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Bioactivity of Gold Nanoparticles Synthesized from Lion's Mushroom on Multidrug-Resistant (MDR) ESKAPE Bacterial Isolates

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ABSTRACT

The current study aims is to evaluate the antibacterial activity of gold nanoparticles manufactured using the green method from aqueous extract of lion's mane fungus against multidrug-resistant (MDR) ESKAPE group isolates, which included *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Enterococcus faecalis, Escherichia coli, and klebsiella pneumoniae*. Antibiotic resistance is one of the biggest threats to public health around the world, and to treat this, many therapeutic alternatives are being used, including the use of nanotechnology to create nanoparticles. During this study, isolates from the ESKAPE group were diagnosed and a sensitivity test to five antibiotics was performed, in addition to the synthesis of gold nanoparticles and their examination using several techniques, including an ultraviolet-visible spectroscopy device, an Fourier transmission infrared (FTIR) spectroscopy device, and a scanning electron microscope (SEM). And determine the inhibitory activity of nanoparticles against bacterial isolates. The green synthesis of gold nanoparticles (AuNPs) was accomplished using an aqueous extract of lion's mane mushroom. Tests confirmed that the formed particles have high absorbance at a wavelength of (540 nm). It was observed using a scanning electron microscope that the nanoparticles are spherical in shape and with nano sizes ranging from (20.77 to 29.37 nm). As for the FTIR examination, the range is between (447.49 cm^{-1} - 3398.57 cm^{-1}). The results of the current study showed that the biosynthesized AuNPs possess antibacterial activity against ESKAPE group isolates, with inhibition diameters ranging from (9 mm – 21 mm).

Keywords: AuNPs, Nanoparticles, Lion's mane mushroom, Meropenem, ESKAPE

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التصنيع الحيوي لجسيمات الذهب النانوية بوساطة فطر عرف الاسد وتأثيره على بكتريا مجموعة ESKAPE متعددة المقاومة

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الملخص

الهدف من الدراسة الحالية هو تقييم النشاط المضاد للبكتيريا لجسيمات الذهب النانوية المصنعة بالطريقة الخضراء من المستخلص المائي لفطر عرف الاسد ضد عزلات مجموعة ESKAPE المقاومة للأدوية المتعددة (MDR) والتي شملت *Staphylococcus aureus* ، *pseudomonas aeruginosa* ، *Acinetobacter baumannii* ، *Escherichia coli, Enterococcus faecalis* ، *klebsiella pneumoniae*. تعد مقاومة المضادات الحيوية واحدة من أكبر التهديدات للصحة العامة في جميع أنحاء العالم ولعلاج ذلك يتم استخدام العديد من البدائل العلاجية منها استخدام تقنية النانو لتخليق الجسيمات النانوية. خلال هذه الدراسة تم تشخيص عزلات مجموعة ESKAPE واجراء اختبار الحساسية لخمس مضادات حيوية بالإضافة الى تخليق جسيمات الذهب النانوية وفحصها باستخدام العديد من التقنيات منها جهاز التحليل الطيفي للأشعة فوق البنفسجية المرئية، جهاز التحليل الطيفي FTIR والمجهر الإلكتروني الماسح (SEM). وتحديد النشاط المثبط للجسيمات النانوية ضد العزلات البكتيرية. تم إنجاز التخليق الأخضر لجسيمات الذهب النانوية (AuNPs) باستخدام المستخلص المائي لفطر عرف الاسد. أكدت الفحوصات أن الجسيمات المتكونة تمتلك امتصاصية عالية عند طول موجي (540 nm). وقد لوحظ باستخدام المجهر الإلكتروني الماسح أن الجسيمات النانوية تكون على شكل كروي وبأحجام نانوية تتراوح بين (20.77 – 29.37 nm). اما فحص FTIR الذي تراوحت نطاقاته بين($3398.57\ cm^{-1}$ - $447.49\ cm^{-1}$) . أظهرت نتائج الدراسة الحالية أن AuNPs المُصنَّعة حيوياً تمتلك نشاطًا مضادًا لعزلات مجموعة ESKAPE وبأقطار تثبيط تتراوح بين (9 mm – 21 mm).

1. INTRODUCTION

Antimicrobial resistance (AMR) has now emerged as a chronic public health problem globally, with 10 million people expected to die annually by 2050[(1)]. Antimicrobial resistance (AMR) results when microorganisms including bacteria evolve to the point where they eventually become resistant to antimicrobial drugs, such as antibiotics[(2)]. The emergence of multidrug-resistant bacteria poses a major challenge in the treatment of bacterial infections. Rapid detection and effective killing of pathogenic bacteria are of great importance in the targeted treatment of bacterial infections[(3)]. Therefore, alternative therapeutic options to antibiotics, including antibodies and bacteriophages, should be considered, but some other strategies such as vaccination, immunostimulation, antibacterial peptide, or probiotics could also achieve encouraging results in the near future. Because bacteria can eventually develop and become resistant to almost any therapeutic agent, it is important to continue to use antibiotics and their alternatives wisely[(4)]. The field of nanotechnology has gained significant interest recently due to the potential uses of biosynthesized fungi-derived nanoparticles including gold nanoparticles (AuNPs). Due to their ability to produce biomolecules and enzymes, which can act as effective reducing and stabilizing agents for the biosynthesis of various nanoparticles through extracellular or intracellular actions, the use of

mushroom extracts is thought to be noteworthy. They have demonstrated high productivity, enhanced enzymatic activities and high tolerance to heavy metals and these exceptional attributes thus facilitate their potential uses in industrial, biomedical, agricultural and environmental fields[5]. In the midst of the microbial community, fungi are considered the best for biosynthesis due to their ability to release a large number of enzymes and proteins that ensure better production levels[6]. Nitrate reductases, proteases, glucan, xylanase, amylase, cellulase, lignin peroxidase, laccase, and sulfite reductase are good examples of enzymes that have a role in the synthesis of nanoparticles[7, 8]. Research is currently progressing on the use of lion's mane mushrooms in the manufacture of gold nanoparticles. Studies have shown that lion's mane mushrooms can be used to manufacture gold nanoparticles with therapeutic properties[9]. Research has indicated that lion's mane mushroom contains a wide range of medicinal properties, such as stimulating nerve cell growth, antioxidant, antihypertensive, and antidiabetic properties. Recently, for years, the green synthesis of gold nanoparticles (AuNPs) has attracted great interest due to their potential use in biomedical applications[10]. These nanoparticles have shown significant antibacterial, antifungal, and antioxidant effects, in addition to their ability to promote wound healing and neuronal growth[11]. The surface area to volume ratio is large the smaller the size of the nanoparticles, which allows the binding of a large number of high-affinity ligands, providing the nanoparticles with multivalency in eliminating bacterial cells[12]. AuNPs have shown antibacterial ability against both Gram-positive and Gram-negative bacteria[13]. Electrostatic interaction between bacterial membranes and gold nanoparticles due to opposite surface charges, can lead to rupture of bacterial membranes[14]. The current study aims to use gold nanoparticles to eliminate the ESKAPE group, which has multi-drug resistance.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

A total of 24 ready ESKAPE pathogenic isolates were obtained from Tikrit Teaching Hospital. The bacterial isolates were distributed as 6 isolates for each of *P. aeruginosa* and *K. pneumoniae*, 4 isolates for each of *S. aureus* and *E. coli*, 3 isolates for *A. baumannii*, and one isolate for *E. faecalis*, and these isolates were re-diagnosed to confirm them using the Vitek-2 device [15].

2.2. Preparation of lion's mane mushroom extract

The method[16] was used with some slight modifications. A (10 gm) of lion's mane mushroom powder (AL-Emad, Iraq) was dissolved in (100 ml) of deionized water, then left after shaking well for (24 hours). After that, the solution was purified using filter paper and Millipore (size 0.22) was used. The solution was then stored at (4 °C) until use.

2.3. Preparation of gold nanoparticles using lion's mane mushroom

Gold nanoparticles were prepared by adding (5 ml) of aqueous extract of lion's mane mushroom to (45 ml) of a solution of gold chloride salts ($HAuCl_4.3H_2O$) (Sigma Aldrich, Germany) prepared at a concentration of (5 mM) with continuous stirring using a magnetic stirrer at a temperature of (70 °C) for (30 minutes), where the color change to purple is evidence of the formation of gold nanoparticles began [17].

2.4. Nanoparticle characterization techniques

2.4.1. UV-Vis spectroscopy

The characterization of AuNPs synthesized by the green method was tested using a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). After the color change occurred in the medium containing the filtrate of the lion's mane mushroom with the gold chloride solution, (2 ml) of the reaction mixture was transferred to confirm the formation of gold nanoparticles to an ultraviolet spectroscopy device at wavelengths between (200-

800 nm), and deionized water was used as blank solution [18, 19] .

2.4.2. Scanning electron microscopy (SEM)

The shape, size, and distribution of gold nanoparticles were determined using a scanning electron microscopy device. (100 ml) of secondary gold particles were dried in a convection oven (Gallen kamp, England) at a temperature of (45 °C), and then a powder was formed that was placed in the tube of the device. Covering it with a special layer of gold and examining the model [20, 21].

2.4.3. Fourier Transmission Infrared Spectroscopy (FTIR)

This device was used to detect the functional groups and structural structures present in the lion's mane mushroom, which are responsible for reducing the ions of metallic gold salts to gold nanoparticles. FTIR (IRAffinity-1- Shimadzu, Japan) spectroscopy of the gold nanoparticles was performed by adding (0.5 mg) of the sample with (150 mg) of potassium bromide Kbr (Schuchard, Germany). The mixture was compressed into discs and inserted into a sample holder to display the result in the form of a spectrum using an FTIR spectrometer that scans the samples at wavelengths between (8000 and 300 cm^{-1}) [22, 23].

2.5. Antibiotic sensitivity test

The disc diffusion method was used to examine the sensitivity of ESKAPE isolates to five types of antibiotics, as shown in (Table 1). Single colonies of the bacterial species under study were transferred to tubes containing (5 ml) of normal saline solution. Then the turbidity of the solution was adjusted compared to a standard McFarland tube at a concentration of 0.5, then the Mueller-Hinton media (Oxoid, England) was inoculated with the bacterial suspension using a sterile cotton swab, then the inoculated dishes were left for (5 minutes), after which the antibiotic tablets were distributed using sterile forceps. The plates were then incubated for (24 hours) at a temperature of (37 °C), and then the inhibition zones were measured and compared with standard tables [24].

Table 1: The antibiotics used in this study with their manufacture company.

Type of antibiotic	Symbol	Concentration	The manufacture company
Cephalexin	CL	30 mg	Bioanalyse
Ampicillin	Am	25 mg	Bioanalyse
Meropenem	MEM	10 mg	Bioanalyse
Gentamicin	CN	10 mg	Bioanalyse
Ciprofloxacin	CIP	10 mg	Bioanalyse

2.6. Antibacterial activity of AuNPs

The bacterial suspension was prepared by culturing the bacterial samples in nutrient broth and incubating for (24 hours) at (37 °C). The number was then stabilized through comparison with a standard 0.5 McFarland solution (1×10^8). Then, (0.1 ml) of the bacterial suspension was spread on Mueller-Hinton agar medium using a sterile cotton swab. Five holes with a diameter of 6 mm were made using a cork drill, then (100 µl) of each concentration of gold nanoparticles (25, 50, 75, and 100 %) were transferred to wells. In addition to transferring (100 µl) of lion's mane mushroom extract (control sample) to one of these wells, the plates were then incubated for (24 hours) at a temperature of (37 °C), after which the diameters of the inhibition zone that appeared around the wells were measured [25, 26].

3. RESULTS AND DISCUSSION

3.1. Diagnosis of bacterial isolates

Bacterial isolates from the ESKAPE group were identified using the Vitek-2 device according to the manufacturer's instructions [15].

3.2. Susceptibility tests for ESKAPE group isolates

The results of the current study showed that all bacterial isolates belonging to the ESKAPE group were (100 %) resistant to all antibiotics used in the current study, except for meropenem, which showed varying resistance to it. *P. aeruginosa* and *A. baumannii* showed the highest resistance to meropenem with a percentage of 2 (33.33 %) and 1 (33.33 %) respectively. *K. pneumoniae* showed least resistance to meropenem, at a rate of 1 (16.67 %), while *E. faecalis* did not show any resistance to it, as shown in (Table 2). A previous study indicated that the resistance of *P. aeruginosa* to the antibiotic meropenem was 16 (27.1 %), and this is consistent with results of the current study[27]. The results of this current study agreed with what the researcher[28] found while conducting a study in Latin America, whose results showed that 40% of *A.baumannii* isolates were resistant to meropenem. In addition, another study conducted by the two researchers[29] showed that *A. baumannii* is resistant to the antibiotic meropenem at a rate of (45 %), which is similar to the results of the current study. The current study also agreed with the findings of the researcher[30], who indicated that the rate of resistance of *K. pneumoniae* to the antibiotic meropenem reached 3 (15 %). Other studies conducted by researchers[31, 32] showed that *S.aureus* is resistant to the antibiotic meropenem at a rate of 6 (18.8 %) and 2 (7 %), respectively, which are similar to the results of the current study. Bacteria have the ability to resist many antibiotics, and this resistance is either natural or acquired through mutations in the chromosome or through the acquisition of resistance genes through one of the methods of genetic transmission, which includes conjugation, transformation, and transduction[33]. The ability of ESKAPE pathogens to resist many antibiotics is due to their possession of many different mechanisms, such as changing the permeability of the outer membrane, the formation of a biofilm, the production of broad-spectrum beta-lactamases, their possession of efflux pumps, and their possession of resistance plasmids, R-plasmids, which carry genes resistance to various antibiotics[32].

Table 2: The resistance of bacterial isolates to antibiotics.

Antibiotic type	Bacterial isolates and antibiotic resistance rate					
	S. aureus	***P. aeruginosa***	***E. coli***	***K. pneumoniae***	***A. baumannii***	***E. faecalis***
CL	4 (100 %)	6 (100 %)	4 (100 %)	6 (100 %)	3 (100 %)	1 (100 %)
CIP	4 (100 %)	6 (100 %)	4 (100%)	6 (100 %)	3 (100 %)	1 (100 %)
Am	4 (100 %)	6 (100 %)	4 (100 %)	6 (100 %)	3 (100 %)	1 (100 %)
MEM	1 (25 %)	2 (33.33 %)	1 (25 %)	1 (16.67 %)	1 (33.33 %)	0 (0 %)
CN	4 (100 %)	6 (100 %)	4 (100 %)	6 (100 %)	3 (100 %)	1 (100 %)

Cephalexin = CL, Ampicillin = Am, Meropenem = MEM, Gentamicin = CN, Ciprofloxacin = CIP

3.3. Synthesis of Au-nano through Lion's mane mushroom extract

Gold nanoparticles, AuNPs, were prepared biologically using an aqueous extract of lion's mane mushroom as a reducing agent. Au^{+3} is reduced to Au^{0} as a result of adding the fungal extract to a solution of gold chloride ($HAuCl_4.3H_2O$). The formation of nanoparticles was indicated by the color of the solution changing to Purple, as shown in Figure (1). This color change results from surface plasmon excitation of gold nanoparticles[33]. The results of the current study were also identical to what the researchers found [34, 35].

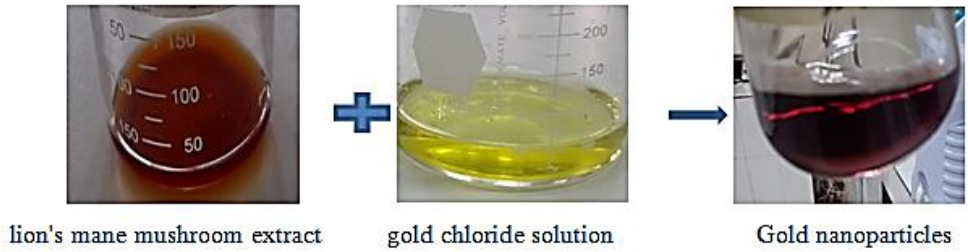

Fig. 1: The stages of synthesis of gold nanoparticles.

3.4. Characterization of gold nanoparticles

3.4.1. UV-Vis analysis

The properties of gold nanoparticles were tested using UV spectroscopy. The electronic spectrum of the gold nanoparticles showed a broad peak at (540 nm) as shown in Figure (2), which corresponds to a particle size of 28.37 nm, indicating the formation of gold nanoparticles. This result was consistent with [36].

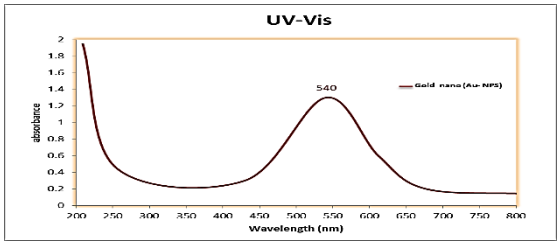

Fig. 2: The absorption spectrum of gold nanoparticles manufactured by Lion's mane mushroom extract.

3.4.2. SEM analysis

The SEM device showed through the current study, that the gold nanoparticles were characterized by cluster-like and spherical shapes, and it is worth noting that they are not in close contact with each other. This is a clear indication that the gold nanoparticles manufactured using lion's mane mushroom are completely stable, and this is consistent with the study conducted by researchers[36, 37]. Through the results of the study it was found that the nanoparticle sizes ranged between (20.77 – 29.37 nm), with an average of (26.003 nm). As in Figure (3). Therefore, this study was consistent with what the researchers found [37, 38].

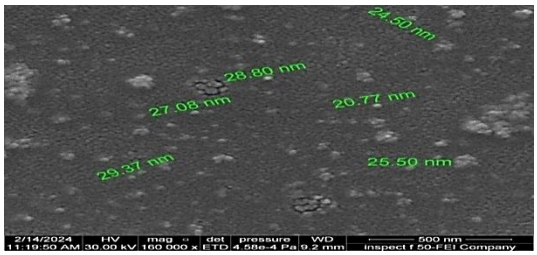

Fig. 3: The nanoscale sizes by SEM of AUNPs.

3.4.3. FTIR analysis

FTIR measurements were performed to identify potential biomolecules responsible for the effective coating and stabilization of nanoparticles[39]. The results showed that the gold AuNPs synthesized by the green method had effective aggregates that were determined between a range of (3398.57 cm^{-1} to 447.49 cm^{-1}) as shown in Figure (4), and may be attributed to OH/NH, CC and from this it was inferred that Functional groups are the reason for the reduction or stabilization of gold nanoparticles[40]. Previous studies indicated that active groups are the reason for the synthesis of Nano bodies, and this is consistent with the results of our current study[41, 42].

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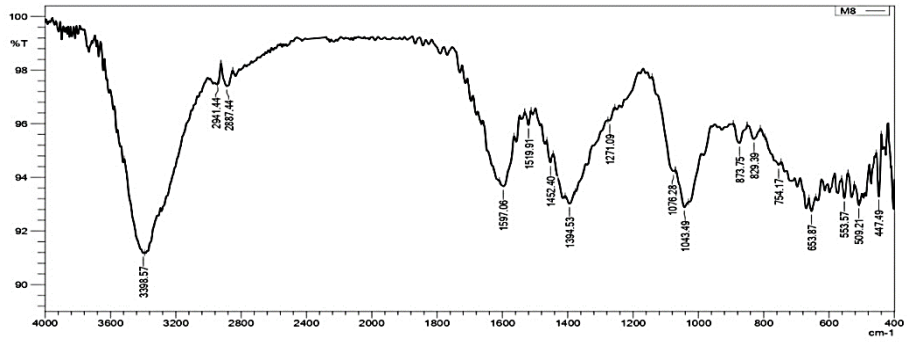

Fig. 4: The absorption spectrum of gold nanoparticles biosynthesized by Lion's mane mushroom extract using an FTIR device.

3.5. Inhibitory effectiveness of gold nanoparticles manufactured using lion's mane mushroom

The results of testing the inhibitory activity of gold nanoparticles manufactured using lion's mane mushroom showed strong activity against isolates of the ESKAPE group, using four different concentrations (25, 50, 75, and 100 %), using the wells method. Whereas the results of the current study showed different diameters of inhibition at a concentration of (100 %) against *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *E. faecalis* and *A. baumannii* with sizes of (21, 20, 19, 19, 18, 18 mm), respectively. It was also shown through the study that the concentration of gold particles have a significant impact on the inhibitory effectiveness, as the higher the concentration, the larger the inhibition zones, and vice versa. In addition, the results showed that there is a clear variation for each concentration depending on the type of bacterial strain in terms of inhibitory effectiveness, as shown in (Table 3) and Figure (5). A previous study showed that gold particles manufactured using the green method showed a good inhibitory ability against *P. aeruginosa* with an inhibitory diameter of (16 mm), and this agrees with the results of the current study[43]. The researcher[44] also indicated the high effectiveness of gold nanoparticles against *S. aureus* and *k. pneumoniae* with an inhibitory diameter of (20 and 22 mm), respectively, and this is consistent with the results of the current study. Another study conducted by the researcher [45] was close to the results of current study, as it indicated that gold nanoparticles manufactured using lion's mane fungus showed good inhibitory activity against *E. faecalis* with a diameter of (14 mm). The results of the present study also agreed with the researcher[46], whose results revealed that the bio-prepared Au-NPs had significant antibiotic activity against *A. bumannii*, with an inhibition diameter of (20 mm). A previous study conducted by the researcher[47] also indicated that gold nanoparticles showed high inhibitory activity against *E. coli*, with an inhibitory diameter of (27 mm), which is similar to the results of the current study. The antibacterial effect of nanoparticles produced with very low concentrations of Au was the mechanism by which nanoparticles interact with bacterial cells is that the bacterial cell carries a negative charge while the nanoparticle metal oxides carry a positive charge, which creates an electromagnetic attraction between the bacteria and the nanoparticle surfaces and that the nanoparticles release ions that interact with the thio group (S-H) of nutrient-transporting proteins that protrude from the bacterial cell membrane and reduce membrane permeability, leading to bacterial cell death[48]. The mechanism of gold nanoparticles inhibiting DNA replication and gene expression of proteins, as well as various cellular proteins and enzymes necessary in the production of the energy compound ATP, therefore becomes ineffective, leading to the death of the bacterial cell [49].

Table 3: Shows the inhibitory effectiveness of gold nanoparticles on ESKAPE group isolates.

Bacterial isolates	Diameters of inhibition according to each concentration			
	25 %	50 %	75 %	100 %
Escherichia coli	12 mm	14 mm	16 mm	19 mm
Staphylococcus aureus	11 mm	18 mm	19 mm	21 mm
Klebsiella pneumonia	11 mm	15 mm	18 mm	19 mm
Pseudomonas aeruginosa	13 mm	16 mm	18 mm	20 mm
Acinetobacter bumannii	9 mm	13 mm	15 mm	18 mm
Enterococcus faecalis	10 mm	13 mm	16 mm	18 mm

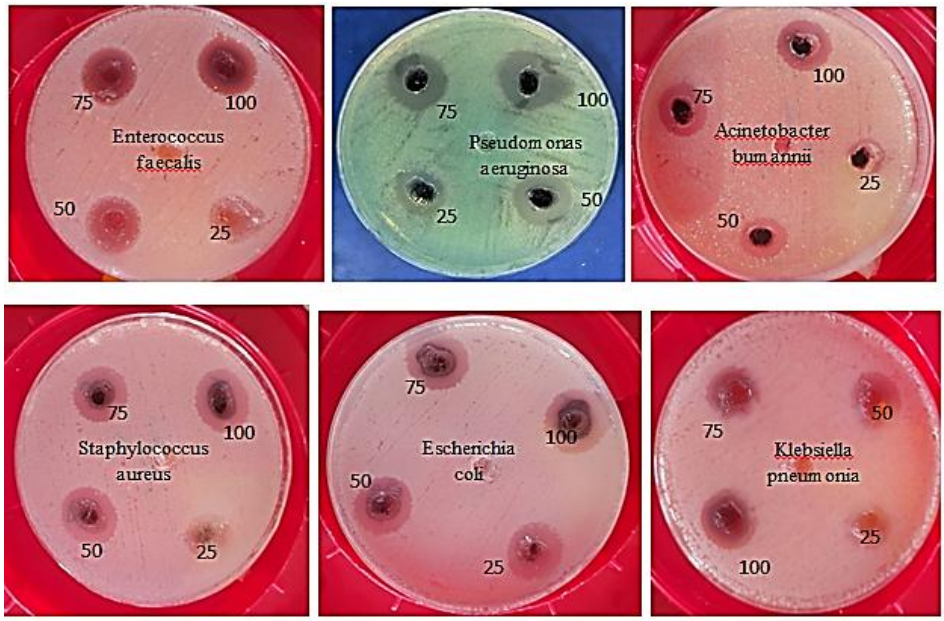

Fig. 5: The inhibitory effectiveness of gold nanoparticles against ESKAPE group isolates.

4. CONCLUSION

In this study, gold nanoparticles (AuNPs) were synthesized in a simple and inexpensive way, which is the biological method, using lion's mane mushroom extract. The use of the green method in preparing nanocomposites is very effective and convenient, as the bio-prepared particles have good properties and inhibitory effectiveness against ESKAPE pathogens. The antibacterial activity of biosynthesized gold nanoparticles increases with concentration, showing its effectiveness against the multidrug-resistant ESKAPE group.

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Author contribution: Authors contributed equally in the study

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