ECO-FRIENLY SYNTHESIS OF GOLD NANOPARTICLES AND STUDY THEIR EFFECT WITH ANTIBIOTICS AGAINST *ACINETOBACTER BAUMANNII*

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ABSTRACT

This study was aimed to produce AuNPs biologically using *Klebsiella pneumoniae* and study their synergistic effect with some antibiotics.Technologies of nanoparticles are quick and are employed in many applications in biomedicine. The potential of metallic nanoparticle as an anti-microbial agent is greatly investigated which considered as an alternative method to reduce the challenges of multi-drug resistance microbes. The present study discusses the novel approach to synthesize nanoparticles involving eco-friendly synthesis of gold nanoparticles using *Klebsiella pneumoniae* and study their effect as antimicrobial spectrum .Also study synergism effect of gold nanoparticles with antibiotic against *Acinetobacter baumannii*. These approaches are used to enhance antimicrobial efficacy of nanoparticles by modification of surfaces and to investigate activity of antibiotic delivery.

Keywords: nanomaterials, biomedicine, synergism, *Klebsiella pneumoniae*, transmission electron microscope.

انتاج جسيمات الذهب النانوية بطريقة صديقة للبيئة ودراسة تأثيرها مع المضادات الحيوية ضد *Acinetobacter baumannii*

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المستخلص

هدف البحث هو انتاج جسيمات ذهب نانويه بايلوجيا باستخدام بكتريا الكلبسيلا الرئويه ودراسة تاثيرها تازريا مع بعض المضادات المايكروبيه. تقنيات الجسيمات النانوية سريعة وتستخدم في كثير من التطبيقات في الطب الحيوي. للجسيمات النانوية المعدنية امكانات كبيرة كعامل مضاد للاحياء المجهرية والتي تعتبر وسيلة بديلة للحد من تحديات الاحياء المجهرية المقاومة للادوية المتعددة. تناقش الدراسة الحالية وسيلة جديدة صديقة للبيئة لتصنيع جسيمات الذهب النانوية باستخدام بكتريا كلبسيلا الرئوية ودراسة تأثير تلك الجسيمات النانوية كمضاد ميكروبي. كذلك دراسة التأثير التأزري بين جسيمات الذهب النانوية وبعض المضادات الحيوية ضد بكتريا المحفظة البومانية.تستخدم هذي الوسائل لتعزيز فعالية مضادات المايكروبات في الجسيمات النانوية عن طريق تعديل الاسطح ولتحقيق ايصال المضادات المايكروبيه.

الكلمات المفتاحيه : مواد نانوية، الطب الحيوي، التأزر، بكتريا الكلبسيلا الرئوية، المجهر الالكتروني النافذ.

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INTRODUCTION

Acinetobacter baumannii have become a worldwide health problem because of developing and resistant isolates of bacteria. It has made classical treatment of infectious diseases difficult, thus discovering new alternative classes of antimicrobial agents (nanomaterials) that can treat resistant isolates is dominant(27,30). Nanoparticles have developed as novel alternatives to broad spectrum bacterial multi-drug resistance fortified worldly because of misuse of the antibiotic ;therefore, the production of alternative new nanoparticle-antimicrobial agents that can treat resistant strains is paramount(11,19,21,32).The antimicrobial agents nanoparticles exhibit different mechanisms to reduce bacterial resistance as the microbicidal nature of NPs resulted from direct adjacency with the cell wall of bacteria, without the need to break through cells (8). The gold nanoparticle (AuNP) is an excellent candidate to any medical application because of its shape and size dependent physiological and optical properties. AuNPs are being formed by chemical and physical methods with controlled size and uniform dispersion. The microbe-assisted AuNPs is environment-friendly and has bigger advantages over other approaches(4,6,15,23). The biologically synthesis of NPs by the microorganism is an eco-friendly, nontoxic, and green technology. These technique used different microorganisms, such as eukaryotes and prokaryotes for biosynthesis of gold nanoparticles(10,12). Green chemistry have emanated as a viable and simple alternative to more complex chemical synthetic methods for obtaining AuNPs. The green synthesis is achieved by using bacteria as media to synthesize inorganic materials. The synthesis of NPs may be extracellular or intracellular depending to the NPs site (20,22). The nanomaterial has exhibited a wide spectrum anti-microbial efficacy toward Gram negative and positive microbes. AuNPs are exhibiting bacteriostatic or bactericidal activity to microbial cells. However, antibiotics combined AuNPs are shown to have strong bactericidal effect against the drug resistant bacteria. The AuNP has unique chemical and physical characteristics and has strong binding attraction to the disulfides, thiol, carboxylic acid, and proteins, which provide strong drug loading; drug is loaded on nano carriers by non-covalent interaction or through covalent conjugation with the help of prodrug, which is treated by cells(16,25,28). The current study was aimed to produce AuNPs biologically using *Klebsiella* sp and study their synergistic effect with some antibiotics.

MATERIALS AND METHODS

Chemicals and media

Gold chloride ($HAuCL_4.4H_2O$) and Tri sodium citrate was purchased from Merk Germany. All media used throughout this study were purchased from Oxoid Ltd., England.

Isolation and identification of *A. baumannii*

A total of 260 clinical specimens have been taken from various anatomical sites mid-stream urine, wounds, burns, blood, CSF and sputum were collected from patients attending hospitals of Baghdad Medical city (Gazi Al Harery Hospital, Burns Hospital and Teaching Laboratories). Specimens were cultured on MacConkey agar and blood agar and incubated at 37°C for 18-24 hr. Afterward, the grown colonies were identified by Vitek 2 compact system using GN card depending on instructions of the manufacturer (3).

Synthesis of AuNPs

According to Al-Taee(1) with some modification (bio- chemical reduction method) was adopted for preparing the colloidal of the gold nanoparticles at a concentration of 150.0 µg/ml as follow:

Solution A

The stock solution of $HAuCL_4.4H2O$ at concentration 0.49mol/l has been made by dissolving 605mg of $HAuCL_4.4H2O$ into 3ml of 10% HCl. A diluted 0.2mM of $HAuCL_4.4H2O$ solution was prepared by adding 40 µl (19.6 µmol) of the stock solution into 50 ml of deionized water as for producing solution A.

Solution B

This solution was prepared according to the procedure described by Srinath *et al.* (29) in brief: An overnight culture of *Klebsiella pneumoniae* was inoculated in 250ml-flask containing 100ml Luria–Bertani broth and incubated at 37°C for 24 hr. Subsequently, the turbidity of the culture was adjusted to 0.5 McFarland unit (1-1.5 × 10^8 cfu/ml) using

densicheck (BioMerieux, France). Thereafter, the cultures were centrifuged and at 4000 rpm for 15 min. The supernatant obtained after centrifugation was applied to produce solution B. Solution A was put to boiling at 150 °C with stirring vigorously to obtain a homogeneous volume solution of AuNPs. About 5 ml of solution B was rapidly added into the vortex of the solution A. The color of solution changed from pale yellow to purple. Continue boiling and stirring for another ten minutes. Then, heating was removed and stirring was continued for an additional fifteen minutes.When the solution cools to the room temperature, it is filtered through a 0.8μm a microfilter paper. The made solution was preserved in the refrigerator at 4 ° C and measured using ultraviolet-visible at 400-800 nm, Transmission electron microscope and Atomic force microscope spectrum.

Antibiotic sensitivity test

Five antibiotics disks Ceftazidime (CAZ), Ceftriaxone (CRO), Imipeneme (IMP), Gentamicin (GM), and Ciprofloxacin (CIP), manufactured by Bioanalyse (Turkey) were tested for efficacy against *A. baumannii* depending on disk diffusion method by Bauer & Kirby(2) and CLSI(5).

Antibacterial activity of AuNPs against *A. baumannii* and determination of MIC

The anti-bacterial efficacy of the biosynthesized AuNPs was estimated by disk diffusion method which suggested by Jarosz and Lipa (13). The minimum inhibitory concentration of AuNPs was assumed using method of NCCLS (18) by preparing serial concentrations of AuNPs (150,75,37.5, 18.75,9.37 and 4.68 μg/ml) and the lowest concentration inhibits the bacterial growth considered MIC.

Synergism effect of AuNPs and antibiotic against *A. baumannii*

Synergism Effect of AuNPs and five antibiotics(CAZ,CRO,IMP,GM and CIP) against *A. baumannii* was evaluated using disk diffusion method proposed by Al-Taee (1). The bacterial suspension was prepared and compared with the standard McFarland No. 0.5. Five ml from tryptone soya broth inoculated with a loopful of *A. baumannii* from overnight culture grown on nutrient agar, incubated for 24h at 37 °C. About twenty milliliters of medium of Mueller Hinton Agar has been poured in each Petri dish and each strain has been swabbed in a uniform way in dishes by using a sterile swab. Antibiotic disk soaked with the AuNPs solution for 1h, then placed for drying. Disks placed onto each bacterium inoculated agar plate by using sterile plastic forceps. The bactericidal efficacy has been assumed by a clear zone of inhibition around the disks after dishes incubation overnight at 37°C. According to Fayaz *et al*.(7) the percentage of excess after synergism was calculated using formula B _ A/A * 100.

A= Inhibition zone of antibiotic

B= Inhibition zone synergism

Statistical analysis

All data were analyzed by test of Chi-square (Cross tabulation) or Mann-Whitney. All graphics of this study (dot chart, bar chart or scatter diagram) have been achieved by Microsoft Excel ver.2016.

RESULTS AND DISCUSSION

Isolation and Identification of *A. baumannii*

A total of 260 clinical specimens have been taken from various anatomical sites mid-stream urine, wounds, burns, blood, CSF and sputum, 80 (30.76%) were identified as *A. baumannii* **.** The isolates obtained from MacConkey agar were identified by VITEK® 2 system. *A. baumannii* appeared on MacConkey agar as small, pale and lactose non fermenter colonies

Synthesis of AuNPs

The detailed research on extracellular bio-synthesis of AuNPs by *K. pneumoniae* was achieved in this study. *K. pneumoniae* biomass (solution B) was added to the solution A after boiling at 150 °C with stirring has led to color change of reaction mixture from pale-yellow to dark-purple color. Such color change indicates gold chloride reduction toAuNPs(Fig.1).

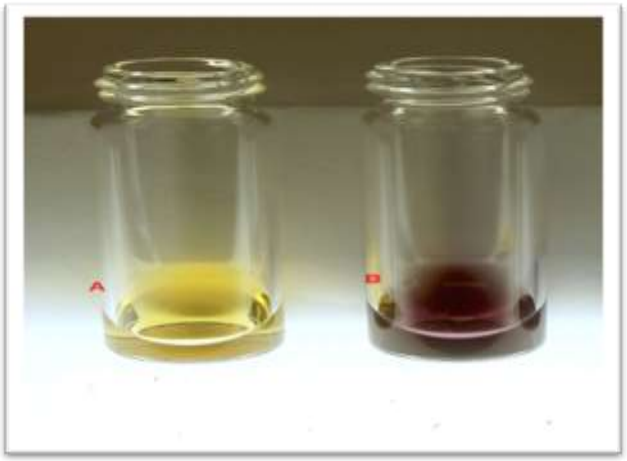

Figure 1. Color change of gold chloride (pale-yellow to dark-purple color) denoting formation of gold nanoparticles

The results of current research exhibited that the formation of AuNPs by extracellular enzymes and biomolecules secreted from *K. pneumoniae* as reduction agent of the gold chloride solution instead of citrate reduction, and appearance of dark-purple color. The current study was agreement with study by Al-Taee(1) and study by Chelladurai *et al.*(4) and study by Shegal *et al.*(24).

UV-vis spectra of AuNPs

UV-visible spectrum is one of the most significant techniques for identifying the stability and formation of the AuNPs in watery solution, the absorption peak gave at wavelength 522 nm (Fig. 2), which shows the absorption peak of gold. The current study is agreeing other several researches(1,4, 20,24).

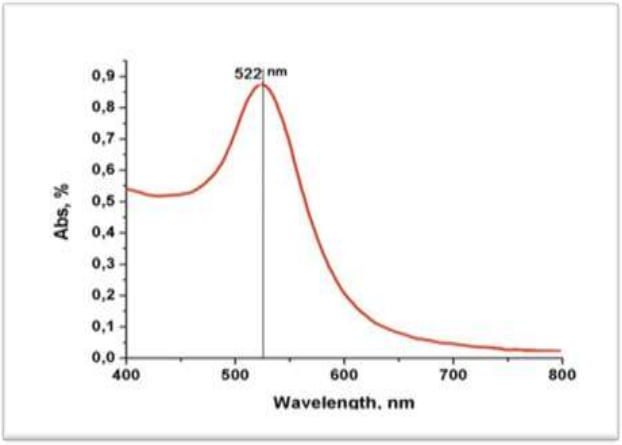

Figure 2. UV- visible spectrum of AuNPs synthesized by *Klebsiella pneumoniae*

Transmission electron microscope analysis: Analysis of TEM (Transmission Electron Microscope) is achieved to define the shape and size of the bio-synthesized AuNPs using *K. pneumoniae* and distribution of AuNPs. The present study revealed that this AuNPs is mono-dispersed and its size range was 11.5-28.5 nm (Figure 3a, b).

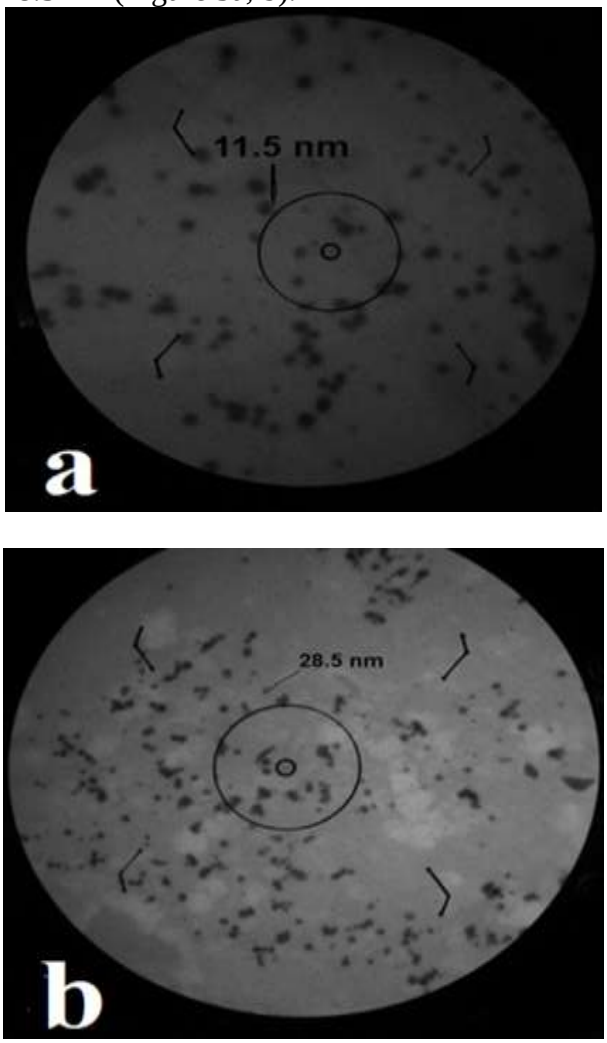

Figure 3. Transmission electron micrograph of gold nanoparticles prepared in this study. a) 245000 X magnification force and size 11.5 nm. b) 130000 X magnification force and size 28.5 nm

Analysis of atomic force microscopy (AFM)

Atomic force microscopy was achieved for colloidal solution of the AuNPs to see the finding of NPs, size, and size distribution. Analysis of AFM was achieved in tapping method and 3D (three dimensional) image of NPs was taken with a scan area of 1240.58nm × 1266.56 nm×20.36 nm as shown in figure 4a, and a scan area of 1243.65nm × 1248.05 nm×14.40 nm as exhibited in figure 4b. The present study revealed that the maximum particle size ranges between 15–25 nm (Fig. 5). This result agreed with the results reached by Singh *et al.* (28) who reported that analysis of AFM exhibited a range between 13nm to 17nm.

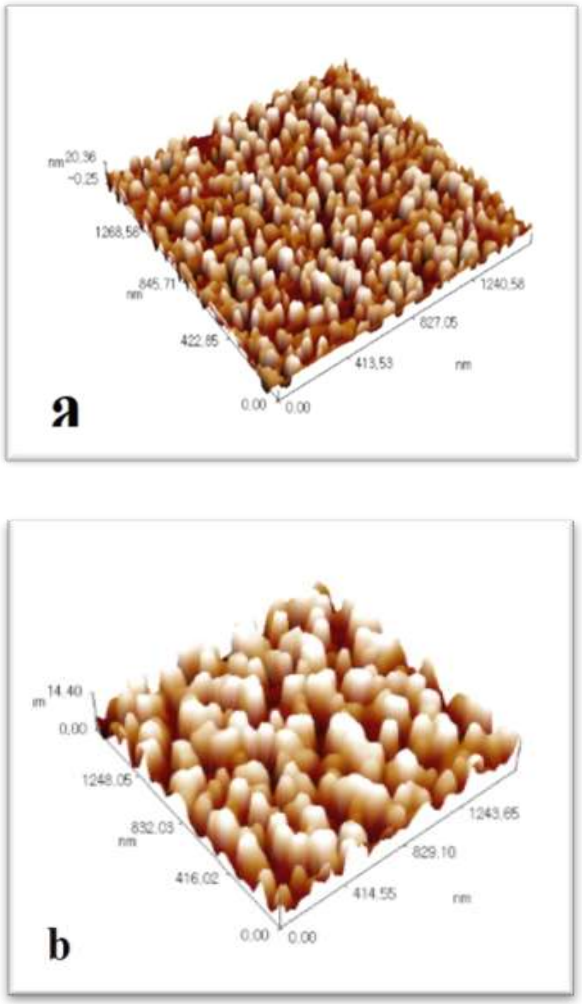

Figure 4. Atomic force microscopic image of gold NPs prepared in this research. a) scan area of 1240.58nm × 1266.56 nm×20.36 nm. b) scan area of 1248.05 nm × 1243.65 nm × 14.40 nm

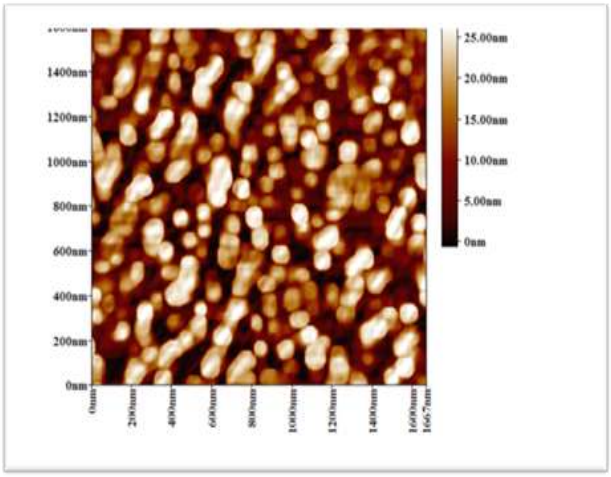

Figure 5. Size of AuNPs in AFM ranges from 15 to 25 nm

Antibacterial activity of AuNPs against *A. baumannii* and determination of MIC

The results illustrated in Table 1 and Figure 6 revealed significant effective (P< 0.01) of AuNPs toward *A. baumannii* that exhibits the decreasing in diameters of zone of the inhibition (28.1, 25.6, 23.4, 21.2,19.1 and 13.5 mm) with the decreasing AuNPs concentrations (150, 113.5, 75, 37.5, 18.75 and 9.36 µg/ml, respectively). Similarly, Al-Taee (1) stated that the effect of gold nanoparticals was dose dependent.

Table 1. Impact of AuNPs with various concentrations on growth of *A. baumannii* growth (n= 20)

AuNPs concentration (µg/mL)	Mean of Inhibition zone ± standard deviation (mm)
150.0	**28.1 ± 0.49**
113.5	**25.6 ± 0.55**
75.0	**23.4 ± 0.39**
37.5	**21.2 ± 0.43**
18.75	**19.1 ± 0.34**
9.36	**13.5 ± 0.46**

Likewise, study by Senthilkmar (25) and co-worker recorded that the maximum anti-bacterial efficacy in 300 µl/ml concentrations of the biosynthesized AuNPs was 19 mm for Gram negative pathogens such as *E. coli*, 17 mm for *P. aeruginosa*, and 16 mm for Gram positive pathogens such as *B. subtilis*; by use different concentrations of 100, 200, and 300 µl/ml. Another study by Teimuri-Mofrad (31) reviewed that the formation of AuNPs by *Mentha piperita* extracts exhibited a significant anti-bacterial efficacy toward medically pathogens isolated from human like *Staphylococcus aureus* and *E. coli*. Moreover, a study by Penders(19) showed that the effect of AuNPs of different shapes (involving stars, flowers, and spheres), with similar dimensions showed antibacterial effects toward *Staphylococcus aureus*.

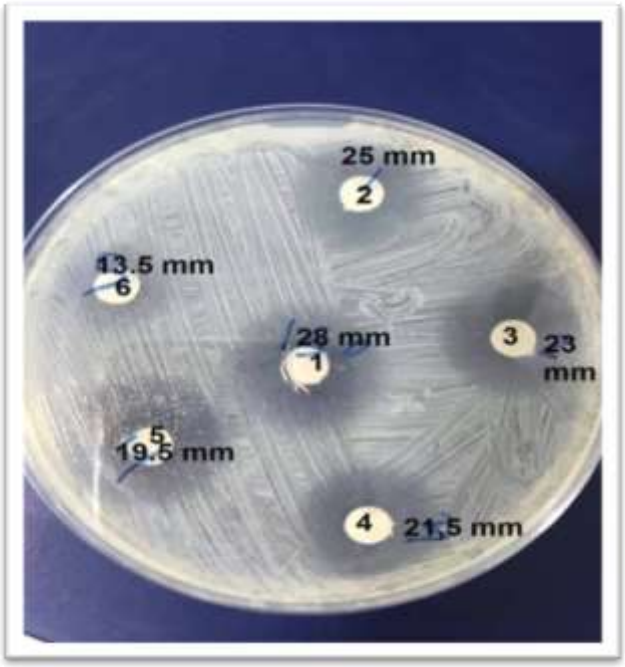

Figure 6. The inhibitory effect of AuNPs with difference concentrations ((1)150, (2) 113.5,(3) 75.0, (4)37.5, (5)18.75 and (6) 9.36 µg/ml) against *A. baumannii* isolates

Synergism Effect of AuNPs and Antibiotic against *A. baumannii* isolates

The results summarized in Table 2 showed that significant influence (P<0.05) of synergistic effect of AuNPs and antibiotic against *A. baumannii* of each isolate in terms of between groups, within groups, and location at (18.75 µg/ml) MIC of AuNPs. The present results showed (24.79%) excess of inhibition zone after synergism of AuNPs and Ceftazidim, 26.39% percentage excess of inhibition zone after synergism of AuNPs and Ceftriaxone, 22.99% percentage excess of inhibition zone after synergism of AuNPs and ciprofloxacin, 31.1% percentage excess of inhibition zone after synergism of AuNPs and imipenem, and 10.65% percentage excess of inhibition zone after synergism of AuNPs and Gentamicin(Fig.7).

Table 2. Effect of AuNPs and Antibiotic against *A. baumannii* isolates

Antibiotics	without AuNPs Mean of Inhbition zone (mm)	Std. Error	with AuNPs Mean of Inhbition zone (mm)	Std. Err	Perce. excess of synergism (%)
Ceftazidim	9.05	1.45	11.35	1.81	24.79
Ceftriaxone	11.20	1.30	13.50	1.58	26.39
Cipro.	17.75	2.14	20.82	2.48	22.99
Imipenem	18.0	0.89	24.15	1.30	31.1
Gentamicin	13.22	1,50	15.33	1.75	10.65

A study by Gad El-Rab *et al.*(9) showed that the ceftriaxone-AuNPs were highly effective against *A. baumannii* strains compared to ceftriaxone and AuNP alone. *A. baumannii* showed zones of inhibition of 6 mm for ceftriaxone, 6 and 7 mm respectively, for AuNPs, and 27 mm and 29 mm inhibition zones respectively, for ceftriaxone-AuNPs. However, a study by Shaikh *et al.*(26) showed a similar mechanism of cefotaxime coated gold nanoparticles toward drug resistant bacteria that generate extended spectrum β-lactamase. Another research worked by Kalita *et al.*(14) demonstrated that the Amoxicillin combined AuNPs revealed enhanced broad spectrum bactericidal efficacy toward Gram-negative.

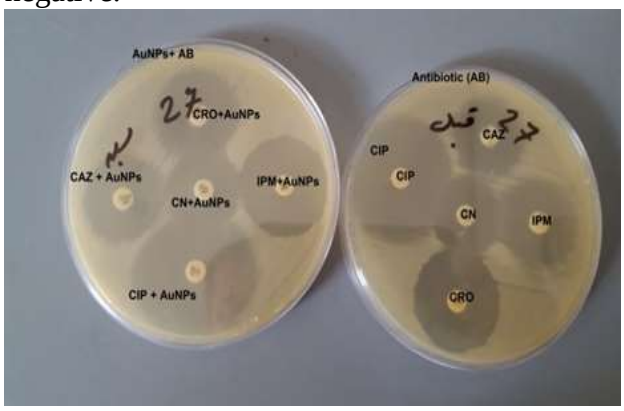

Figure7. Inhibition zone by AuNPs, antibiotics and their synergistic efficacy(mm).

However, antibiotics combined to AuNPs exhibited an increased and more targeted local concentration (of antibiotics) and help destroy microbes more efficiently than antibiotics alone, antibiotics combined to gold nanoparticles pass to the bacterium they facilely stop all regulatory functions of the membrane, effectively inactivating the bioactive blockage of protein synthesis, sulfur including proteins, and interaction with the phosphorous element in the nucleic acid structure, combination of ampicillin, streptomycin, and kanamycin to AuNPs made these drugs more heat tolerant and stable, also reduced their MICs to *Staphylococcus aureus*, and *E. coli*, and *Micrococcus luteus* recorded by Gad El-Rab *et al.*(9) and Miller(17).

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