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The effect and characterizations of silver nanoparticles on biofilm formation in Pseudomonas aeruginosa isolated from UTIs patients

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Abstract---Background: Urinary tract infection (UTI) is a common health problem in both community and nosocomial settings, affecting both men and women equally.*Pseudomonas aeruginosa* is an opportunistic human pathogen causing devastating acuteand chronic infections in individuals with compromised immune systems. Biofilmis an architecture built mostly by autogenic extracellular polymeric substances which function asa scaffold to encase the bacteria together on surfaces, and to protect them from environmentalstresses, impedes phagocytosis and thereby conferring the capacity for colonization and long-termpersistence.The present study aim to evaluate the antibacterial activity of chemo-synthesized AgNPs onbiofilm-forming XDR *Pseudomonas aeruginosa*isolated from UTIs patients. Methods: These study was conducted in Al-Qadisiyah province, Iraq at five major hospitals (AL-Diwanyia Teaching Hospital,Feminine and children teaching hospital, Afak General Hospital, AL-Hamzah General Hospital and AL-Shamiya General Hospital) during the period from (November, 2020 to June, 2021). A total of 800 urine samples were collected from male and females referring to five major hospitals. The age of the patients ranged from (1 to 80) years-old. Results:Sixty isolates were showed positive and identified as *P. aeruginosa* by using selective media, biochemical test system and VITEK-2 compact system. Genetically, in the present study, a total DNA was extracted from all clinical *P.aeruginosa* isolates. The results revealed a good response toinhibit biofilm-forming *P. aeruginosa* growth by silver

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nanoparticles antibacterialactivity by chemical preparation methodsand in combination with amikacin, ceftriaxone, ciprofloxacin and cefotaxime; the antimicrobialeffect was increased and enhanced. In the present study, it was found that the chemicalsilver nanoparticles were considered a novel and decisive solution against biofilm and extensive- drugresistance *P. aeruginosa*. Conclusion:In this study, the chemicalsilver nanoparticles were considered a good anddecisive solution against biofilm and extensive-drugresistant bacteria. Also, the antibacterial effectof chemo-synthesized of AgNPs and amikacin, ceftriaxone, ciprofloxacin and cefotaximeexhibited a higher synergistic effect than AgNPs orantibiotic alone at a significant value.

***Keywords*---**UTIs, P. aeruginosa, silver nanoparticles, biofilm.

Introduction

Urinary tract infections (UTIs) are defined as the presence of bacteria in urine along with symptoms of infection. There are three basic categories of UTI are cystitis, pyelonephritis and asymptomatic bacteriuria (1). This infection is the most commonly experienced by humans after respiratory and gastrointestinal infections and predominant cause of both hospital acquired(nosocomial) infections and community acquired for patients admitted to the hospitals (2).*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that causes nosocomial infections, as well as fatal infections in immunocompromised individuals, such as patients with cancer, post-surgery, severe burns or infected by human immunodeficiency virus (HIV) (3). It normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, from water and soil to plant and animal tissues (4). *P. aeruginosa* is a well-known biofilm former, which makes it an excellentmodel to study biofilm formation (5). A resilient biofilm is a critical weapon for *P. aeruginosa* tocompete, survive and dominate in the cystic fibrosis lung polymicrobial environment (6). *P. aeruginosa*also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants,contact lenses, etc.) (7). Common antimicrobial agents like antibiotics frequently exhibit limited efficacy due to adaptability and high intrinsic antibiotic resistance of *P. aeruginosa*, thus increasing mortality (8). Additionally, treatment ofthese infections is also hindered by the *P. aeruginosa* ability to form biofilms which protect themfrom surrounding environmental stresses, impedes phagocytosis and thereby confers capacityfor colonization and long-term persistence (9).

Silver-ions have been reported to possess strongbiocidal effects. The silver-compounds are usedas disinfection agents from the ancient time.Nanoparticles have dimension less than 100 nm.Silver nanoparticles (AgNPs) contain compoundswhich act as antimicrobial agents (10). Recently, silvernanoparticles have been considered as powerfulantimicrobial agents, especially with increasingincidence of diseases associated with biofilm andmulti-drug resistance pathogens which are necessarilyrequired to find a novel path to eradicate that challenge.Moreover, there are wide range of studies focusingon AgNPs antimicrobial activity (11). They possess ahigh activity against microorganisms (bacteria, fungi,and virus) but the mechanism of action still mostlyunknown.Silver has long standing antibacterial compound, andsilver nanoparticles are more potent in antimicrobialeffect than normal scale (12). Silver nanoparticlesincrease bacterial susceptibility to antibiotics whencombined with them as synergistic effect especiallyin biofilm infection, for example, nitrofurazoneincreased its effect in silver present (13).Studies have pointed out that silver is non-toxic,safe, and may not accumulate or cause harmful effectsto human body, so silver nanoparticles have been usedin the medical field as wound dressing, heart valvesand face mask (14). Many methods such as chemicaland biological methods have been used to synthesize silver nanoparticles (15).The chemical method used in nanoparticlessynthesis is the chemical reduction, which reducesthe metal ions to nano-sized particles by reductionagents such as sodium citrate, sodium borohydride,elemental hydrogen, ascorbate, etc. (16).The medical world is in urgent need of a newway to eradicate and kill biofilm-forming bacteria.AgNPs are the most promising antimicrobial agents tofill this role (17).

Materials and Methods

Study Design and Sample Collection

These study was conducted in Al-Qadisiyah province, Iraq at five major hospitals (AL-Diwanyia Teaching Hospital,Feminine and children teaching hospital, Afak General Hospital, AL-Hamzah General Hospital and AL-Shamiya General Hospital) during the period from (November, 2020 to June, 2021). For each patient, medical records were included name, gender, age, hospitalization, address, and antibiotic receiving. A total of 800 urine samples were collected from male and females referring to five major hospitals. The age of the patients ranged from (1 to 80) years-old.

Inclusion and Exclusion Criteria

Inclusion criteria included male and females gender, positive microbiological evidence of UTI (bacterial growth of higher than 10^5 CFU/mL), and willingness to be recruited in the study, all the *P. aeruginosa* isolates included in present study were obtained from patients specimens has UTIs without any additional substances. The study did not included any specimens with incomplete information and patients who have been on antibiotics for at least three days, and forbidden biological materials or genetically modified organisms.

Ethical Approval

All subjects involved in this work were informed and theagreement required for doing the experiments and publicationof this work was obtained from each one prior the collectionof samples. The study protocol and the subject informationand consent form were reviewed and approved by alocal ethics committee at (College of Medicine Universityof Al-Qadisiyah).

Identification of *P. aeruginosa* Isolates

Depending on its morphological properties (colony form, size,color, borders, and texture), a single colony from each primarypositive culture on blood, MacConkey and nutrient agar andclassify it and examine it by light microscope after being stainedwith Gram's stain. Biochemical tests were performed on eachisolate after inspection to complete the final identificationaccording to (18) and it usedthe VITEK-2 compact system for *P. aeruginosa* final identification.

Antibiotic Susceptibility Testing

Antibiogram testing was performed with the automated VITEK-2 compact system based on the MIC technique determination by using AST-N222. Any isolates of bacteria that were resistance to at least one antibiotic in three or more classes called Multi Drug Resistance (MDR), isolates were resistant to at least one antibiotic in all but one or two classes of antibiotics called Extensive Drug Resistance (XDR), the isolates exhibited resistance to all classes of antimicrobial agents called Pan Drug Resistance (PDR) (19).

Quantitative test for biofilm formation using microtiter plate(MTP) assay

Quantitative determination of biofilm formation by selectedisolates was performed using microtiter plate (MTP) assay of 96 wellsflat bottom dish. It was performed by a spectrophotometric method,which measures the total biofilm biomass (bacterial cells andextracellular matrix). Biofilm formation assay was conducted by Banerjee, (20).

Prepareof Silver Nanoparticles (AgNPs)

The silver nanoparticles (AgNPs) were synthesized by wet method. The spherical AgNPs were prepared according to the procedure reported by Babiker*et al.,* (21). In this method, AgNPs were synthesized by using NaBH4 as reducing agent. An aqueous solution of trisodium citrate (0.5 mL, 6 mM) was added into a flask containing (50 mL of deionized water), and then, an aqueous solution of AgNO3 (1 mL, 1 mM) was added. Freshly prepared NaBH4 aqueous solution (0.5 mL, 10 mM) was quickly added, and the suspension immediately turned a light-yellow color. After 10 second, the suspension changed to a darker yellow or brown color after reaction had proceeded for another 20 second.

Characterization of AgNPs

Characterization was performed using UV visible spectroscopy, Fourier Transform Infrared (FTIR) spectroscopy, X-ray diffraction (XRD) and scanning electron microscope (SEM).

UV-Vis spectroscopy analysis

The detection of synthesized SNPs was primarily carried out by visual observation of color change via UV–visible spectroscopy at a resolution of 1 nm and explored spectrum in series of wavelength endorse 200-1100 nm at 1% at 25 °C and controlled by beer lambert low and plotted the best fitted curve absorbance (22).

Fourier transforms infrared spectroscopy (FTIR) analysis

Fourier Transform Infrared (FT-IR) spectroscopy analysis was performed using an FTIR spectrometer in attenuated complete reflective mode and a spectral range of 400 - 4000 cm-1 with a resolution of 4 cm. The powder samples for FTIR analysis were prepared by centrifugation of SNPs solution at 10000 rpm for 15 minutes, the solid pellet then washed with deionized water for three times to remove any unattached molecules to the surface of SNPs. The residues then dried at 40°C before subjecting to the FTIR analysis. The samples then mixed with KBr (the binding agent) and put into discs at high pressure (hydraulic pressure), finally the discs were scanned to obtain the FTIR spectra (23).

Field Emission Scanning Electron Microscopic (FESEM)

The synthesized silver nanoparticles were monitored morphologically using SEM (Hiyachi S-4500). A thin film of each synthesized silver nanoparticles samples were dispersed on a slide and then coated with platinum in an auto fine coater, after that the material was subjected to analysis. The micrograph and procedure were performed in RAZI Applied Research Foundation in Iran (24).

X-Ray Diffraction (XRD)

The X-ray diffraction (XRD) analysis was performed using XRD system (Phillips PW 1830) operated at a voltage of 40 kV and current of 20 mA to determine the crystallinity, metallic nature, and cubic structure of prepared sample. Analysis was carried out in in RAZI Applied Research Foundation in Iran (25).

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) Tests of AgNPs

The bacteriostatic and bactericidal potential of prepared silver nanoparticles was assessed using broth macro-dilution method. Twofold serial concentration (3.12, 6.25, 12.5, 25, 50, 100, 200, 400, 800, and 1600μg/ml) of silver nanoparticles was prepared in broth medium using sterile 10 test tubes with three control test tubes (C1: tube of broth medium that are free of bacteria, C2: tube with silver nanoparticles only, C3: tube was inoculated with the test organism only as shown in table (3-2), afterward, 18-24 hours bacterial inoculum with turbidity adjusted according to 5×10^5 CFU/ml was dispensed in tubes containing different concentrations of AgNPs and incubated at 37^o C overnight. Following incubation, the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of drug materials were determined. MIC is lowest concentration that can cause invisible growth. MBC is the lowest concentration that can prevent the growth of bacteria as confirmed by subculture.The experiment was performed in triplicate for each isolate and negative and positive control was also included (26).

Determination of Killing Time for AgNPs

Assays for the rate of killing of *P. aeruginosa* by prepared silver nanoparticles was carried out according to the method described by Zhang*et al.,* (27). The drug material was incorporated into 10 mL Mueller Hinton broth at MBC. Controls, contain Mueller Hinton broth without drugs inoculated with activated test organisms with inoculums density, approximately 10^5 cfu/mL. The tubes were incubated at 37°C. The bacterial count was made at 0, 2, 4, 6, 8,12,18,20 and 24 hrs., for the determination of cfu/mL by the plate count technique.

Determination Antibacterial Effect of Combined Antibiotics and Prepared AgNPs

The agar well diffusion method was employed to screen the antibacterial activity of different concentrations of silver nanoparticles AgNPs (250, 500, 750, 1000 µg/mL), antibiotics alone (Amikacin 30 µg/mL, Ceftriaxone 30 µg/mL, Ciprofloxacin 5 µg/mL, Cefotaxime 30 µg/mL) and combination with silver nanoparticle (500 µg/mL) against 10 different isolates of pathogenic *P. aeruginosa* (2, 6, 18, 31, 34, 37, 41, 44, 55 and 59) that are sensitive for these antibiotics. Inoculum was prepared using fresh cultures of bacteria strains cultured on nutrient agar. A loop full of bacteria culture was inoculated into a nutrient broth medium and incubated for 24h at 37°C. The size was adjusted to 0.5 McFarland standard turbidity, approximately 10^8 colony-forming units (CFU/ml). Cell suspensions (100µl of target strain) were introduced into the nutrient agar plates and spread thinly on the plates using a glass spreader, wells of 6mm diameter were impregnated with 100µl of each prepared silver nanoparticle concentration or antibiotics and DMSO as control. The petridishes were then incubated at 37°C for 24h under aerobic conditions. The diameter of the inhibition zones (in millimeters) around the wells was measured after 24h, tests were performed in duplicate (28).

Statistical Analysis

The results in the present study were evaluated statistically via T test and Chi square using Statistical Package for Social Sciences (SPSS) program version 23 at a probability of ($P \leq 0.05$) as a significant level between the parameters of the present study such as gender, age, and source of samples. *P* value of <0.05 was considered significant (29).

Results and Discussion

Isolation and Identification of *P. aeruginosa*

A total of 800 samples were collected from patients suffering from urinary tract infections were admitted and visited five major hospitals in Al-Qadisiyah province, during the period from (November, 2020 to June, 2021),sixty (7.5%) isolates were showed positive and identified as *P. aeruginosa* by using selective media, biochemical test system and VITEK-2 compact system. During this study, the origin of *P. aeruginosa* isolates according to the gender, age and hospitalization are presented in Table (3-1). The number of *P. aeruginosa* isolates recovered from patients was 23 (38.34%), followed by 14 (23.34%), 10 (16.67%), 7 (11.66%) and 6 (10.0%) in the age group 16-30 years, 31-45 years, 46-60 years, less than 15 years, and more than 60 years respectively. The relation between the age groups wise profiles of *P. aeruginosa* infection was found statistically not significant (P= 0.081). Out of 60 patients from whom *P. aeruginosa* were isolated 38 (63.4%) were female patients and 22 (36.6%) were male patients which was statistically significant (*P*= 0.05) when compared to gender-distribution of patients from whom *P. aeruginosa* isolated. It was observed that hospitalized patients (24, 40.0%) were less infected with *P. aeruginosa* infection as compared to outpatients (36, 60.0%).Belal, (29) in Najaf and Abdul-Wahid, (30) in Thi-Qar reported (9.8) and (8.9%) of *P. aeruginosa* among the urine samples of patients with UTI, respectively.

Table (3-1): Frequency distribution of patients with *P. aeruginosa* infections (n=60)

Patientsprofile	Status	No. (%) ofsamples (n=800)	No. (%) of *P.aeruginosa*(n=60)	*P*-value
Age group(year)	<15	113 (14.13)	7 (11.66)	¥ P=0.081 NS
	16-30	235 (29.37)	23 (38.34)	
	31-45	217 (27.12)	14 (23.33)	
	46-60	130 (16.25)	10 (16.67)	
	>60	105 (13.12)	6 (10.0)	
Gender	Male	330 (41.25)	22 (36.6)	¥ P=0.001 HS
	Female	470 (58.75)	38 (63.4)	
Hospitalization	Outpatient	513 (64.13)	36 (60.0)	¥ P= 0.640 NS
	Inpatient	287 (35.87)	24 (40.0)	

¥: Chi-square test; HS: Highly significant; NS: not significant at P ≤ 0.05

Sensitivity Profile of *P. aeruginosa* Isolates

All the 60 confirmed isolates of *P. aeruginosa* were evaluated for susceptibility to 17 selected antibiotics that are belonging to the ten generic classes of antimicrobial antibiotics according to (31). The Antibiotic susceptibility test was assessed by automated VITEK-2 compact system using AST-N222 cards. The sensitivity and resistance pattern of the isolates showed in (Table 3-2).

Table 3-2: Antibiotic susceptibility test of *P. aeruginosa* isolates by VITEK-2 compact system

Antibioticagent	No.of isolates(n=60)			P-value
	Resistance (R)	Intermediate(I)	Sensitive(S)	
Piperacillin	45(75.0%)	0(0%)	15(25.0%)	
Ticarcillin	50(83.34%)	0(0%)	10(16.66%)	
Ticarcillin/clavulanic	41(68.34%)	0(0%)	19(31.66%)	
Piperacillin/tazobactam	15(25.0%)	0(0%)	45(75.0%)	
Ceftazidime	40 (66.66%)	7 (11.66%)	13 (21.68%)	

Ceftriaxone	43 (71.66%)	7 (11.66%)	10 (16.68%)	¥ P= 0.001 HS
Cefotaxime	42 (70.0%)	6 (10.0%)	12 (20.0%)	
Meropenem	20(33.34%)	9(15.0%)	31(51.66%)	
Ciprofloxacin	35(58.34%)	14(23.33%)	11(18.33%)	
Pefloxacin	36(60.0%)	3(5.0%)	21(35.0%)	
Amikacin	26(43.34%)	7(11.66%)	27(45.0%)	
Tobramycin	28(46.66%)	3(5.0%)	29(48.34%)	
Gentamicin	37(61.66%)	5(8.34%)	18(30.0%)	
Aztreonam	33(55.0%)	5(8.34%)	22(36.66%)	
Colistin	12(20.0%)	0(0%)	48(80.0%)	
Minocycline	54(90.0%)	2(3.34%)	4(6.66%)	
Trimethoprim+sulfamthoxazole	57(95.0%)	0(0%)	3 (5.0%)	

¥: Chi-square test; HS: Highly significant

Resistance of *P. aeruginosa* to most antibiotics develops very rapidly and arises from the combination of unusually restricted outer-membrane permeability and secondary resistance mechanisms such as chromosomally or plasmid encoded periplasmic β-lactamase and energy- dependent multidrug efflux (32).In the current study, the highest antibiotic resistance of (75.0%, 83.34%, 90.0%, and 95.0%) was topiperacillin, ticarcillin, minocycline and trimethoprim+sulfamthoxazole,respectively.The moderate antibiotic resistance of (66.66%, 65.0%, 58.34%, 60.0%, 61.66%, and 55.0%) was to ceftazidime, cefepime, ciprofloxacin, pefloxacin, gentamicin and aztreonam,respectively. While, the lowest antibiotic resistance of (40.0%, 33.34% and 20.0%) was to imipenem, meropenem and colistin, respectively.A study in Iraq reported nearly similar results, wherethe resistant rates for penicillin antibiotics including ticarcillin, piperacillin and minocycline and trimethoprim+sulfamthoxazole were 76.7%, 81.0%, 91.0% and 93.5%, respectively (33). Also, Abdul-Wahid, (30) in Iraq reported similar results wherethe resistant rates for ceftazidime, cefepime, ciprofloxacin, pefloxacin, gentamicin and aztreonam (64.0%, 63.5%, 59.0%, 58.5%, 63.0% and 57.0%),respectively. While, the other study in Iraq by Al-kazrage(34) reported the resistant rates forimipenem, meropenem and colistin (35.0%, 31.0% and 21.8%),respectively. The variation in the level of resistance between different studiesmay be attributed to the difference in geographical distribution, typeand number of samples collected in each study and the difference inantibiotic policies implemented in each country.

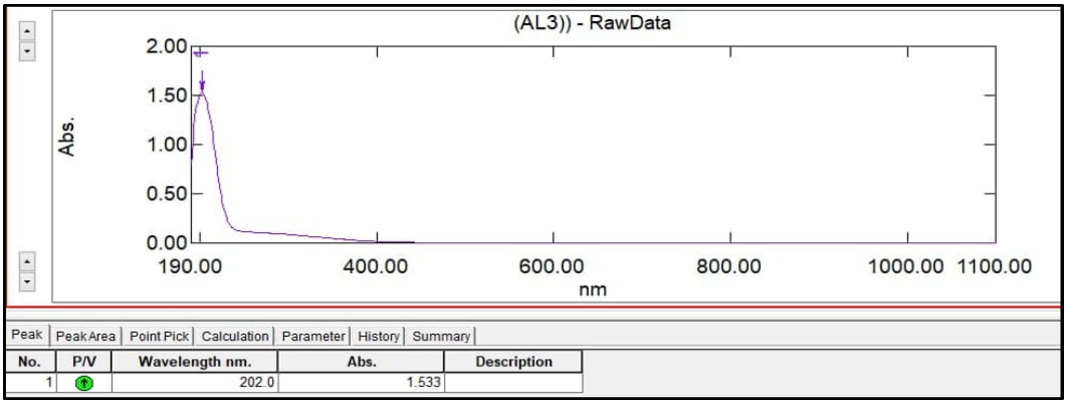
Quantitative Determination of Biofilm by MTP Test

MTP assay which issime-quantitative indirect indicator to the comparing among isolates incapability of biofilm establishment. It was found that 54 (90.0%) of the total isolates were biofilmformation in which 31 (51.67%) were strong biofilm, 15 (25.0%) were moderate and weakbiofilm producing isolates were 8 (13.33%) and 6 (10.0%) of the total isolates were non-biofilmformation. The results show significant differences at ($P \leq 0.01$) of thebiofilm formation for the strong and moderate and ($P \leq 0.05$) for the weak biofilm.Many studies highlight similar results of biofilm formation ranged from 83.75%-90% (35) while low frequency (24%-27%) of biofilm formation stated by another studies (36). Biofilm style of *P. aeruginosa* represent the aggregates encased in a self-produced extracellular matrix and are difficult or impossible to eradicate with antibiotic treatment. Biofilms are estimated to be responsible for over 65% of nosocomial infections and 60% of all human infections. Additionally, the biofilm risk originated from the fact that, it is the main driver of persistence of chronic infections (37).The results in the current study were corresponding with the results mentioned by Al-kazrage, (34) it was found that 90% of the total isolates were biofilm formation in which 50% were strong biofilm.

Characterization of AgNPs

UV-Spectroscopy

The presence of AgNPs was confirmed by using UV visible spectrophotometer and by obtaining a spectrum in the visible range of 250 nm to 800 nm. From this analysis, absorbance peak was found at around 450 nm as shown in figure (3-1) which served as visual confirmation for the formation of nanoparticles.The UV–Vis absorption band in the current visible light region (420–450) nm is an evidence of the presence of SPR of AgNPs (38). Moreover, narrow peak indicates that the narrow size range of nanoparticles is less than100 nm (39).



No.	P/V	Wavelength nm.	Abs.	Description
1	●	202.0	1.533	

Figure 3-1: UV-VIS Absorbance Spectroscopy for AgNPs

Fourier Transform Infrared Spectroscopy (FTIR)

The results illustrated FTIR spectrum of biological synthesized green AgNPs in the wavelength range from 500 cm^{-1} to 4000 cm^{-1}. The band detected at 3286.70 cm^{-1} assigned to hydroxyl (OH) group and, the slight bands observed at 2110.12 cm^{-1} symbolize the methyl (CH) groups, the band detected at 1631.78 cm^{-1}, the ester group (C=O) and the band detected at 972.12 cm^{-1} referred to amine group (NH), the band detected at 771.53 cm^{-1} represents alkenyl (C=C) stretch. The bands at 663.51 cm^{-1} related to carboxylate. While, 582.50 cm^{-1} represents secondary alcohol (CO) stretch as shown in figure (3-2).FTIR spectra were analysed to detect biomolecules that involve in Ag+ reduction (40). The FTIR analysis in this study indicated the involvement of carboxyl, amino, amides groups and polyphenols in the synthesized green AgNPs. It is well known that there are amino acid, polyphenols, and protein in tea, the tea extract’s organic compounds could assign to the reduction of AgNO3 and the stabilization of AgNPs by the surface bound by the organic compounds (41).This result was very close to Gavamukulya *et al.,* (42). That included the functional groups responsible for AgNPs formation; alkenyl, alcohol groups, carboxylic acids, amides, and polyphenols.

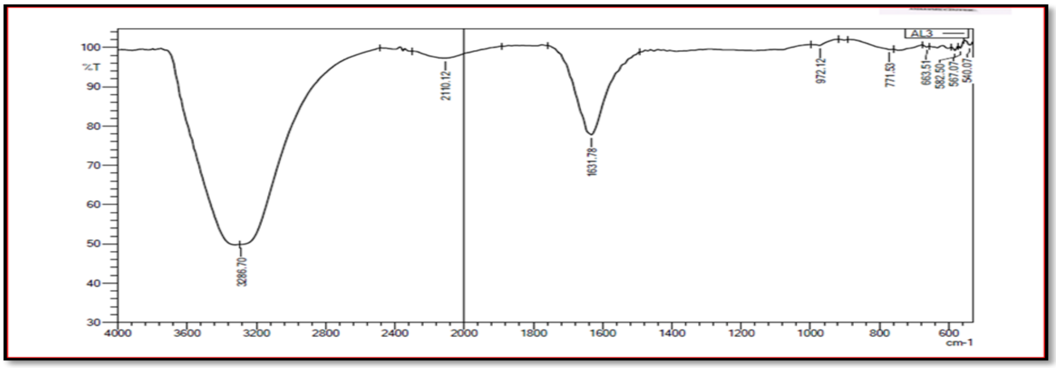


Figure 3-2: FTIR Spectra Pattern of for AgNPs

Scanning Electron Microscope (SEM) analysis

Another technique used to determine the distribution, shape and size of synthesized green AgNPs is SEM. Figure (3-3) illustrated the particles were spherical in shape and ranged (64.27–78.76 nm), in terms of size, particles were different, which implies the fact that the process introduced for AgNPs green synthesis is flexible in producing the nanoparticle with different size distribution profile and spectrum of particle size.Particularly, using sonication and stirring, both crack the particles' agglomeration and makes them (43). The SEM image of AgNPs was resulted from the interactions of hydrogen bond and electrostatic interactions between the bio-organic capping molecules bound to the AgNPs (44).

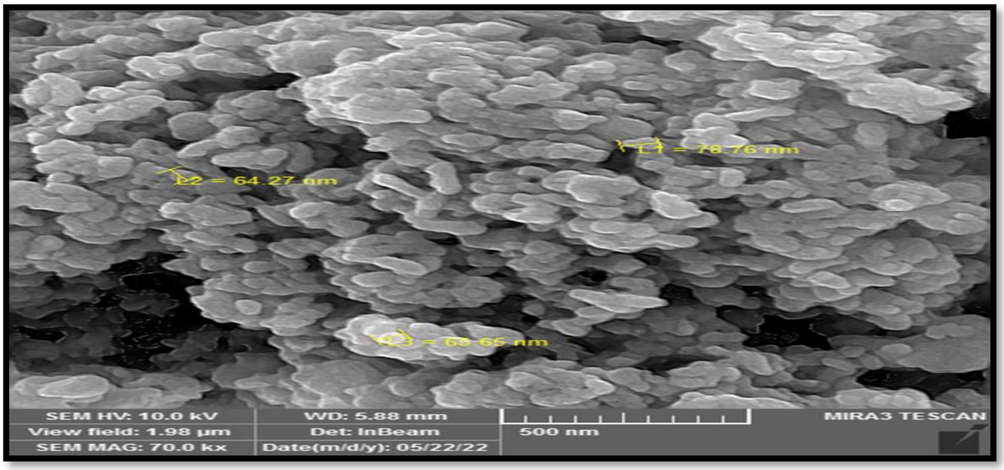


Figure 3-3: SEM image of for AgNPs

X-Ray Diffraction (XRD)

The XRD analysis of AgNPs shown in figure (3-4), which proposed that the reactionmixture of silver ions was reduced to a silver element. Approximately at 3keV, the most principal sharp signal was observed to be silver, which is distinguishing the crystalline absorption nature of biosynthesized AgNPs.Some of the weak peaks for Si, O, C, Cl, S and Al were also found. The presence of the weak signal may possibly bedue to the biomolecules that are bound to the surface of AgNPs (45). In another study, the synthesis of AgNPs using Pterocarpus *santalinus* leaf extract showed peak at the energy of 3 ke V for silver, and also some of the weak peaks for C, O, Cl, Al, and Na (46).

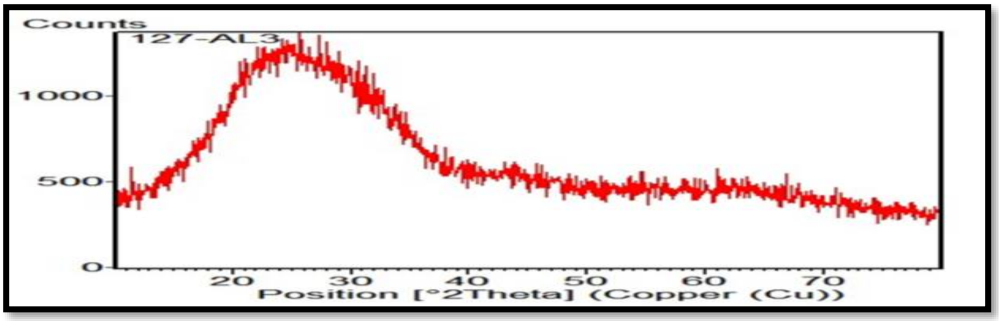


Figure 3-4: XRD report, lattice planes resulted by AgNPs

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of AgNPs against *P. aeruginosa*

The inhibitory effects of different concentrations of AgNPs on *P. aeruginosa*isolates were examined and described in table (3-3). MIC of AgNPs is ranged between (25 - 100 µg/ml) on *P. aeruginosa* isolates, the lowest concentration of 3.12 µg/ml showed the smaller inhibition zone. While, the higher concentration of 1600 µg/ml revealed the larger inhibition zone, the effect of green AgNPs was clear with a highly significant difference P-Value= 0.001.

Table (3-3): The MIC and MBC values of AgNPs against *P. aeruginosa*

Category	Mean	SE
MIC value	80	32.97
MBC value	220	73.48

These results of this study were accordant with the results of a study done in Babylon by Al-Tameme, (47), which found that the green AgNPs MBC value for *P. aeruginosa* was (200 µg.mL^{-1}), while, Raheem *et al.*, (48) established the MIC of green AgNPs ranged (16-125 µg.mL^{-1}) and MBC (125-500 µg.mL^{-1}) against *E. coli, K. Pneumoniae, S. typhi, S. aureus, P. mirabilis* and *P. aeruginosa.* This result was also agreed with the result of Masoumi and Shakibaie, (49) reported that the values of MIC for AgNPs were between (20- 150 µg/ml) that showed high concentration in MIC and MBC values against *P. aeruginosa.*

Antibacterial Activity of Silver Nanoparticles (AgNPs) by Agar well diffusion method

The results showed that the antibacterial activity of prepared silver nanoparticles was examined towards *P. aeruginosa* bacteria as shown in table (3-4) and figure (3-5). The prepared AgNPs effected on the growth of pathogenic bacteria by zone of inhibition ranging between (10-15mm).

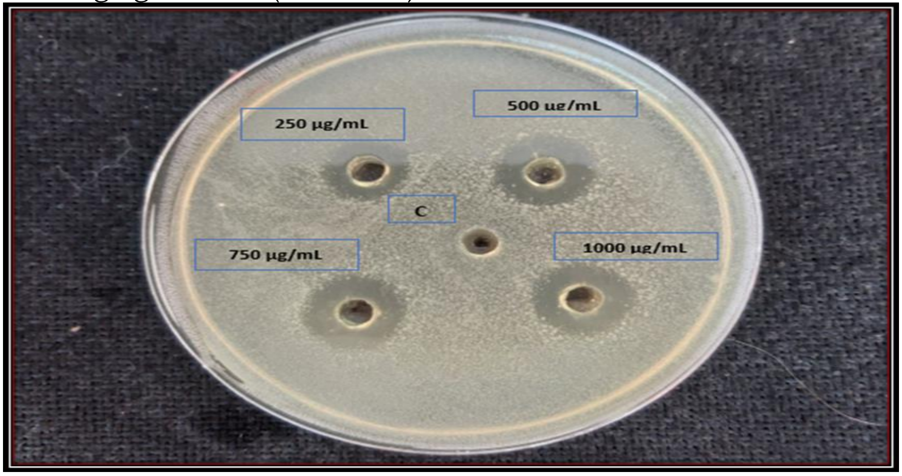


Figure 3-5: Zone of inhibition of AgNPs against *P. aeruginosa*

Table 3-4: Inhibition Zone of AgNPs against *P. aeruginosa*

Drug materials	Concentration	Mean	SE
SNPs	250 µ/ml	11.3A	0.37
	500µ/ml	12.6B	0.24
	750µ/ml	13.7C	0.3
	1000µ/ml	13.8C	0.37
Negative control DMSO	-----	0D	0
LSD(P<0.05)	0.865		

Excellent antibacterial activity was revealed by the biologically synthesized AgNPs against bacterial pathogens. The negatively charged bacterial cell wall may attach to the AgNPs discharge silver ions and rupture it, consequently leading to the denaturation of protein and death of the cell. These massive changes in the bacteria membrane structure as a result of the silver cations' interaction cause the increased of the bacteria membrane permeability (50).

Determination of Killing Time for AgNPs

The kill time test of AgNPs was performed to estimate its bactericidal effect against *P. aeruginosa*. The results showed that the time of killing of *P. aeruginosa* by AgNPs was (8-10) hr. as shown in table (3-5).

Table (3-5): Killing Time for AgNPs against *P. aeruginosa*

Category	Mean	SE
Killing Time	10.8	1.85

The mechanism of the nanoparticles action on bacteria is not well understood, and may be due to the interaction between these particles and the groups of sulfur and phosphorus found in the bacterial cell membrane because the proteins of cell membrane are the preferred sites for the work of these particles, which lead to the destruction of the cell and death (51).

Antibacterial of Combined Antibiotics and AgNPs

In this study, the effects of AgNPs with some antibiotic combination were studied. Antibiotics, such as Amikacin (AK), Ceftriaxone (CRO), Ciprofloxacin (CIP) and Cefotaxime (CTX) against 10 different isolates of *P. aeruginosa* that are sensitive for these antibiotics. For Amikacin (AK) the inhibition zone ranging between (22.6±0.74Aa), while the inhibition zone of combined AgNPs with Amikacin ranging between (28.2±0.44Ab) as shown in table (3-6) and figures (3-6a, 3-6b).For Ceftriaxone (CRO) the inhibition zone ranging between (26±0.31Ba), while the inhibition zone of combined AgNPs with Ceftriaxone (CRO) ranging between (30±0.63Bb) as shown in table (3-6) and figures (3-6a, 3-6b). For Ciprofloxacin (CIP) the inhibition zone ranging between (23.8±0.48Aa), while the inhibition zone of combined AgNPs with Ciprofloxacin ranging between (31±0.44Bb) as shown in table (3-6) and figures (3-6a, 3-6b).For Cefotaxime (CTX) the inhibition zone ranging between (25.6±0.24Ba), while the inhibition zone of combined AgNPs with Cefotaxime ranging between (31±0.37Bb) as shown in table (3-6) and figures (3-6a, 3-6b).

Table (3-6): Antibacterial activity of combination antibiotics and AgNPs

Type of antibiotics	Zone of inhibition (mm)	
	Antibiotic alone	Combination with AgNPs
Amikacin (AK)	22.6±0.74Aa	28.2±0.44Ab
Ceftriaxone (CRO)	26±0.31Ba	30±0.63Bb
Ciprofloxacin (CIP)	23.8±0.48Aa	31±0.44Bb
Cefotaxime (CTX)	25.6±0.24Ba	31±0.37Bb
Negative control DMSO	0±0Ca	0±0Ca
LSD(P<0.05)	1.54	

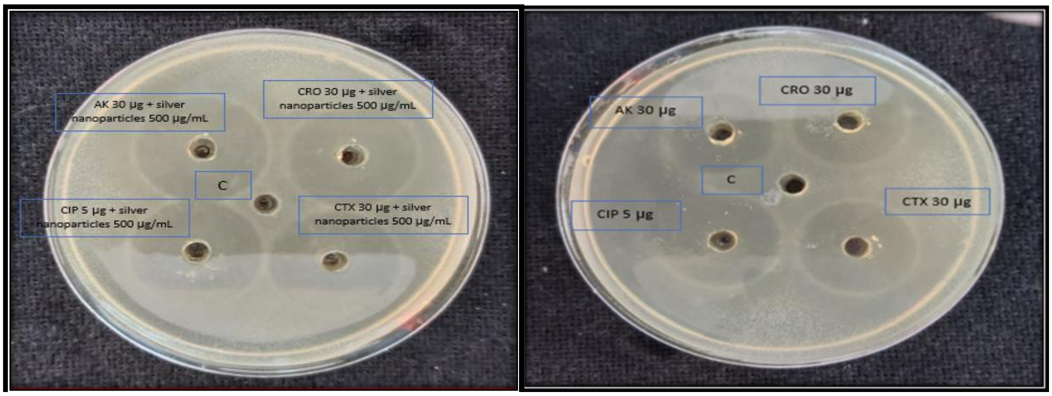


Figure (3-6a, 3-6b): Antibacterial activity of a number of antibiotics alone and combined with AgNPs against *P. aeruginosa* isolates

The synergistic effect between antimicrobial drugs and AgNPs has already been tested, and it was proved that it potentiates antimicrobial action against antibiotic-sensitive and MDR *P. aeruginosa* strains. Studies have associated AgNPs with chloramphenicol, kanamycin, vancomycin, ciprofloxacin, and polymyxin B. In this sense, AgNPs optimize and facilitate the infiltration of antibiotics, allowing maximum efficiency, as well as promoting damage to the microorganism (52). The antibacterial effect of combination AgNPs and antibiotic exhibited higher than AgNPs or antibiotic alone, this result corresponded with. Moreover those researchers pointed out that combined antibiotics with AgNPs make it as greater anti- biofilm activity and elevated bacterial cell death level, so treatment with combination of antibiotics and AgNPs consider more potent effectiveness as antibacterial and ant- biofilm (53).Recently reported that combination between AgNPsand Vancomycin increase inhibit biofilm activity to 55% and 75% to gram positive and negative bacteria respectively. AgNPs as other NPs may disrupt the bacterial cell wall formation and cause damage to the cellular proteins and nitrogenous bases in the bacterial components (54).

Conclusion

Quorum sensing system take a critical part in pathogenicity of *P. aeruginosa*, and it’s an excellent was for bacteria to increase growth and resistant of antibiotics.

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References

1. Foxman, B.(2020). Urinary tract infection syndromes: occurrence, recurrence, bacteriology, risk factors, and disease burden. Infect. Dis. Clin. North America., 28 (1):1-13.
2. Dunne, M. W., Puttagunta, S., Aronin, S. I., Brossette, S., Murray, J., &

Gupta, V. (2022). Impact of Empirical Antibiotic Therapy on Outcomes of Outpatient Urinary Tract Infection Due to Nonsusceptible Enterobacterales. *Microbiology spectrum*, *10*(1), e02359-02321.

3. Gomila, A.; Carratalà, J.; Badia, J.M.; Camprubí, D.; Piriz, M.; Shaw, E.; Diaz-Brito, V.; Espejo, E.; Nicolás, C.; Brugués, M.; et al. (2018). Preoperative oral antibiotic prophylaxis reduces *Pseudomonas aeruginosa* surgical site infections after elective colorectal surgery: A multicenter prospective cohort study. *BMC Infect. Dis*, *18*, 507.
4. Galdino, A. C. M., Branquinha, M. H., Santos, A. L., & Viganor, L. (2017). *Pseudomonas aeruginosa* and its arsenal of proteases: weapons to battle the host. *Pathophysiological Aspects of proteases*, 381-397.
5. Ghafoor, A.; Hay, I.D.; Rehm, B.H. (2011).Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formationand architecture. *Appl. Environ. Microbiol.*, *77*, 5238–5246.
6. Crespo, A.; Blanco-Cabra, N.; Torrents, E. (2018).Aerobic Vitamin B12 Biosynthesis Is Essential for *Pseudomonasaeruginosa* Class II Ribonucleotide Reductase Activity During Planktonic and Biofilm Growth. *Front. Microbiol.*, *9*, 986.
7. Oluyombo, O.; Penfol. M d, C.N.; Diggle, S.P. (2019).Competition in Biofilms between Cystic Fibrosis Isolates of*Pseudomonas aeruginosa*is Shaped by R-Pyocins. *mBio*, *10*, e01828-18.
8. Pang, Z.; Raudonis, R.; Glick, B.R.; Lin, T.J.; Cheng, Z. (2019).Antibiotic resistance in *Pseudomonas aeruginosa*: Mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.*, *37*, 177–192.
9. Moradali, M.F.; Ghods, S.; Rehm, B.H. (2017).*Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front. Cell Infect. Microbiol,7*, 39.
10. Romling, U.; Balsalobre, C. (2012).Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med*, *272*, 541–561.
11. M. Radzig, V.A. Nadtochenko, O.A. Koksharova, et al., (2013). Antibacterial effects of silver nanoparticles on gramnegative bacteria: influence on the growth and biofilms formation, mechanisms of action. *Colloids and Surfaces B: Biointerfaces*, 102: 300-306.
12. M. Prabakaran, K.V. Nithya, P. Gajendiran, et al., (2018). Green synthesis of piperine/triton X-100/silver nanoconjugates:antimicrobial activity and cytotoxicity. Nano Biomedicine and Engineering, 10(2): 141-148.
13. S.O. Hasson, M.J. Al-Awady, A.H. Al-Hamadani, et al., Boosting antimicrobial activity of imipenem incombination with silver nanoparticles towards S. fonticolaand Pantoea sp. Nano Biomed. Eng., 2019, 11(2): 200-214.
14. Theivasanthi, M. Alagar, (2011). Anti-bacterial studies of silver nanoparticles. *arXiv.org,*: arXiv:1101.0348 [physics.gen-ph].
15. M.J. Al-Awady, A.A.J.N.B.E. Balakit, (2019). Investigation of anti-MRSA and anticancer activity of eco-friendly synthesized silver nanoparticles from palm dates extract. *Nano Biomed. Eng.*, 10(2): 157-169.
16. G. Sharma, A.S. Kumar, S. Naushad, et al., (2017). Novel development of nanoparticles to bimetallic nanoparticles and their composites: A review. *Journal of King Saud University-Science*.
17. S. Tang, J. Zheng, Antibacterial activity of silver nanoparticles: Structural effects. *Advanced Healthcare Materials*, 2018: 1701503.

18. MacFaddin, J.F. (2004).Biochemical tests for identification of medical bacteria (7rd ed.), Lippincott Williams and Wilkins, USA.
19. Daragon, B., Fournier, D., Plésiat, P., & Jeannot, K. (2021). Performance of disc diffusion, MIC gradient tests and Vitek 2 for ceftolozane/tazobactam and ceftazidime/avibactam susceptibility testing of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*, *76*(10), 2586-2592.
20. Banerjee, G. (2014). Comparative Study of Biofilm Formation in *Pseudomonas aeruginosa* isolates from Patients of Lower Respiratory Tract Infection. Journal of clinical and diagnostic research, 8(5), 9–11.
21. Babiker, K.S.; Idrees, D.A.; Alumagadam, T. M.(2020). Efficacy of propolis extract against *S. aureus, P. aeruginosa, and K. pneumoniae* strains isolated from patients with wound infection. Pharmacy & Pharmacology International Journal. 8(5): 269-272.
22. Ahamad, I., Aziz, N., Zaki, A., & Fatma, T. (2021). Synthesis and characterization of silver nanoparticles using *Anabaena variabilis* as a potential antimicrobial agent. Journal of Applied Phycology, 33(2), 829-841.
23. Bajracharya, A.; Pawar, D., Mourya, P. and Patil, A. (2018). Bacterial synthesis of silver nanoparticles. World Journal of Pharmaceutical research. 7(6):872-887.
24. Prasad, K.S.; D.Pathak; A.Patel; P. Dalwadi; R. Prasad; P. Patel, and K. Selvaraj. (2011). Biogenic synthesis of silver nanoparticles using *Nicotiana tobaccum* leaf extract and study of their antibacterial effect. *Afr J Biotechnol., 10* (41): 8122.
25. BAKIR, Ö., Güller, P., & KURBANOĞLU, E. B. (2022). Synthesis, characterization, antibacterial, antioxidant activity, and lipoxygenase enzyme inhibition profile of silver nanoparticles (AgNPs) by green synthesis from Seseli resinosum Freyn & Sint. *International Journal of Plant Based Pharmaceuticals*, *2*(2), 182-189.
26. Zhang, X.-F.; Liu, Z.-G.; Shen, W. and Gurunathan, S. (2016). Silver Nanoparticles: Synthesis, Characterization, Properties, Applications, and Therapeutic Approaches. International Journal of Molecular Sciences, 17(9), 1534.
27. Aabed, K., and Mohammed, A. E. (2021). Synergistic and Antagonistic Effects of Biogenic Silver Nanoparticles in Combination with Antibiotics Against Some Pathogenic Microbes. Frontiers in Bioengineering and Biotechnology, 9, 249.
28. McDonald, J.H. (2014). Handbook of Biological Statistics. 3rd ed. USA: Sparky House Publishing.
29. Belal, E. J. K. (2010). Investigation of some B-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa* in Najaf City. M.Sc. Thesis. Edication of Girls/ University of Kufa. Iraq.
30. Abdul-Wahid, Ali, A. (2020). Antibiotic Sensitivity and Molecular Profile of Carbapenemase-Producing *Pseudomonas aeruginosa* from hospital-acquired infections in Thi-Qar province. PhD. Thesis, College of Medicine, University of Al-Qadissiyah.
31. Clinical and Laboratory Standards Institute (CLSI).(2020). Performance standards for antimicrobial susceptibility testing. CLSI supplement M100, 30th. Ed. Clinical and Laboratory Standards Institute, Wayne, PA.

32. Muderris, T.; Durmaz, R. and Ozdem, B. (2018). Role of efflux pump and OprD porin expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. J Infect Dev. Ctries. 12(1):1–8.
33. Al-Janahi, Haider C. L. (2020).Occurrence and Molecular Characterization of Metallo-β-Lactamase (MBLs)-Producing *Pseudomonas aeruginosa* in Najaf Hospitals. MSc. Thesis, Faculty of Medicine, University of Kufa.
34. Al-kazrage, Hussein A. D. (2021).Inhibition of Virulence Factors in *Pseudomonas aeruginosa* Isolated from Clinical Samples Using Galardin Loaded AgPEG Nanocomposite. PhD. Thesis, College of Biotechnology, University of Al-Nahrain.
35. Kamali E, Jamali A, Ardebili A, Ezadi F, Mohebbi A (2020). Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of Pseudomonas aeruginosa. BMC Research Notes 13(1): 27.
36. Abdelraheem, W. M., Abdelkader, A. E., Mohamed, E. S., & Mohammed, M. S. (2020). Detection of biofilm formation and assessment of biofilm genes expression in different *Pseudomonas aeruginosa* clinical isolates. Meta Gene, 23, 100646.
37. Ciofu O, Tolker-Nielsen T (2019). Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents-how *P. aeruginosa* can escape antibiotics. Frontiers in microbiology 10: 913.
38. Muthukrishnan, S., Bhakya, S., Senthil Kumar, T. and Rao, M.V. (2015) 'Biosynthesis,characterization and antibacterial effect of plant-mediated silver nanoparticles usingCeropegiathwaitesii – An endemic species', *Industrial Crops and Products,* 63, pp. 119-124.
39. De Leersnyder, I., Rijckaert, H., De Gelder, L., Van Driessche, I., & Vermeir, P. (2020). High variability in silver particle characteristics, silver concentrations, and production batches of commercially available products indicates the need for a more rigorous approach. Nanomaterials, 10(7), 1394.
40. Ulagesan, S., Nam, T.-J., & Choi, Y.-H. (2021). Biogenic preparation and characterization of Pyropia yezoensis silver nanoparticles (Py AgNPs) and their antibacterial activity against Pseudomonas aeruginosa. *Bioprocess and Biosystems Engineering*, *44*(3), 443-452.
41. Zhang, X., Zhang, Z., Wu, W., Yang, J., & Yang, Q. (2021). Preparation and characterization of chitosan/Nano-ZnO composite film with antimicrobial activity. Bioprocess and Biosystems Engineering, 44(6), 1193-1199.
42. Gavamukulya, Y., Maina, E. N., Wamunyokoli, F., Meroka, A. M., Madivoli, E. S., El-Shemy, H. A., & Magoma, G. (2019).Synthesis and Characterization of Silver Nanoparticles from Ethanolic Extracts of Leaves of *Annona muricata*: A Green Nanobiotechnology Approach. *Biotechnol. J. Int*, *4*, 1-18.
43. Samuggam, S., Chinni, S. V., Mutusamy, P., Gopinath, S. C., Anbu, P., Venugopal, V., Reddy, L. V., & Enugutti, B. (2021). Green Synthesis and Characterization of Silver Nanoparticles Using Spondias mombin Extract and Their Antimicrobial Activity against Biofilm-Producing Bacteria. *Molecules*, *26*(9), 2681.
44. Pham, T. T. H., Vu, X. H., Trinh, D. K., Nguyen, D. D., Luong, D. T., Nguyen, Q. H., & Luong, V. L. (2021). Synthesis and Characterization of Silver Nanoparticles for Antibacterial Application against *Bacillus Subtilis* and *Pseudomonas Aeruginosa*. VNU Journal of Science: Mathematics-Physics, 37(2).

45. Zubair, M., Azeem, M., Mumtaz, R., Younas, M., Adrees, M., Zubair, E., Khalid, A., Hafeez, F., Rizwan, M., & Ali, S. (2022). Green synthesis and characterization of silver nanoparticles from *Acacia nilotica* and their anticancer, antidiabetic and antioxidant efficacy. *Environmental Pollution*, *304*, 119249.
46. Beeler, E., Choy, N., Franks, J., Mulcahy, F., & Singh, O. V. (2020). Extracellular Synthesis and Characterization of Silver Nanoparticles from Alkaliphilic Pseudomonas sp. *Journal of Nanoscience and Nanotechnology*, *20*(3), 1567-1577.
47. Al-Tameme, S.A. (2017). The Antimicrobial Effect of Silver NanoParticles (AgNPs) Against *Pseudomonas aeruginosa* Isolated from Clinical Samples. M.Sc.
48. Raheem, H.Q., Al-Thahab, A. and Abd, F.G. (2018). 'Antibacterialactivity of silver nanoparticles extracted from *Proteus mirabilis* andhealing the wound in rabbit', *Biochemical and CellularArchives,* 18(1), pp. 97-104.
49. Masoumi, S., and Shakibaie**,** M. R. (2018). Evaluation Synergistic Effect ofTiO2, AgNPs Nanoparticles and Amphiphilic Peptides (Mastoparan-B, Indolicidin)Against Drug-Resistant *Pseudomonas aeruginosa, Klebsiella pneumoniae* and*Acinetobacter baumannii.*
50. Singh P, Pandit S, Garnæs J, Tunjic S, Mokkapati VR, Sultan A, Thygesen A, Mackevica A, Mateiu RV, Daugaard AE, Baun A (2018). Green synthesis of gold and silver nanoparticles from Cannabis sativa (industrial hemp) and their capacity for biofilm inhibition. Int J Nanomedicine 13:3571–3591.
51. Garg, D., Sarkar, A., Chand, P., Bansal, P., Gola, D., Sharma, S., Khantwal, S., Mehrotra, R., Chauhan, N., & Bharti, R. K. (2020). Synthesis of silver nanoparticles utilizing various biological systems: mechanisms and applications—a review. Progress in Biomaterials, 9(3), 81-95.
52. Al-Obaidi H, Kalgudi R, Zariwala MG (2018). Fabrication of inhaled hybrid silver/ciprofloxacin nanoparticles with synergetic effect against *Pseudomonas aeruginosa*. Eur J Pharm Biopharm 128:27–35.
53. Bose D, Chatterjee S. (2016). Biogenic synthesis of silver nanoparticles using guava (*Psidium guajava*) leaf extract and its antibacterial activity against *Pseudomonas aeruginosa*. Appl Nanosci6 (6):895–901.
54. Erjaee, H., Rajaian, H., & Nazifi, S. (2017). Synthesis and characterization of novel silver nanoparticles using Chamaemelum nobile extract for antibacterial application. Advances in Natural Sciences: Nanoscience and Nanotechnology, 8(2), 025004.
55. Parmin, P., Suarayasa, K., & Wandira, B. A. (2020). Relationship between quality of service with patient loyality at general polyclinic of kamonji public health center. *International Journal of Health & Medical Sciences*, *3*(1), 86-91. https://doi.org/10.31295/ijhms.v3n1.157
56. Widana, I.K., Sumetri, N.W., Sutapa, I.K., Suryasa, W. (2021). Anthropometric measures for better cardiovascular and musculoskeletal health. *Computer Applications in Engineering Education*, *29*(3), 550–561. https://doi.org/10.1002/cae.22202