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

O. S. HAMID **S. S. MAHMOOD**
Researcher **Prof.**
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.
Suhadsaad22@gmail.com

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 investigated. The result showed that ceftazidium-loaded nanoparticles have superior effectiveness compared to native ceftazidium against *pseudomonas aeruginosa*.

Keywords:Nanoparticles ,Antimicrobial effect, biological method .

مجلة العلوم الزراعية العراقية -2021 :52 (4):828-835 **حامد ومحمود**

الفعل التآزري لدقائق الذهب النانوية مع المضادالحيوي ceftazidime ضد بكتريا *Pseudomonas aeruginosa* متعددة المقاومة للمضادات*الحيويه*

علا سرمد حامد **سهاد سعد محمود**
باحث **أستاذ**
قسم التقنيات الاحيائية–كلية العلوم –جامعة بغداد–بغداد– العراق

المستخلص

ركزت هذه الدراسة على تقيم الفعالية الضد مايكروبيةلجزيئات الذهب النانوية المحضره بطريقةالحيوية بأستخدام مستخلص الصبار ثم التأكد من المظهر الخارجي للدقائق النانوية المحظرة بواسطة(AFM) بينما المجاميع الفعالة الموجوده في الذهب تم تحديدها بواسطة (FTIR) ثم دراسة الفعالية المضادة للذهب ضدالعزلات البكتيرية المتعددة المقاومة لمختلف المضادات الحياتيه للبكتريا *Pseudomonas aeruginosa* .حيث اظهرت النتائج تأثيرا كبيرا على هذه العزلات.ثم تحميل جزيئات الذهب الناوية بالمضاد الحيوي ceftazidium لغرض تحسين الفعالية المضادة للبكتريا وكفاءة نقل الدواء .ثم التحقق ايضا من الفعل التأزري لدقائق الذهب النانويةمحملة بالمضاد الحيوي ضد العزلات البكتيرية المتعددة المقاومة لمختلف انواع المضادات الحيويه.واظهرت النتائج ان المضاد الحيوي المحمل بواسطة الدقائق النانوية له تأثير كبير مقارنة مع المضاد وحده ضد بكتريا *Pseudomonas aeruginosa*.

الكلمات المفتاحية:الدقائق النانوية ,الفعل الضد ميكروبي ,الطرق النباتية

Received:14/7/2020, Accepted:11/10/2020

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 invasionmaking 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). GNPs 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 synthesize gold nanoparticles by biological method and evaluate their antimicrobial activity against multi-drugresistance 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 aeruginosa*isolates.

MATERIALS AND METHODS

Samples collection

One hundred and fifty samples were collected randomly from burn wounds . During the period ofsep 8th to feb2nd 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 andKligler's Iron agar).Other Biochemical tests using the API 20 E Were employed to confirmed the diagnosis for *pseudomonas aueruginosa*.

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 aerugi*nosa 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 usedto 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 purchasedfromHimedia , 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 ofsterile distilled water and boiled for 10 minutes at 70-80°C,then filtering and centrifuging the extract and stored at 4°C.2 ml of 1.5mM Aqueous chloroauric acid (HAuCl4) solution was added to 10 ml of extract at ratio 1:5.and put the solution in the stirrer for2 hr, Within a

particular time(overnight) ,the color of solutionwas changed from yellow to red color which is 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

Inoculumpreparation: The bacterial inoculum was prepared according to CLSI instruction: A loop full of single bacteria isolate was inoculated in10 ml tube of Muller Hinton broth and incubated over night 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 anabsorbency of (600nm), 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 mlof 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

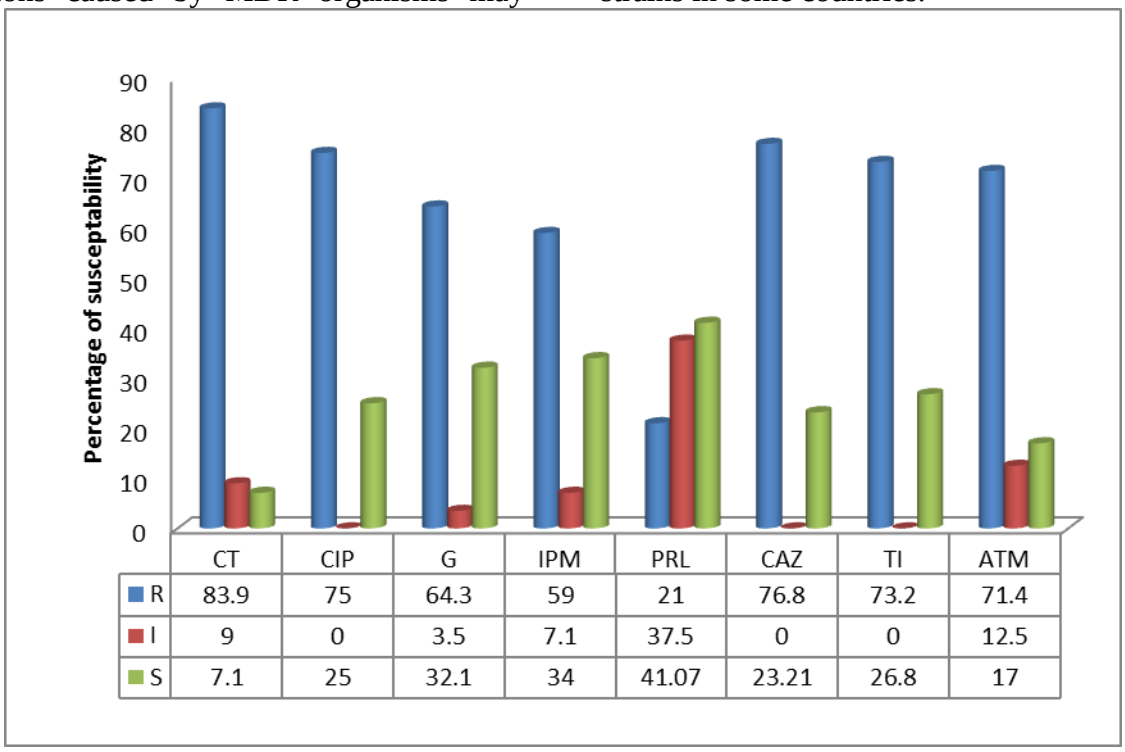
Isolation and Identification of bacterial isolate

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%),azetronem(71.4%) and imipenem (59%) while the isolates showed low resistance to pipracillin(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 pipracilin 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.



	CT	CIP	G	IPM	PRL	CAZ	TI	ATM
R	83.9	75	64.3	59	21	76.8	73.2	71.4
I	9	0	3.5	7.1	37.5	0	0	12.5
S	7.1	25	32.1	34	41.07	23.21	26.8	17

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.

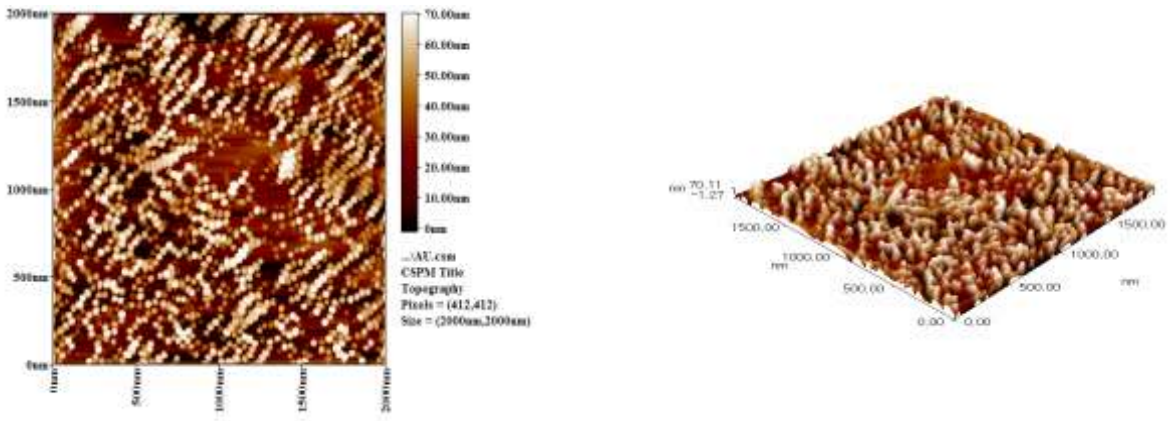


Figure2. Atomic Force Microscopy of gold nanoparticle illustrate 2D and 3D topological of gold nanoparticles

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 differentbands 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 2929.67cm-^{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 groupsthat present in ketones, aldehydes, and carboxylic acids(24).And, The 1568.02 cm-^{1}band is attributed to amine groups,while 1423.37cm^{-1}related to the symmetric bending of CH3, 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 ,2362.64, 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} to1737.74 cm-1that 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 CH3 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 rolein the stabilization and capping of the gold NPs.

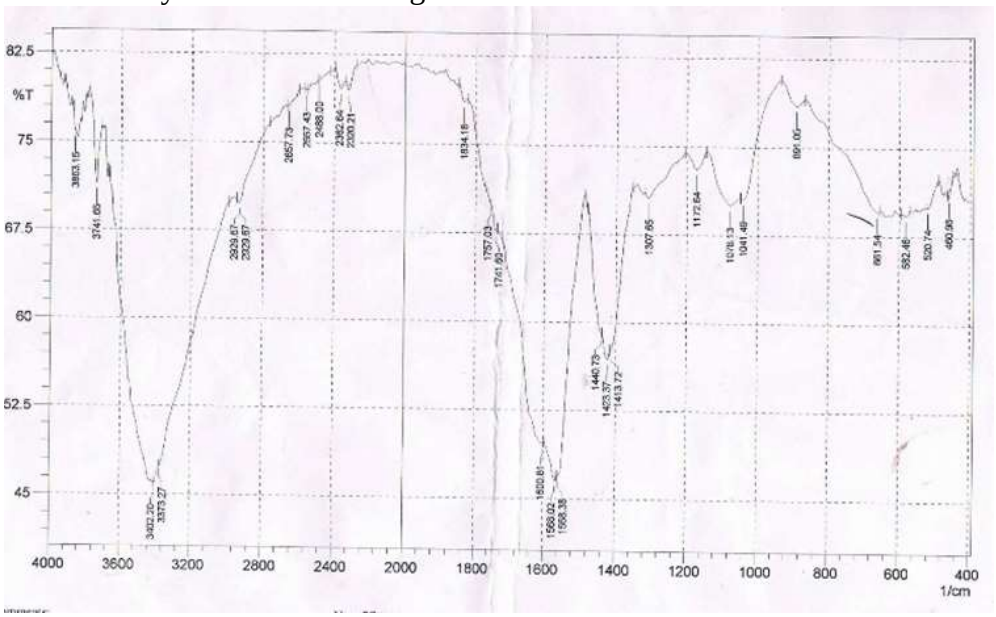


Figure 2. The Fourier transforms infrared (FT-IR) spectroscopy measurement of aloe vera

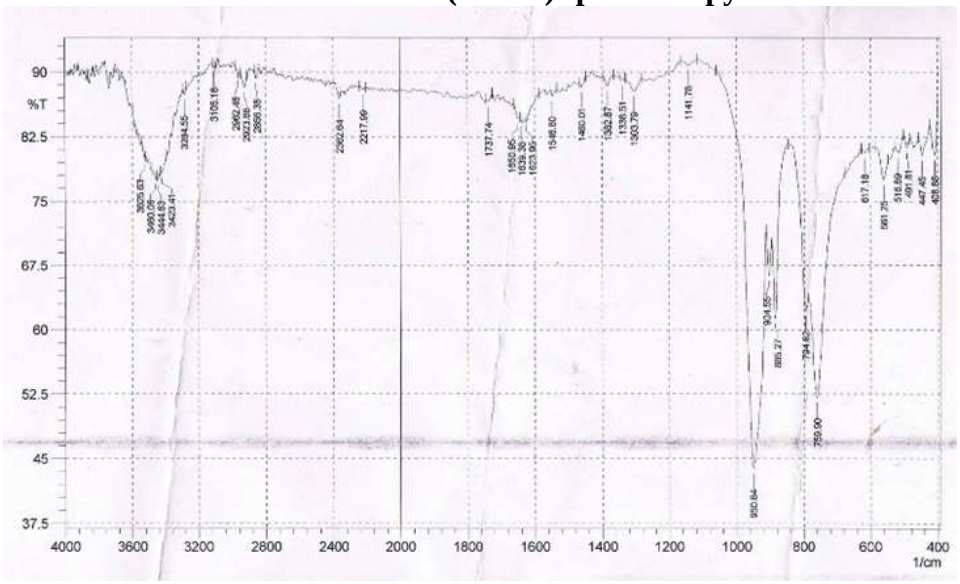


Figure 3.The Fourier transforms infrared (FT-IR) spectroscopy measurement of gold nanoparticle

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 auerginosa* isolates.While the lowest concentration (7.8 µg/ml) gave also a good antibacterial activity by killing about the half of bacterial growth 47.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 differents concentration of gold nanoparticle and percentage death for each one for *Pseudomonas aeruginosa*

NPs µg/ml	%f 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

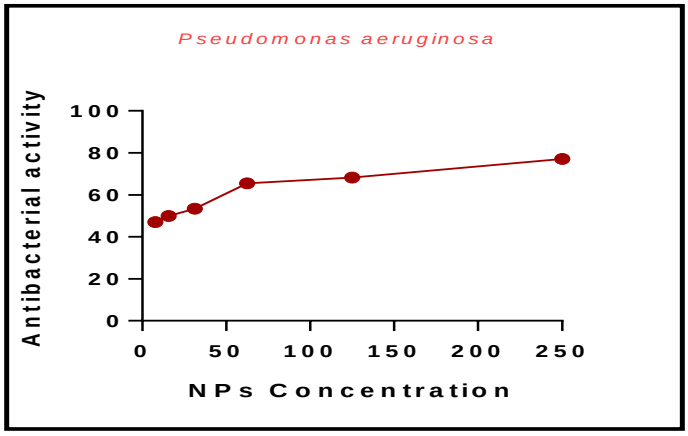
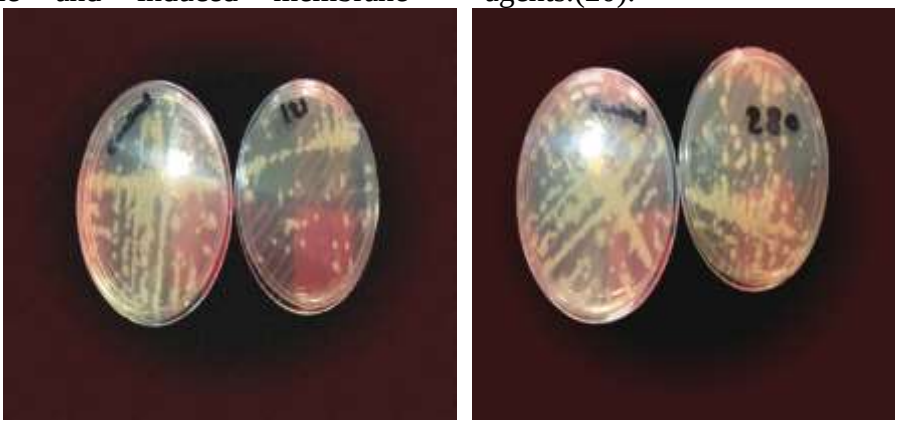


Figure4. Antibacterial activity of gold nanoparticle at different concentration

Among these different concentration, 250 µg/ml NPs concentration was considered the best antibacterial concentration .NPs are considered as next-generation antibioticsand 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(6)(8).Gold nanoparticles (AuNPs) represent a revolution in drug delivery, and are considered safe and non-toxic antimicrobial agents.(20).



A **B**

Figure5.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 nanoparicle (7.8 µg/ml)

The synergistic effect of gold nanoparticle loaded with ceftazidime antibiotic: The results of synergestic effect to determine the potency of the combination of AuNPs and antibioticsshowed 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 differents 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 GNPs with antibiotics showed synergistic effects against bacteria, prohibit biofilm formation, and have been utilized to combat MDR bacteria .Inadditon ,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).

REFERENCES

1.Al-Azzawi, S.N.A. and R.M, Abdullah, 2018. Study of the resistance of P. aeruginosa isolated from wounds and burns for some disinfects and antiseptic from some Baghdad hospitals. Journal of Pharmaceutical Sciences and Research. 10(6): 1481-1484

2.Asghar A.H, and H.S. Faidah 2003. Frequency andantimicrobial susceptibility of gram-negativebacteria isolated from 2 hospitals in Makkah,Saudi Arabia. Saudi Med J; 30: 1017-23

3. Bang R. L, R. K, Gang, S. C, Sanyal, E. Mokaddas, and M. K. Ebrahim 1998. Burn septicaemia: an analysis of 79 patients. Burns 1998 Jun; 24(4): 354–61

4. Baptista, P. V., M.P Mccusker, A. Carvalho, D.A. Ferreira, N.M. Mohan, Martins, and A. R. Fernandes1 2018. Nano-strategies to fight multidrug resistant bacteria—"a battle ofthe titans". Front. Microbiol.9, 1441. doi:10.3389/fmicb.2018.01441

5 .Clinical and Laboratory Standards Institute. 2018. Performance Standards for Antimicrobial Susceptibility Testing. 28th ed. CLSI supplement M10012.Sumit S Lal et al., IJSID, 2012, 2 (3), 325-350

6. Duran N., M. Duran., M. B. De Jesus, A. B., Seabra, W. J., Favaro, and G. Nakazato 2016. Silver nanoparticles:a new view on mechanistic aspects on antimicrobial activity. Nanomedicine 12, 789–799

7.Golshani Z, A. M. Ahadi, and A. Sharifzadeh 2012. Antimicrobial susceptibility pattern of Pseudomonas aeruginosa isolated from patients referring to hospitals. Arch HygSci;1:48-53

8. Hemeg H. A. (2017). Nanomaterials for alternative antibacterial therapy. Int. J.Nanomed. 12, 8211–8225. 10.2147/IJN.S132163

9.Ismail, S. J., and S.S. Mahmoud 2018. First detection of New Delhi metallo-β-lactamases variants (NDM-1, NDM-2) among Pseudo-monas aeruginosa isolatedfrom Iraqi hospitals. Iranian journal of microbiology. 10(2): 98–103

10. Lari A. R, H. B Honar, and R. Alaghehbandan 1998 . Pseudomonas infections in Tohid Burn Center, Iran. Burns; 24: 637-641

Lima E,R.Guerra, V. Lara, and A. Guzmán 2013. Gold nanoparticles as.11efficient antimicrobial agents for Escherichia coli and Salmonella typhi Chemistry Central Journal 7(1): 11

12. Lyles, V. D., W. K., Serem, J. J., Yu, amp; and J. C. Garno, 2013. Surface characterization.using atomic force microscopy (afm) in liquid environments. In Surface Science Techniques Springer, Berlin, Heidelberg. (pp. 599-620).

13.Muzammil, S., S., Hayat, M., Fakhar, E.a. B., Aslam, M. H., Siddique, and M. A.Nisar,.

2018. Nanoantibiotics: future nanotechnologies to combat antibiotic resistance. Front. Biosci. (Elite Ed) 10, 352–374. doi:10.2741/e827

14..Natan, M., and E. Banin 2017. From nano to micro: using nanotechnology tocombat microorganisms and their multidrug resistance. FEMS Microbiol. Rev.41, 302–322. oi:10.1093/femsre/fux003

15..Nikokar, I., A., Tishayar, Z., Flakiyan, K., Alijani, S., Rehana-Banisaeed, M., Hossinpour, S. Amir-Alvaei, and A. Araghian, 2013. Antibiotic resistance and frequency of class 1 integrons among Pseudomonas aeruginosa, isolated from burn patients in Guilan, Iran. Iranian journal of microbiology.5(1): 36

16. .Podsiadlo P, V.A. J. H. Sinani, N. W. S. Bahng, N. W. Kam, J. Lee, N. A. Kotov, 2008. Gold nanoparticles enhance the anti-leukemiaaction of a 6-mercaptopurinechemotherapeutic agent. Langmuir 24(2): 568-574

17..S.M. Magana, P. Quintana, D.H. Aguilar, J A. Toledo, C. Angeles Chavez, M. A. Cortes 2008. Antibacterial activity of montmorillonites modified with silver, 281 pp. 192-199

18-Sader, H. S., Flamm, R. K., Carvalhaes, C. G., amp; M. Castanheira, 2018. Antimicrobial Susceptibility of Pseudomonas aeruginosa to Ceftazidime-Avibactam, Ceftolozane-Tazobactam, Piperacillin-Tazobactam, andMeropenem Stratified by U. S. Census DivisionsAntimicrobial agents and chemotherapy, 62(12), e01587-18. doi:10.1128/AAC.01587-18

19.Salimi, H., B., Yakhchali, P. Owlia, and A. R. Lari, 2010. Molecular epidemiologyand drug susceptibility of Pseudomonas aeruginosa strains isolated from burn patients. Laboratory Medicine. 41(9): 540-544

20.Shaikh, S., N., Nazam, S., Rizvi, K., Ahmad, M. H., Baig, E. J., Lee, & and I. Choi, 2019. Mechanistic Insights into the Antimicrobial Actions of Metallic Nanoparticles and Their Implications for Multidrug Resistance. International journal of molecular sciences, 20 (10), 2468. doi:10.3390/ijms20102468

21. Shankar, S. S.; A.; Rai, B.; Ankamwar, A.; Singh, A.; Ahmad, M. Sastry, 2004. Biological synthesis of triangular gold nanoprisms.Nat.Mater. 3, 482-488

22.Singh, G., B. Wu, M. S. Baek, A. Camargo, A. Nguyen, N. A.Slusher, R.Srinivasan, J. P. Wiener-Kronish and S.V. Lynch 2010. Secretion of Pseudomonas aeruginosatype III cytotoxins is dependent on pseudomonas quinolone ignal concentration. MicrobPathog., 49,196–203

23. Sumit.S. Lal and P. L.Nayak, 2012. IJSID, 2 (3), 325-350

24. Suresh A. K, D. A, Pelletier, W. Wang M.L, Broich, J.W,Moon, B,Gu D.P, Allison, D. C, Joy T. J, Phelps, and M.J. Doktycz, 2011. Biofabrication of discrete spherical gold nanoparticles using the metal-reducing bacterium Shewanellaoneidensis Acta Biomater. 7(5):2148–2152

25. Vaez, H., H. G., Safaei, and J. Faghri, 2017. The emergence of multidrug-resistant clone ST664 *Pseudomonas aeruginosa* in a referral burn hospital, Isfahan, Iran. *Burns trauma*.*5*(1).

26. Vindenes H and R. Bjerknes 1995. Microbial colonisation of large wounds. Burns 1995; 21(8): 575-579

27. Yayan, J., B., Ghebremedhin, amp; and K. Rasche, 2015. Antibiotic Resistance of Pseudomonas aeruginosa in Pneumonia at a Single University Hospital Center in Germany over a 10-Year Period. PloS one, 10(10), e0139836. doi:10.1371/journal.pone.0139836