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Antibacterial Potential of Green Synthesize Gold Nanoparticles Using Pomegranate Peel Extract on MDR Uropathogenic Bacteria

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UTI, Gram-negative, Gram positive, AuNps, antibiotic resistance.

Abstract

Urinary tract infections have become a public health concern in recent years due to antibiotic resistance. The purpose of this study is to assess the antibacterial activity of green synthesize gold nanoparticles on MDR bacteria. One hundred urine samples were collected from patients with urinary tract infections, the identification and antibiotic susceptibility tests of bacterial isolates were achieved by using the Vitek 2 compact system. The green AuNPs was synthesized by using extract of pomegranate peel, then detected visually by changing in color, and characterized by using Ultra-Violet/ Visible spectroscopy and SEM. The antibacterial activity of AuNPs is determined by agar well diffusing. Out of 100 urine samples, seventy-three bacterial isolates were 38 (52%) Gram-negative bacteria and 35 (47.9%) were Gram-positive bacteria. Among Gram-negative bacteria, *E. Coli* 26 (68.42%) was the most common species. While the most common Gram-positive bacteria *Streptococcus epidermidis* 15 (42.8%). Gold Nanoparticles were detected visually by changing in color to violet and characterized using Ultra-Violet/ Visible spectroscopy (UVVis) that showed absorbance peak (550 nm). Scanning Electron Microscopy (SEM) analyzer showed the size ranged from (64-76nm) with spherical shape. The gold nanoparticles have the ability to inhibit the growth of *E. Coli* (13mm) and *Staphylococcus epidermidis* (12mm) bacteria. Green gold nanoparticles can be used eco-friendlily (more safety, less toxicity) as an alternative to antibiotics against antibiotic resistance bacteria.

Introduction

Urinary tract infection (UTI) is a prevalent infectious illness, especially in underdeveloped nations that face significant healthcare and economic limitations [1]. This illness is affecting over 150 million individuals each year on a global scale [2]. Therefore, the expenses related to healthcare for this

ailment are substantial with an approximate annual expenditure of $3 billion only on UTIs [3]. The incidence of urinary tract infections (UTIs) differs according to gender and age, with women and elderly individuals being more prone to UTIs compared to males and younger individuals [4].

Urinary tract infections (UTIs) are often caused by bacteria, particularly Gram-negative bacteria, including *E. coli*, *Proteus species*, *P. aeruginosa*, and *Klebsiella* species. *Staphylococcus aureus*, *Enterococcus* species, *Staphylococcus* species, and coagulase-negative *staphylococci* are often identified Gram-positive bacteria that are expected to cause urinary tract infections (UTIs) [5]. The discovery of antibiotics was a significant breakthrough in modern medicine. However, the widespread availability and excessive use of antibiotics have resulted in the progressive development of bacteria-resistance against them [6]. Urinary tract infections are often managed with broad-spectrum antibiotics. Treatment is typically beginning empirically, without the need for culture and sensitivity testing. The imprudent and indiscriminate use of antibiotics has led to the formation of antibiotic resistance in bacteria on a global scale, resulting in the creation of multidrug-resistant (MDR) strains of bacterial pathogens [7]. Antimicrobial resistance is a significant medical issue characterized by microbes using many resistance mechanisms, including horizontal gene transfer (such as plasmids and bacteriophages), genetic recombination, and mutations [8]. Prior research has shown a possible connection between antibiotic resistance and the production of biofilms [9]. Biofilms act as a barrier that prevents antibiotics from penetrating, thereby reducing the amount of antibiotic that can reach the intended target and promoting the development of resistance [10]. Prolonged antibiotic resistance may impose financial pressures on government agencies, since prolonged hospital stays result in increased medical costs and mortality rates [11].

Nanoparticles provide a versatile foundation for therapeutic applications because of their distinctive physical and chemical characteristics, and they may effectively cure drug-resistant bacteria [12]. The antimicrobial properties shown by nanomaterials such silver, gold, copper, titanium, zinc oxide, and magnesium oxide are anticipated to serve as a replacement for antibacterial agents [13]. AuNPs possess distinctive characteristics, including their modifiable size, shape, surface properties, optical properties, biocompatibility, little toxicity, remarkable stability and versatile capabilities, which make them very attractive in several medical domains [14]. The objective of this study is to assess the potential antibacterial activity green synthesis AuNPs against MDR bacteria.

Materials and Methods

1. Sample collection:

From October 2022 to January 2023, sterile test tubes were used to collect about 5 ml of non-repetitive, clean catch-mid-stream urine from (100) individuals suspected of having a urinary tract infection (UTI) and exhibiting clinical signs of UTI. All patients were hospitalized in Babylon Hospitals in Iraq and had received an antibiotic treatment for a minimum of 3 days.

2.Identification of bacteria and antibiotic susceptibility

The urine samples were cultured in routine medium to promote bacterial growth by using nutrient and MacConkey agar (Oxoid, UK). They were then incubated at 37°C for 24 hours. The bacterial isolates were identified using the automated VITEK 2 system, following the manufacturer's directions, and using the Vitek 2 GN ID card and Vitek 2 GP ID card kits (Biomerx/France) to get the final diagnosis and identification. Antibiotic susceptibility testing was also performed using the Vitek 2 system following the instructions of the Vitek 2 Compact System, using multi-antibiotic AST-Cards for both gram-negative and gram-positive bacteria (Biomerx/France). (16) antibiotics were tested against gram-positive bacteria, while (18) antibiotics were tested against gram-negative bacteria.

3. Preparation of Green synthesize gold nanoparticles using extract of pomegranate peel:

Two kg of fresh pomegranates were collected from the garden of the house and then washed well with distilled water. After drying the peels, they were ground into powder. The extract was prepared by dissolving (10 g) of pomegranate peel powder in (100 ml) of deionized water and placing it on a heater for 2 hours at 60 °C and stirring continuously with a magnetic stirrer. After that, impurities were removed by using filter papers, and the filtrate is stored in the refrigerator. Gold nanoparticles were prepared by adding 33 ml of aqueous extract of pomegranate peels to 100 ml with a concentration of (0,0039 g) of gold chloride acid solution and placed on a heater for a week at 37 °C with continuous stirring using a magnetic stirrer. Then, the color changes to purple, and the chemical reaction process is complete. After that the filtrate is disposed of and the precipitate is washed several times with deionized water using a centrifugal cooling device. Then the precipitate containing the gold nanoparticles is dried and preserved until used [15].

4.Characterization of Gold Nanoparticles:

The optical characteristics of blended pomegranate-strip-extract-mediated gold nanoparticles were measured by using the UV-VIS spectrophotometer. The size and form of the AuNPs synthesised by

green methods were assessed through Scanning Electron microscopy (SEM). Fourier transform infrared spectroscopy (FTIR) was used to identify the potential presence of biomolecules that may be linked to the creation of gold nanoparticles (AuNPs). X-ray diffractometers were used to examine the composition and structure of green AuNPs.

5.Measurement of Antimicrobial Activity of AuNPs

The antibacterial activity of AuNPs at various doses was evaluated by using the agar-well-diffusion technique. Muller Hinton plates were infected with tested bacteria at an inoculum density of 1,5 x 108 CFU/mL. A cork borer was used to create wells in the centre of a plate. These wells were then filled with 100 µL of filtered AuNPs at various concentrations (32.25, 62.25, 125, 250 and 500µg/mL^{-1}) and incubated at 37°C for 24 hours under dark circumstances. Subsequently, the diameter of the inhibitory zone was assessed.

6.Statistical analysis

Statistical analysis was conducted with the SPSS-Statistics 24.0-Programme.

Results and Discussion

1. Identification of uropathogenic bacterial isolates from UTI patients

Among the 100 urine samples collected from individuals suspected of having UTI, 73 (73%) included bacteria. These bacteria isolates were further categorized as 38 (52%) gram negative and 35 (48%) gram positive. Additionally, 27 (27%) of the samples showed no growth, indicating the presence of a causal factor other than bacteria. Among the Gram-negative bacteria, the most common was *E. coli*, with a prevalence of 26 (68.42%). Among gram positive bacteria, *Staphylococcus epidermidis* accounted for the highest abundance at 15 (42.8%). (Table 1)

Table 1: occurrence bacteria isolates from urine samples of UTI patients.

Gram negative bacteria							
Genus & sp.	*E.COLI*	*Klebsiella pneumoniae*	*Enterobacter cloacae*	*Pantoea agglomeras*	*Enterobactwe aerogens*	*Acinetobacter baumannii*	*Citrobacter freundii*
No.& percentile	26 (68.42)%	4 (10.5)%	3 (7.9)%	2 (5.26)%	1 (2.6)%	1 (2.6)%	1 (2.6)%

Gram positive bacteria								
Genus & sp.	*Staphylococcs **epide**rmidis*	*Staphylococcs hominis*	*Staphylococcs haemolyticus*	*Enterococcus faecalis*	*Staphylococcs aureus*	*Staphylococcs saprophyticus*	*Streptococcus agalactiae*	
No.& percentile	15 (42.8)%	5 (14.28)%	5 (14.28)%	4 (11.42)%	3 (8.57)%	2 (5.7)%	1 (2.85)%	

2.The antibacterial susceptibility testing against different species of gram negative and gram-positive bacteria

Staphylococcus epidermidis was the most common isolate comprising about (45.45%) of Gram-positive isolates. It was highly resistant to benzylpenicillin, piperacillin /tazobactam, oxacillin and clindamycin 15 (100%) and Fusidic Acid 14 (93.3%). As shown in figure (1)

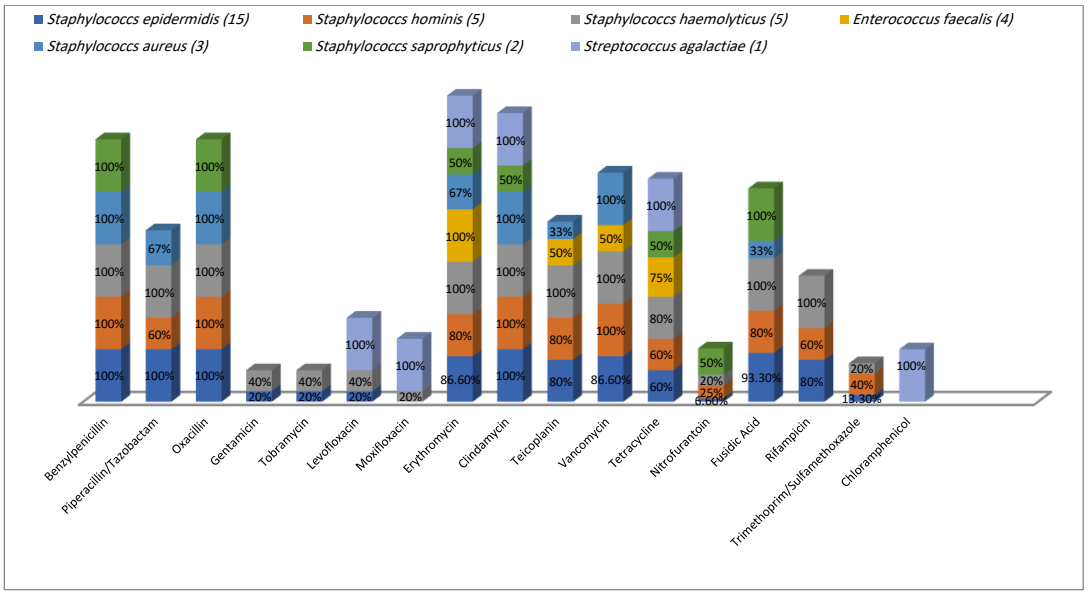

Figure 1: Ratio of uropathogenic Gram-positive resistance among prevalent antibiotics.

Escherichia coli was the most common isolate comprising about (74.28%) of Gram-negative isolates. It was highly resistant to Norfloxacin (100%), ampicillin (73%) and Trimethoprim/ Sulfamethoxazole (69%), as shown in (2).

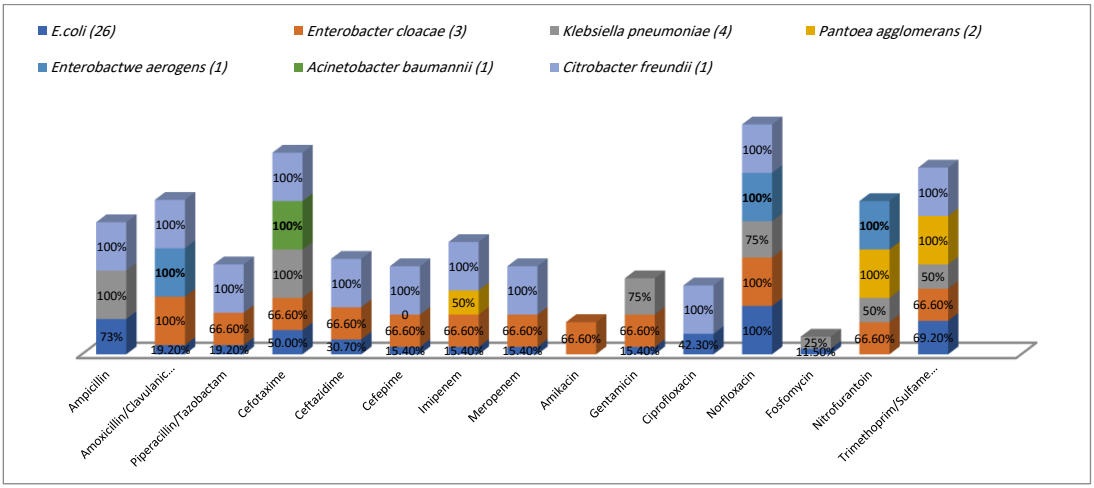

Figure 2: Ratio of uropathogenic Gram-negative resistance among prevalent antibiotics.

3.Multi drug resistance (MDR) rate among uropathogenic bacteria

The majority of the gram-negative bacterial isolates exhibited multi-drug resistance (MDR), indicating resistance to at least one antibiotic from three or more classes, as per the antimicrobial susceptibility testing guidelines set by the Clinical and Laboratory guidelines Institute (CLSI), as shown in (3).

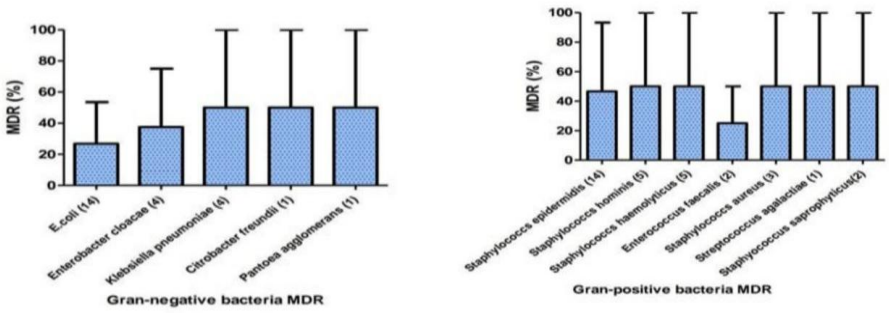

Figure 3: Percentage of MDR uropathogenic gram-negative and gram-positive bacteria.

The majority of the gram-positive bacteria isolates exhibited resistance to three or more antibiotics within each class, as shown by figure (3), in accordance with the antimicrobial susceptibility testing guidelines set by the Clinical and Laboratory guidelines Institute (CLSI).

4. Characterization of green synthesized AuNPs

4.1 Visual observation

By mix pomegranate peel extract to the gold salt, the extract functions as a catalyst, transforming the gold salt into gold nanoparticles. This transformation is visibly identifiable by a color change from yellow color of gold salt to purple color. The color change indicates the formation of AuNPs , which occurs as a result of the activation of surface plasmon vibrations inside the gold nanoparticles, as seen in figure (4).

Figure 4: Represent the color changing before and after adding plant extract to gold salt (A) pale or yellow color of gold salt (3H2O.HAuCl4), (B) brown color of pomegranate peel extract (C) purple color of AuNPs after adding aqueous pomegranate peel

4.2 UV-Vis spectroscopy

The characteristics of the produced nanoparticle solutions were analyzed by using UV-Vis spectroscopy. The electronic spectra of gold nanoparticles exhibited a wide peak at a wavelength of 550 nm, which signifies the presence of gold nanoparticles (AuNps) as seen in figure (5).

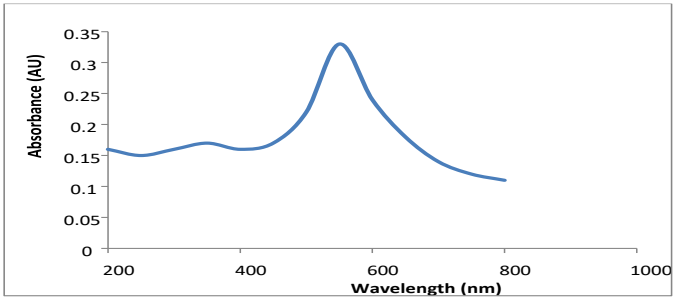

Figure 5: UV – Vis spectroscopy analysis of green synthesis AuNPs.

4.3 Scanning Electron Microscopy (SEM)

The scanning electron microscopy (SEM) technique reveals the characteristic surface of green gold nanoparticles (AuNPs) produced from pomegranate peels utilizing the green methods. The scanning

electron microscopy (SEM) revealed that the gold nanomaterials had a mostly spherical form, with a diameter ranging from 64 to 76 nm. This falls within the desired range of 70 nm (figure (6)).

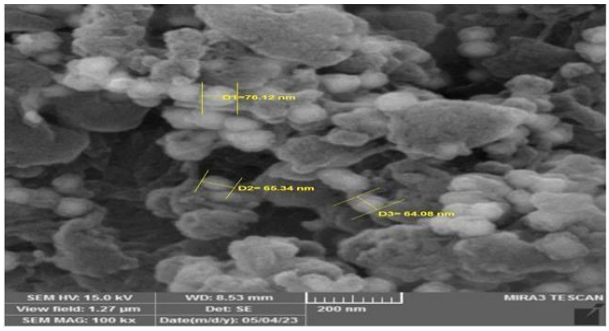

Figure 6: SEM image of green AuNPs synthesized from pomegranate peels with average size 70nm.

4.4 X- ray diffraction (XRD)

The X-ray diffraction analysis reveals that the gold nanoparticles display atomic arrangements corresponding to the [111], [200], [220], and [311] crystal planes with diffraction angles of 38.2°, 44.6°, 64.6°, and 77.47°. The X-ray diffraction of the sample is shown in figure (7).

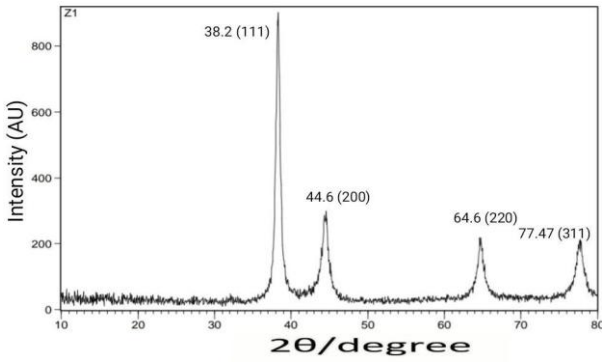

Figure 7:XRD of green synthesized AuNPs mediated by pomegranate peel extract.

4.5 Fourier Transform Infrared Spectroscopy (FTIR)

The spectrum exhibited a prominent peak at around 3305 cm^{-1}, indicating the presence of the hydroxyl (OH) functional group. The bands seen at 2923.98 cm^{-1} and 2855.13 cm^{-1} corresponded to methyl (CH) groups, whereas the band detected at 1614.91 cm^{-1} corresponded to an amine group (NH). The bands seen at 1540.01 cm^{-1} and 1435.19 cm^{-1} correspond to the carboxylate group, whereas the band at 1319.33 cm^{-1} corresponds to the (CN) groups, as shown in figure (8).

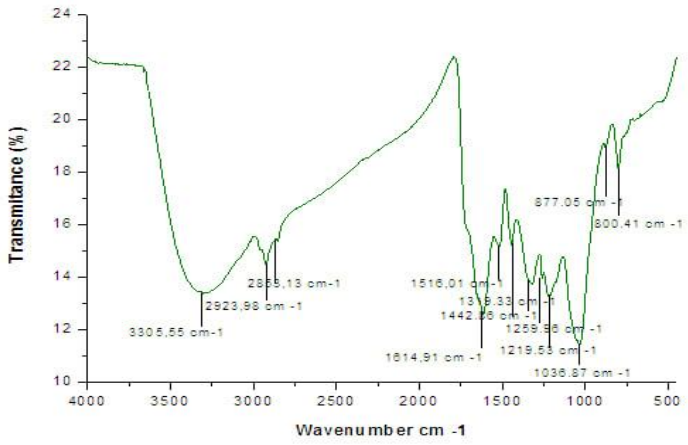

Figure 8: FTIR spectra of AuNPs and pomegranate peel extract.

5. Detection the antibacterial activity green AuNPs:

The antimicrobial efficacy of green AuNPs against multidrug-resistant uropathogenic *Staphylococcus epidermidis* and *E. coli* isolates was assessed by using agar well diffusion method. Using Various concentrations of green AuNPs (62.5, 125, 250 and 500µg.mL^{-1}). The highest level of inhibition was observed at a concentration of 500 µg.mL^{-1} where exhibited the greatest zone of inhibition against *E.coli* bacteria, by measuring 12 mm. Concentrations of 250 µg.mL^{-1} and 125 µg.mL^{-1} exhibited inhibition zones of approximately 11 mm and 8 mm, respectively. The lowest level of inhibition was observed at a concentration of 62.5 µg. mL^{-1}, resulting in a zone of inhibition measuring 7 mm. Figure (9) demonstrates that a concentration of 31.25 µg. mL^{-1} does not have any inhibitory effect on bacterial growth**.**

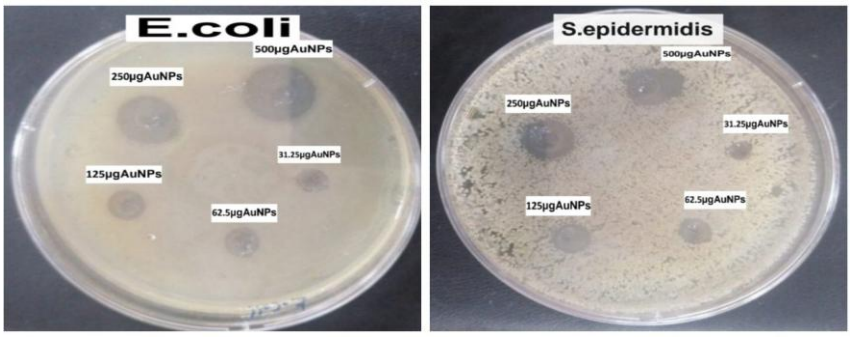

Figure 9:Zone of Bacterial Growth Inhibition According to Green AuNPs Antibacterial Activity against MDR uropathogenic E. coli and Staphylococcus epidermidis.

The study demonstrated that the concentration of 500 µg. mL^{-1} exhibited the greatest zone of inhibition against *Staphylococcus epidermidis* bacteria, measuring 11 mm. In contrast, concentrations

of 250 µg. mL^{-1}, 125 µg. mL^{-1}, and 62.5 µg. mL^{-1} resulted in bacterial growth, with respective zone of inhibition measurements of 10 mm, 8 mm, and 8 mm (figure 9). Based on the aforementioned findings of the agar well diffusion test, it is seen that the proliferation of *E. Coli* and *Staphylococcus epidermidis* bacteria diminishes as the concentration of gold nanoparticles increases, as shown in figure (10).

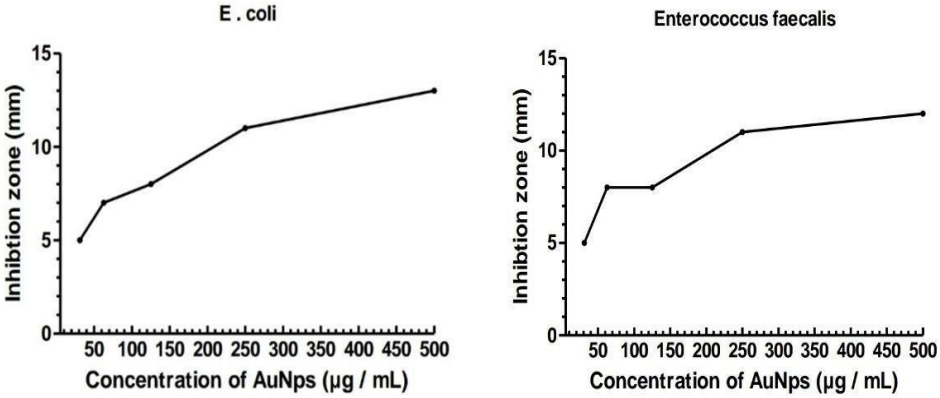

Figure 10: Represent growing that the diameter of the inhibition zone increases compared to increasing concentrations (31.25, 62.5, 125, 250 and 500µg/mL-1) of green synthesize AuNps.

Medical overuse and abuse of antibiotics causes multidrug resistance in UTIs, a major hospital issue. The percentage of Gram-negative bacteria resistant to antibiotics. The susceptibility testing of *E. coli* revealed a significant resistance pattern to several antibiotics, as shown below: Norfloxcin (100) %, Ampicillin (73) %. These results are consistent with relatively similar results all the following (Trimethoprim/Sulfamethoxazole (76.5) %, Ampicillin (76.1) %, Cefotaxime (40) % and these results do not match other results obtained by previous studies: Ampicillin (88.9) %, Norfloxcin (61.9) %, [16]. *Klebsiella pneumoniae* bacteria have shown a high resistance pattern to ampicillin and Cefotaxime (100 and100) % respectively, was similar to that of who found researchers [17,18]. But not consistent with some results shown in previous studies [20]. Through the results, *Enterobacter aerogenes* bacteria showed high resistance to Amoxicillin/Clavulanic acid, Norfloxacin and Nitrofurantoin, each of which was (100) %, almost similar to those results confirmed by previous studies, all of which included Amoxicillin/Clavulanic acid (100) % and Nitrofurantoin (100) %, while Norfloxacin was in an approach proportion (100) % [20]. *Pantoea agglomerans*, the results showed that the resistance of the bacteria to fosfomycin and Nitrofurant (100 and 100) % is similar to that of the studies [21,22]. The results also showed that *Citrobacter freundii* bacteria was high in resistance to many antibiotics, which is consistent with the results reached by the researchers [23].

Antibiotic-resistant rate of Gram-positive Bacteria. Some *Staphylococcus epidermidis* isolates showed high resistance to most antibiotics, with resistance rates reaching all of the following: (Benzylpenicillin, Tazobactam/Piperacillin, Oxacillin, Clindamycin) (100) %, Fusidic Acid (93) %, Which is relatively consistent with previous studies benzylpenicillin (100%), fusidic acid (100,85) % and tetracycline (65,60) % and while it is not consistent with the results reached by some researchers [24]. During the antibiotic sensitivity test, it was found that the *Staphylococcus haemolyticus* bacteria showed high resistance to many antibiotics, including Benzylpenicllin, piperacillin/tazobactam, oxacillin, erythromycin, clindamycin, teicoplanin, vancomycin, fusidic acid and rifampicin, each of which was 100 %.

These results are consistent with the results reached by the researchers regarding those bacteria that showed resistance to Piperacillin/Tazobactam, Oxacillin and erythromycin (99.1, 991.1 and 86) % [25]. While they do not agree with the results reached by previous studies [25,26]. Isolates of the *Staphylococcus hominis* bacteria showed high resistance to a wide range of antibiotics, especially Benzylpenicllin (100) %, oxacillin (100) %, clindamycin (100) % and vancomycin (100) %. This is consistent with scientific research on the resistance of bacteria to Benzylpenicin (88.8) % and erythromycin (85.7) % and while it is not consistent with previous research [27,28]. There is resistance of the bacteria *Enterococcus faecalis* to some antibiotics such as erythromycin and tetracyclin at a rate of (100 and 75) %, and this is consistent with current studies of erythromycin and tetracyclin at a rate of (97,8 and 72 %) [29,30], while not consistent with some results, previous studies showed [29].

Staphylococcus aureus bacteria have been shown to have a high resistance to Benzylpenicllin, oxacillin and clindamycin at (100, 100 and 100 %.) This is confirmed by studies conducted by researchers on Benzylpenicllin (100%) and oxacillin (100%) [31,32]. However, the results confirmed by studies for Clindamycin, fusidic acid, telcoplanin and vancomycin (35.3, 3.9, 0 and 0 %) are not consistent with those results shown by bacteria towards those antibiotics [32]. Susceptibility testing of Gram positive *Staphylococcus saprophyticus* isolates showed a high resistance pattern to many antibiotics, with the rate of Benzylpenicllin, oxacillin and fusidic acid (100, 100 and 100) %. This is consistent with studies in recent years [33]. *Strephylococcus agalactiae*. Studies have shown, through results, the presence of severe resistance to several antibiotics. Resistance of bacteria to Levofloxacin, Moxifloxacin, Erythromycin, Clindamycin and tetracycline, each of which was (100 %). This is relatively similar to what researchers have found regarding bacterial resistance to antibiotics such the tetracycline (98.9%), Erythromycin (78.6%) and Clindamycin (64.3%) [34,35]. While the results of previous studies that showed antibiotic resistance at varying rates do not agree

with study [35]. The majority of Gram-positive and Gram-negative bacteria can resist antibiotics. Resistance can be innate or acquired through chromosome changes or genetic transmission processes like conjugation, transformation, and conjugation [36]. Overproduction of B-lactamase enzymes causes most bacterial isolates to resist B-lactam antibiotics.

A decrease in outer membrane permeability limits antibiotic entry into bacteria, boosting resistance [37]. Some bacteria strains are resistant to Cephalosporin antibiotics because they can inhibit the production of the bacterial cell wall. The resistance to these antibiotics may be due to the antibiotic's limited ability to penetrate the bacterial cell's plasma membrane or the presence of beta-lactamase enzymes along with efflux pumps [38]. Aminoglycosides may exhibit resistance due to the presence of enzymes that hinder the interaction between the antibiotic and the 30s subunits of ribosomes [39]. Bacteria's resistance to vancomycin can be attributed to various factors. One possible reason is alterations in the pathway of cell wall synthesis that are susceptible to antibiotics, resulting in an increase in thickness. Resistance may also arise from the transfer of genes that carry the resistance trait to Vancomycin [40]. These genes can be transmitted through plasmids or mobile genetic elements, such as jumping genes, from different bacterial strains and types.

The bacterial isolates exhibited resistance to Sulfamethoxazole/Trimethoprim by means of the permeability barrier, which is recognized as the primary factor for resistance to sulfamethoxazole/trimethoprim, together with the presence of efflux pumps [41]. Scientists are continually hunting for novel medications to fight MDR bacteria. AuNPs have antibacterial, antifungal, and anti-cancer properties and are widely utilized in medicine delivery and diagnostics. Plant extracts are good reducing agents for AuNP production because they contain active phytochemicals and are safer and more ecologically friendly than chemically generated NPs.

Using UV-Vis spectroscopy, an SPR band was seen at around 550 nm, further confirming the formation of Au-NPs, using (SEM) revealed that the gold nanomaterials had a mostly spherical form, with a diameter ranging almost 70 nm. By examining XRD, it was revealed that the gold nanoparticles created had a face-centered cubic structure and the FTIR spectra revealed the presence of hydroxyl, flavonoid, carboxyl, and phenolic groups, which were previously shown to be functional groups that contributed to the stabilisation and capping of AuNPs [42,43]. The efficacy of gold nanoparticles in inhibiting bacterial growth has been shown. The greatest level of inhibition was observed at a concentration of 500 µg. mL^{-1}, resulting in an inhibition zone with a diameter of approximately 12 mm for *E. coli* and 11 mm for *Staphylococcus epidermidis*. Conversely, the smallest inhibition zone was observed at a concentration of 62,5 µg. mL^{-1}, with a diameter of 7 mm for *E. coli* and 8 mm for *Staphylococcus epidermidis*.

Some studies validate the efficacy of gold nanoparticles in inhibiting bacterial growth. The antibacterial activity significantly diminishes when the concentration of AuNPs decreases. As the concentration of gold nanoparticles rises, the permeability of the bacterial membrane also increases. This process is initiated by the contact between the bacterial cell wall and the gold nanoparticles, resulting in the rupture of the bacterial membrane. Consequently, the proteins inside the bacterium undergo denaturation, ultimately leading to cell death. Gold particles exhibit a stronger impact on negative bacteria compared to positive bacteria due to the presence of a thin peptidoglycan layer in their cell wall. This thin layer allows for easy penetration, whereas positive bacteria possess a cell wall with a thick peptidoglycan layer and a linear-polysaccharide chain that is cross-linked by short peptides. These results in a highly robust structure that hinders the entry of nanoparticles into the bacterial cell and impedes their growth [44].

Conclusion

In this study, plant products from pomegranate peel were used to successfully make green gold nanoparticles. UV–visible spectroscopy and SEM were used to study the nanoparticles that were made. The antibacterial activity of the green AuNPs was studied, and it was found that gold nanoparticles made from pomegranate peel had the strongest antibacterial activity in the bacterial inhibition zone which stopped bacteria from growing. This might have happened because their main crystallite was only 70nm in size, which is smaller than the others. As the quantity of green AuNPs went up, the inhibiting effects got stronger. The results show that green AuNP may bend and hurt the membrane of a bacterial cell, letting inside substances leak out and finally killing the cells.

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