 |
| 7.8             | 16                          | 32                          | 64                          | 128                         | 100%                 |

However, many researchers were mentioned that the conjugated of GNP with antibiotics showed synergistic effects against bacteria, prohibit biofilm formation, and have been utilized to combat MDR bacteria. In addition, NPs possess antimicrobial activity that can overcome common resistant mechanisms, including enzyme inactivation, decreased cell permeability, modification of target sites/enzymes, and increased efflux through overexpression of efflux pumps, to escape from the antibacterial activity of antimicrobial agents (4).

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