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Antibacterial Activity of Silver nanoparticles Against Pathogenic Bacterial Isolates from Diabetic Foot Patients

Duaa R. Ibraheem *, Nehia N. Hussein , Ghassan M. Sulaiman
Division of Biotechnology, Department of Applied Sciences, University of Technology, Baghdad, Iraq

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Abstract

Diabetic foot is a catastrophic complication of diabetes. This study included isolation and identification of three types of bacteria that cause diabetic foot ulcers, fifty-five isolates of *Staphylococcus aureus*, thirty-five isolates of *Acinetobacter baumannii*, and thirty isolates of *Serratia marcescens.* These isolates were obtained from diabetic foot patients at different private clinics in and around Baghdad and Medical City Hospital. The proportion of male patients was greater than females, and it was noted that the age group (51-68 years) was more ages affected by diabetic foot. These isolates showed high resistance to most of the antibiotics used, *Staphylococcus aureus* was resistant to Penicillin, Tetracycline, and Ciprofloxacin in the percentage of 100 %, 65 %, and 26 %, respectively. *Acinetobacter baumannii*, showed high resistance to Penicillin, at a percentage of 80%, and Ciprofloxacin at 60%. *Serratia marcescens* was resistant to most antibiotics that were used in this study, Tetracycline, Penicillin, Cefotaxime, and Amoxicillin in the percent of (100, 95, 91, 88,70, and 64) %, respectively. The chemical reduction method was used in this study to synthesize silver nanoparticles. The characterization of silver nanoparticles was done by Field Emission Scanning Electron Microscope, and transmission electron microscope, which showed particle sizes of 24.56 to 66.2 nm, which proved that silver nanoparticles had nano size and spherical shape. The result of antibacterial activity of silver nanoparticles and silver nitrate against *Staphylococcus aureus* bacteria showed the highest effect of silver nitrate than other bacteria tested in this study, the diameter of the inhibition zone reached 15.66mm. Likewise for silver nanoparticles where the diameter of the inhibition zone of highest effect reached 29.33mm for *Staphylococcus aureus* bacteria. The test of silver nanoparticles' ability to inhibit bacterial growth produced the greatest results for *Staphylococcus aureus* bacteria, which were inhibited after 60 minutes. Based on these research findings, silver nanoparticles have demonstrated their efficacy in this study against isolated bacteria from diabetic feet.

Keywords: Diabetes, Foot Ulcer, Silver nanoparticles, Antibacterial activity, Gram-positive bacteria, Gram-negative bacteria

*Email: as.20.58@grad.uotechnology.edu.iq

الفعالية المضادة لجسيمات الفضة النانوية ضد العزلات البكتيرية الممرضة من مرضى القدم السكري .

دعاء رحمن ابراهيم *, نهاية نعمة حسين , غسان محمد سليمان

فرع التقنيات الاحيائية ، قسم العلوم التطبيقية ، الجامعة التكنولوجية ، بغداد ، العراق

الخلاصة

القدم السكري هي مشكلة صحية عامة لمرضى السكر. تحدث الإصابة نتيجة العديد من المضاعفات. مثل تلف الأعصاب وضعف تدفق الدم . تضمنت هذه الدراسة عزل وتحديد ثلاثة أنواع من البكتيريا المسببة لقرحة القدم السكري. مثل بكتريا *Staphylococcus aureus*، *Acinetobacter baumannii*، و *Serratia marcescens*. تم الحصول عليها من عيادات خاصة مختلفة في بغداد وما حولها ومستشفى مدينة الطب ، وكانت نسبة الذكور أكبر من الإناث ، وقد لوحظ أن الفئة العمرية 51–68 سنة كانت أكثر تأثرًا بالقدم السكري. أظهرت هذه العزلات مقاومة عالية لأغلب المضادات الحيوية استخداماً. تم استخدام طريقة الاختزال الكيميائي في دراستنا لتصنيع جسيمات الفضة النانوية ، لإيجاد حل بديل ضد بكتيريا القدم السكري بدلاً من المضادات الحيوية .تم تشخيصها بالمجهر الإلكتروني الماسح والمجهر الإلكتروني النافذ ، والذي أظهر حجم الجسيمات بمدى حجم 24.56 إلى 66,2 نانومتر ، مما أثبت أن دقائق الفضة النانوية لها حجم نانوي وشكل كروي .أظهرت نتيجة الفعالية المضادة للبكتريا ان دقائق الفضة النانوية ونترات الفضة ضد البكتيريا المعزولة ، أعلى تأثير لنترات الفضة ضد بكتريا *Staphylococcus aureus* مقارنة بالبكتيريا الأخرى(*Acientobacter baumannii*و *Serratia marcescens*) حيث بلغ قطر منطقة التثبيط 15.66ملم .وبالمثل بالنسبة لدقائق الفضة النانوية حيث بلغ قطر منطقة التثبيط ذات التأثير الأعلى 29.33ملم لبكتريا . أفضل نتيجة لتأثير دقائق الفضة النانوية على تثبيط النمو البكتيري كانت لبكتريا *Staphylococcus aureus* والتي ثبط نموها عند 60 دقيقة . لذلك حسب نتائج هذا العمل أثبتت دقائق الفضة النانوية فعاليتها ضد البكتيريا المعزولة من القدم السكري في دراستنا.

Introduction:

Diabetes mellitus (DM) disease and its complications are becoming the leading cause of morbidity and mortality. Whereas, it affects around 382 million people worldwide, with predictions that this figure will rise to 592 million by 2035 [1]. Diabetes is a systemic disease that affects nearly every part of the body, and the feet are frequently the first to suffer [2]. Diabetic foot infection (DFI) was considered to be one of the most common and dangerous diabetes complications [3]. Recent reports estimated that about 10-25 % of diabetic patients at any point in their life live may be exposed to foot ulcers due to uncontrolled or undiagnosed diabetes, which is characterized by Infection, ulceration, and tissue damage of the superficial and deep tissues and gangrene, as a result of restricted blood flow and poor healing caused by vascular disease and nerve damage which caused by diabetic neuropathy. So, the most common cause of hospitalization and non-traumatic lower limb amputation is diabetic foot ulcer (DFU) [4,5].

Different types of microorganisms contribute to the occurrence of foot ulcer infections, and bacteria stand in the way by having several virulence factors and antibiotic resistance [6]. In patients with acute infections, the majority of lesions are caused by aerobic Gram-positive cocci such as *Staphylococcus aureus (S. aureus*) usually as monomicrobial infections, but in deep and chronic infections both aerobic and obligate anaerobic gram-negative bacteria. Whereas chronic infections or previously treated with antibiotics are frequently polymicrobial [7].

Several investigations have shown that *S. aureus* is the most common cause of diabetic foot ulcers. Due to Staphylococcal surface proteins such as (protein A) assist the bacteria to cling to the skin and facilitate the bacterial colonization in the diabetic foot, and then release many of the virulence factors that invade the immune system such as β-hemolysin, and it can be resistance for most antibiotics. The skin′s mucous membranes act as a mechanical screen preventing tissue invasion. When the barrier between the skin and the underlying tissue is breached, microorganisms such as *S. aureus* gain access to the tissue resulting in a local lesion as seen in DF [8]. *Acinetobacter baumannii (A. baumannii*) has been identified as one of the Gram-negative bacillus bacteria are associated with a higher incidence of major amputation, a major human pathogen, and a major source of hospital-acquired illness, particularly in immune-compromised individuals, both bacteria are known to cause different diseases, including pneumonia and serious blood and wound infections[9]. *A. baumannii* has the ability to resist most kind of the Aminoglycoside antibiotics with a high rate, and there is no connection between biofilm production and the resist to the antibiotics [10].

These bacteria considered the most common or serious MDR pathogens have been encompassed within the term "ESKAPE" which stands for *Enterococcus. fascism, S. aureus, K. pneumonia, A. baumannii, P. aeruginosa,* and *Enterobacter spp* [11]. *Serratia marcescens (S. marcescens*)is facultative anaerobic Gram-negative bacilli widely distributed in the environment. Until 1951, when it caused a nosocomial infection outbreak, was considered a nonpathogenic microorganism [12]. *S. marcescens* is a common opportunistic human pathogen that can cause pneumonia, intravenous catheter-associated infections, endocarditis, osteomyelitis, and UTI, among other hospital-acquired infections [13]. It is sometimes distinguished by the produce of the red pigment prodigiosin (as a biological marker), even though most *Serratia* pathogenic strains in humans do not generate this pigment [14].

Many antibiotics are used against DFU microorganisms, but the excessive use of these antibiotics, which is often random and for long periods, allow resistant strains to emerge and the emergence of side effects that harm the health of the individual [15]. Silver metal has been used in medicine since around 4000 B.C, even before it was recognized that bacteria constitute the primary cause of infection [16]. It became possible to make nano-scale silver with dimensions of 1-100nm with the advent of nanotechnology [17,18]. It is utilized as a more potent antibacterial than antibiotics because it creates a strong bond with bacteria across the cell membrane and enters the bacterial cell. When it is ionized with water or tissue fluids, it releases bioactive silver ions, otherwise, it is.

Silver nanoparticles (AgNPs) have no negative effects on human health at low concentrations because of the form, size, stability, and biocompatibility of nanoparticles, but long-term exposure to silver produces serious disorders such as argyrosis [19]. Several theories have been put forward to explain the mechanism of AgNPs on microorganisms, e.g., their binding to the bacterial cell wall, causing changes in the cell membrane and thus the death of the bacterial cell [20], Silver ions also work to inhibit respiratory enzymes, and during the inhibition process, active oxygen species (ROS) are generated that attack the cell itself. And thus its death. AgNPs have the ability to interfere with the bases of sulfur and phosphorous acid DNA and thus lead to their destruction and cell death due to a disturbance in the DNA replication of bacteria and microbes in general [21]. There are numerous conventional methods for producing AgNPs, including chemical reactions, photochemical processes, thermal degradation of different silver compounds, electrochemical processes, radiation, and microwave-assisted methods. [22]. One of the most frequently employed methods to synthesize AgNPs is chemical reduction [23]. The aim of this study is to the

chemical synthesis of AgNPs, study their antibacterial activity against pathogenic DF bacteria *S. aureus*, *A. baumannii*, and *S. marcescens*, and compare the antibacterial activity of silver nitrate and silver nanoparticles.

MATERIAL AND METHODS

Chemicals and reagents

The chemicals were used to synthesize AgNPs, Silver nitrate-AgNO3 99.5% from (BDH, England), Tri-sodium citrate dehydrate, Sodium dodecyl sulphate -SDS were ordered from(CDH, India), Deionized water (Baghdad, Iraq) used as a solvent, The media and reagents were used are: Blood Base Agar medium (BA), MacConkey Agar medium(MAC), Mannitol salt Agar(MSA), Muller-Hinton Agar medium(MHA) were bought from (Difco, USA), Brain-Heart Infusion Broth (BHI)(Biolab, Thailand), Nutrient broth medium (NB)(BDH, England), Normal saline water (PiONEER, Iraq), Gram stain from (Himedia, India), the antibiotics: Penicillin, Tetracycline, Ciprofloxacin, Gentamicin, Amoxicillin, Cefotaxime were purchased from (Bioanalyse, Turkey).

Collection of Bacterial Isolates

Totally, 152 swab samples were collected from patients who suffered from diabetic foot ulcers, using sterile cotton swabs in a circular motion, from different private clinics in and around Baghdad and Medical City Hospital, during the period from 5 October 2021 to 30 January 2022. The Demographic and clinical data of diabetic foot patients such as gender, age, DM duration, and type of diabetes, type of treatment (drug or insulin) were gathered.

Identification of bacterial isolates

The swabs were aerobically cultivated at 37C°, for 24hrs. on Blood and MaCconkey agar medium to isolate Gram-negative and Gram-positive bacteria, the process was repeated twice for the colonies' purification. Initially, the isolates were characterized based on their culture morphology and microscopic examination, as well as the biochemical tests[24], and then confirmed the diagnoses with the VITEK 2 system technology.

Antibiotic sensitivity test

The Kirby-Bauer disc diffusion method was used to determine the sensitivity of isolated bacteria to antibiotics according to the Clinical and Laboratory Standard Institute (CLSI), Mueller-Hinton plates were prepared and inoculated with bacterial suspension, which was compared with 0.5CFU/ml standard McFarland tube, using cotton swabs and permit the dishes to dry at room temperature, then the antibiotic discs (Penicillin (P) 10 µg, Tetracycline(TE) 10 µg, Ciprofloxacin(CIP) 10 µg, Gentamicin(CN) 10 µg, Amoxicillin(AX)25 µg, Cefotaxime(CTX) 30 µg) were placed with sterile forceps at the rate of 6 disks per plate. And the dishes were incubated at 37°C for 24 hrs. Then, measuring the diameters of the inhibition area formed around the antibiotic discs in millimeters with a metric ruler and compared with CLSI, 2021 [25].

Preparation of Silver Nitrate Solution

By dissolving 0.067g of silver nitrate in 100 ml of deionized water, the silver nitrate solution which has a 4 mM concentration was made, stored in darkness to halt oxidation, and utilized to create silver nanoparticles [26].

Synthesis of silver nanoparticles

The chemical reduction method was used to synthesize silver nanoparticles, with slight modification. By using 4mM of silver nitrate, 0.4mM tri-sodium citrate dehydrates as a

reducing agent, and 0.5mM sodium dodecyl sulphate-SDS as a capping agent. Silver nitrate was dissolved in 100 ml of de-ionized water under hot plate magnetic stirring until it reach 80C°, a mixture of tri-sodium citrate dehydrate and SDS was also dissolved in 100 ml of de-ionized water, and was dropped for 30 min under continuous stirring. The final mixture was kept at 80C°, 350 rpm, for two hrs. Changing the color to yellow indicates the AgNPs' formation. And then it was cooled at room temperature, stored in a dark place in a refrigerator [23].

Characterization of silver nanoparticles

Field Emission Scanning Electron Microscope (FE-SEM) analysis was used to study the particle's size, AgNPs shape, and surface morphology by scanning electron microscope (Zeiss, Germany) and transmission electron microscope (TEM)(Zeiss, Germany) used to identify the morphological feature of the silver nanoparticles.

Antibacterial Activity of Silver Nitrate and Silver Nanoparticles against DF Bacterial Isolates

Well-diffusion method was used to test the antibacterial effect, MHA plates were swabbed with bacterial suspension of *S. aureus*, *A. baumannii*, and *S. marcescens* which were adjusted to 0.5 McFarland tube to obtain culture with 0.5 CFU mL^{-1}. Then five wells were punched into the agar using a sterilized well cutter. The wells were loaded with 80µl of different concentrations of 4mM $AgNO_3$ (20, 40, 80, and 100)µgmL^{-1} of the silver nitrate solution and silver nanoparticles for all types of bacteria, de-ionized water was used as a controlling factor. The dishes were incubated at 37 C° for 24hrs. Results were obtained by measuring the inhibition zone [27]. Three replicates were made for each treatment.

Determination of MIC and MBC

Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined based on [28], test tubes containing 0.8 mL of BHI broth were added with 0.1 mL of a suspension of S. aureus, A. baumannii, or S. marcescens, along with 0.1 mL of AgNPs at various concentrations (20, 40, 80, or 100 µgmL^{-1}). These test tubes were then compared to 0.5 McFarland standard tubes. Before turbidity readings were taken, the tubes were given a vigorous shake and left at 37C° for 24 hrs. Afterward, turbidity was used to record the results. 100 l of the mixture was then spread out in a loop on MHA medium and incubated for 24 hrs. at a temperature of 37 C°. The results were then recorded based on whether or not growth appeared on the agar.

Determination of Growth Curve

In sterile test tubes, containing 2ml of NB medium, 0.5ml of AgNPs from a stock concentration (100µgmL^{-1}), and 0.5ml of bacterial suspension for each bacteria *S. aureus, A. baumannii,* and *S. marcescens* which prepared by a suspended individual colony of 24 hrs. culture, and adjusted to 0.5 McFarland. A volume of 0.5ml of the sample was pipette out from each tube and spread on freshly prepared MHA medium at zero time, 30, 60, and 90 min, and incubated for 24 hrs. at a temperature of 37C°. with measurement of absorbance at wavelength 540 nm using an SP-300 spectrophotometer (Optima, Japan) every half hour. After the incubation period, the growth curve characteristics were observed and represented graphically [29].

StatisticalAnalysis

Using the provided statistical program (SPSS.24), the data were examined using Analysis of Variance (ANOVA) in two directions.

RESULT AND DISCUSSION

This study included 152 diabetic foot patients. The male patient percentage was higher than females (85: 67). This ~~is~~ might be due to the fact that in Iraq, men are more exposed to the factors of the outside environment [30]. The mean age of DF patients was between 36-82 years, it was noted that the age group (51-68) was more ages affected by DF. The majority of them were with Type 2 DM and the main treatment was drugs more than insulin. The mean DM duration was 7 to 25 years. These demographic and clinical data are close to study in northern Iraq [31]. However, from these clinical specimens, fifty-five isolates of *S. aureus*, forty isolates of *A. baumannii*, and thirty isolates of *S. marcescens* bacteria were isolated to investigate the antibacterial activity potential of synthesized silver nanoparticles.

Bacterial isolation and Identification

Morphological and Microscopic Examination

To identify the bacterial isolates in addition to diagnosis with the VITEK 2 system, the isolates were cultured on three culture media to determine the shape and size of colonies as shown in Figure (1), as well as the characteristics of each isolate, *S. aureus* can form beta-hemolytic colonies on BA, the colonies showed yellow zones on MSA. This bacteria can grow in a high salt environment and ferment mannitol generating acid products, and changing the color of the phenol red pH indicator from red to yellow [32]. *A. baumannii* appeared pale or bright purple but lactose non-fermenter colonies on MAC agar, while non-hemolytic colonies on BA. Finally, *S. marcescens* appeared motile, with alpha hemolytic colonies on BA, and non-lactose fermenter on MAC agar, these result agrees with [33,14]. After that, using a Gram stain, the cells were examined microscopically, and their sizes, colors, and shapes were determined by looking at the cells under a light microscope.

Figure 1: Bacterial isolates growing on culture media. (**A, D**) growth of *S. aureus* bacteria on Blood agar, Mannitol salt agar, (**B, E**) growth of *A.baumannii* bacteria on Blood agar, MaCconky agar, and (**C, F**) growth of *S. marcescens* bacteria on Blood agar, MacConkey agar.

Biochemical tests

Table (1) shows the results of the biochemical tests for the tested bacteria. The results of *S. aureus* bacteria showed that they were positive for the catalase test, coagulase, Simmon citrate, methyl red, mannitol fermentation, urease hydrolysis, and either the hemolysis test, which produced a β-hemolysis. And negative for the KOH test, oxidase, motility, and swarming test. This agrees with [34]. As for *A. baumanni* was positive for the KOH test, catalase, and Simmon citrate. Negative for oxidase, coagulase, urease, motility, methyl red, mannitol fermentation, lactose, and non-hemolytic (γ-hemolysis). These findings agree with [35]. *S. marcescens* was positive for the KOH test, Catalase, Simmon citrate, urease, motility test, mannitol fermentation, and produced α-hemolysis. A negative result for oxidase, coagulase, methyl red, and lactose fermentation [36].

Table 1: The Results of Biochemical Tests for isolated strains
(+): Positive result, (-): Negative result

Baeteria / Tests	*S. aureus*	*A. baumannii*	*S. marcescens*
KOH test	-	+	+
Catalase	+	+	+
Oxidase	-	-	-
Coagulase	+	-	-
Urease	+	-	+
Simmon citrate	+	+	+
Methyl red	+	-	-
Hemolysin	+ (β-hemolytic)	-(γhemolytic)	+(α-hemolytic)
Motility	-	-	+
Mannitol fermentation	+	-	/
Lactose fermentation	/	-	-
Swarming	-	-	+
Growth at 42C°	+	+	+
Growth condition	Facultative anaerobic	obligatory aerobic	Facultative anaerobic

Antibiotic susceptibility test of DF isolated bacteria

The extent of resistance of microbial isolates to antibiotics was determined using the Kirby-Bauer disc diffusion method, and the results were compared to the standard tables described in CLSI, 2021. Figures (3-A, 3-B) show that *S. aureus* was resistant to Penicillin, Tetracycline, and Ciprofloxacin in the percentage of 100 %, 65 %, and 26 %, respectively, the results are in agreement with previous studies [37,38,30] that proved the highest drug resistance was against Penicillin and Tetracycline, and the lower resistance was with CIP. While it was sensitive to Gentamicin, Amoxicillin.

S. aureus was intermediate sensitive Cefotaxime with an average of 18 mm. The previous studies proved that 88% were methicillin-resistant (MRSA), this finding was similar to that of [39, 35], who discovered that 91 of the 96 *S. aureus* isolates examined were methicillin-resistant. As for *A. baumannii* bacteria, it showed high resistance to Penicillin, at a percentage of 80%, and Ciprofloxacin at 60%. This agrees with [40]. Also, the results showed the highest sensitivity of *A. baumannii* bacteria for Gentamicin with an average inhibition diameter of 20 mm, followed by Amoxicillin with an average inhibition diameter of 18 mm, while Cefotaxime, Tetracycline, showed an intermediate sensitivity with an average inhibition diameter of 17,13 mm, respectively. The result was similar to [41].

Finally, *S. marcescence* bacteria was resistant to most antibiotics that were used in this study, Tetracycline, Penicillin, Cefotaxime, and Amoxicillin in the percent of 100, 95, 91, 88,70, and 64 □, respectively. And it showed high sensitivity toward Gentamicin with 22 mm and intermediate sensitivity for Ciprofloxacin with an inhibition diameter of 21 mm, this result is similar to the study [42], which indicated in their case, the strain was still susceptible to Gentamicin and Fluoroquinolones only.

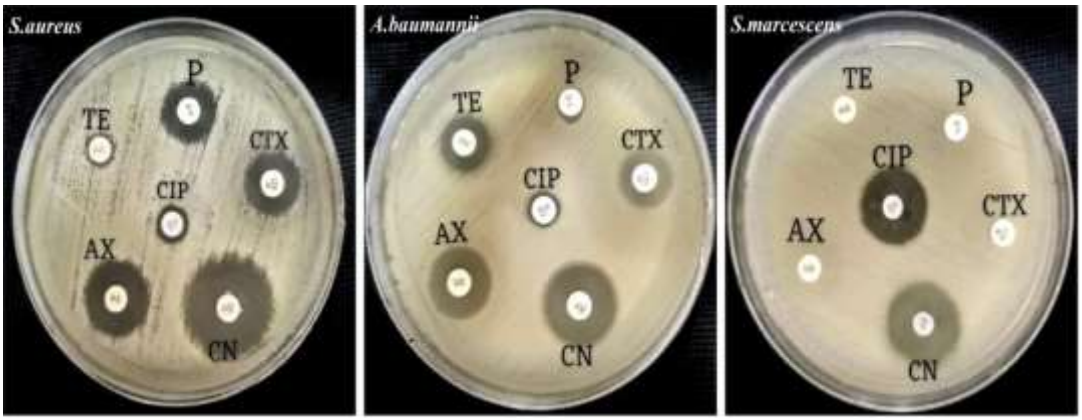

Figure 3-A: Antibiotic susceptibility test on MHA, **CIP:** Ciprofloxacin, **P:** Penicillin, **TE:** Tetracycline, **CN:** Gentamicin, **CTX:** Cefotaxime, **AX:** Amoxicillin

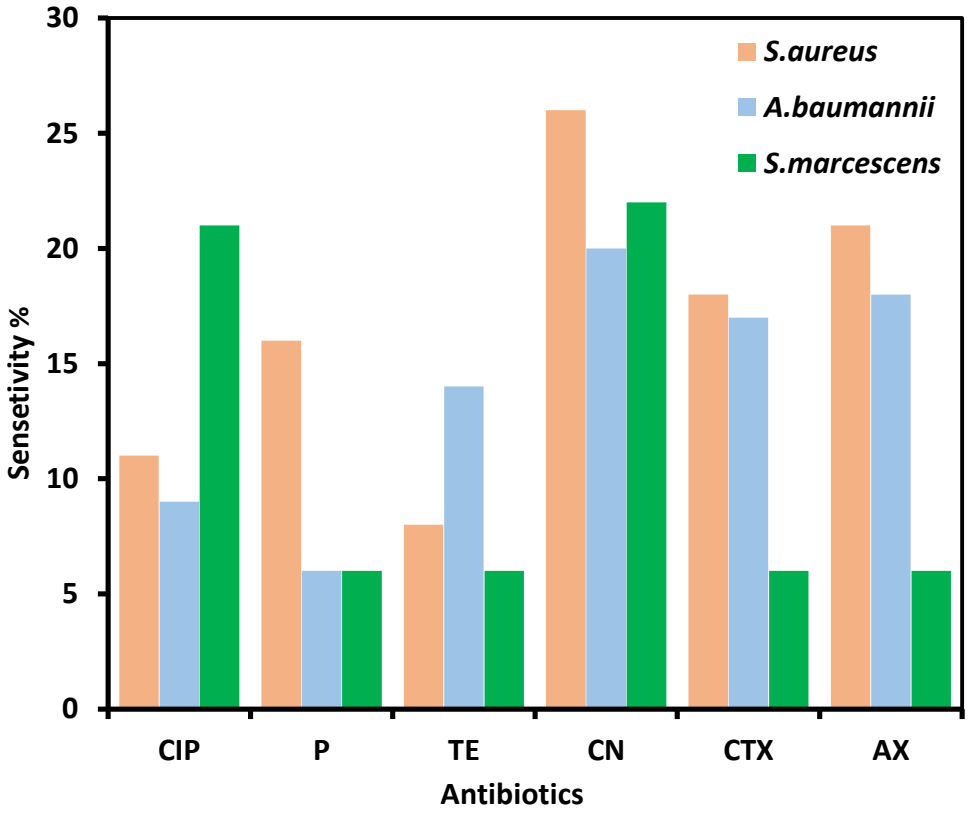

Figure 3-B: Antibiotic susceptibility test, **CIP:** Ciprofloxacin, **P:** Penicillin, **TE:** Tetracycline, **CN:** Gentamicin, **CTX:** Cefotaxime, **AX:** Amoxicillin

Synthesis of silver nanoparticles

Chemical reduction method of the synthesis process was done by adding SDS as a capping agent to control size and stability along with tri-sodium citrate di-hydrate as a reducing agent. The first indication is the appearance of the yellow color characteristic of silver nanoparticle formation as shown in Figure (4). One of the most frequently employed methods to synthesize AgNPs is ~~the~~ chemical reduction due to their high yield, low cost, and simplicity [23].

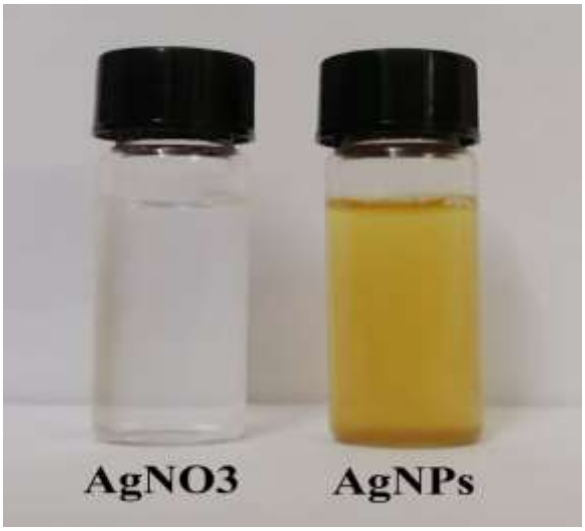

Figure 4: Synthesis of silver nanoparticles. Silver nitrate ($AgNO_3$) at a concentration of 4mM, chemically synthesized silver nanoparticles (AgNPs).

Characterization of silver nanoparticles

Field Emission Scanning Electron Microscope (FE-SEM)

FE-SEM micrographs were used to assess the size, surface morphology, and homogeneity of nanoparticles. It's a technique for obtaining qualitative and quantitative data as well as other approaches to nanoparticle morphology and size [43]. The majority of AgNPs have a more obvious structural arrangement, are spherical, and have smooth surfaces. With particle agglomeration and aggregation saw, with a size range of 24.56 to 66.2 nm, at a magnification power of 50.000KX, Figure (5).

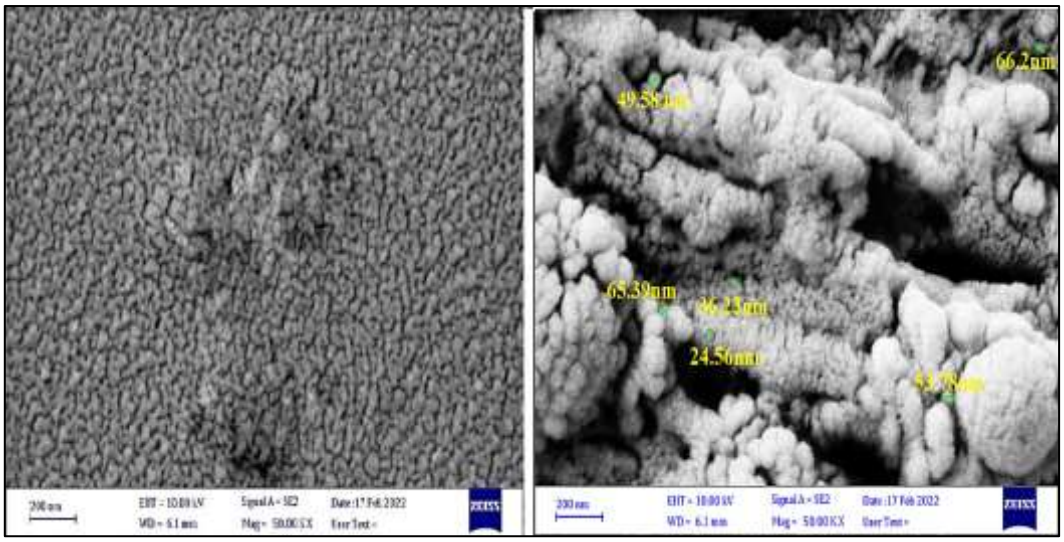

Figure 5: SEM image of Silver nanoparticles

Transmission Electron Microscopy (TEM)

To access The morphology of the chemically synthesized silver nanoparticles (AgNPs) we performed TEM with magnification power at around 27.500 KX, the magnified image result demonstrated that AgNPs have small and spherical shapes with faceted nanoparticles. Individual nanoparticles were not in direct touch with one another, indicating increased stability when a capping agent was used. as shown in Figure (6). The result was close to [44,23].

The histogram result shows the mean size of AgNPs as about 31.38nm at a 40nm scale.

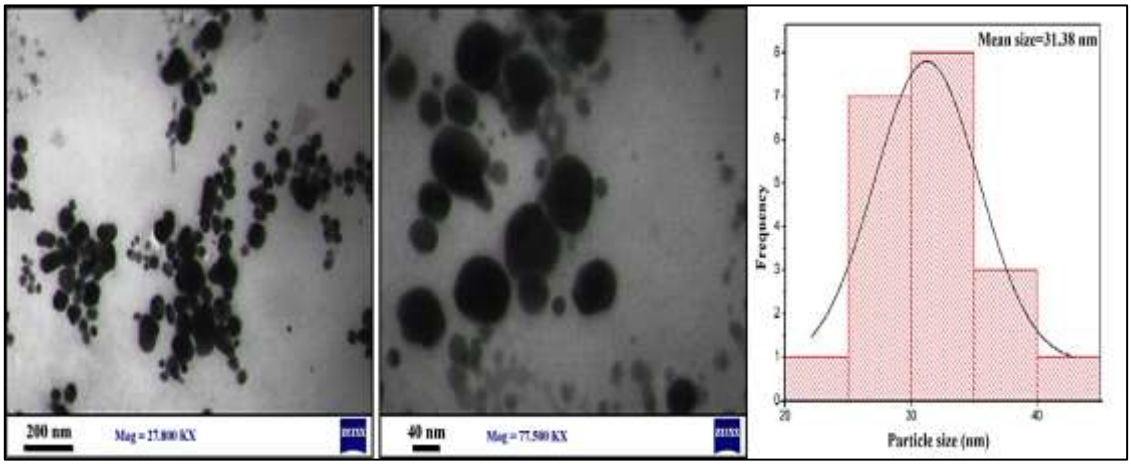

Figure 6: TEM image of silver nanoparticles with a histogram of scale 40nm.

Comparing the antibacterial activity of $AgNO_3$ and AgNPs against DF Bacterial Isolates

Table (2) shows the result of the well diffusion method, $AgNo_3$ exhibited a slight effect on *S. aureus*, *A. baumannii*, and no effect appeared on *S. marcescens*. Bacteria with the highest inhibitory diameter in the current study were *S. aureus* bacteria, with an average inhibition zone diameter of 15.66±1.52 mm at 100 µg mL^{-1} concentration (stock), followed by that *A. baumannii* bacteria with an average inhibition zone diameter of 14.33±1.52mm, respectively. The bacteria with the lowest inhibitory diameter in the current study was *S. marcescens* bacteria, with an average inhibition zone diameter of 10±1.60 mm at stock concentration. The mechanism of Ag ions' microorganism inhibiting actions is still partially understood. According to several researches, the positive charge on the Ag ion is critical for its antibacterial effectiveness because negatively charged microbe cell membranes attract positively charged nanoparticles via electrostatic attraction [45]. These results are in line with the finding of a previous study that $AgNO_3$ was more effective against Gram-positive bacteria than Gram-negative bacteria [46].

Table 2: The antibacterial activity of $AgNO_3$ and AgNPs against DF bacterial isolates.

Bacteria	Inhibition zone in diameter of different concentration(µg mL^{-1})			
	20 µg mL^{-1}	**40 µg mL^{-1}**	**80 µg mL^{-1}**	**100 µg mL^{-1}**
S. aureus	**$AgNO_3$**			
	11.66±1.42	12.66±1.22	13.33±1.30	15.66±1.52
	AgNPs			
	18.33±1.82	21.66±1.52	25.00±1.20	28.22±2.24
A. baumannii	**$AgNO_3$**			
	9.33±1.42	10.33±1.27	12.33±0.08	14.33±1.52
	AgNPs			

S. marcescen	17.66±7.98	21.66±2.08	24.66±2.08	27.33±1.62
	$AgNO_3$			
	8.00±0.02	8.33±0.57	9.00±0.08	10.00±1.60
	AgNPs			
	15.66±0.60	17.33±1.80	22.33±2.12	23.66±1.54

The findings of evaluating the effect of silver nanoparticles demonstrated that they have a much stronger inhibitory effect than silver nitrate, which agrees with [47]. The synthesized silver nanoparticles had the greatest effect on the growth of *S. aureus* bacteria, with an average diameter of the inhibition zone of 28.22±2.24 mm at 100 μg mL^{-1}. Followed by *A. baumannii* with an average diameter of the inhibitory zone of 27.33±1.62 mm. And then *S. marcescens*, the result showed a better effect compared with silver nitrate, whereas at the stock concentration, the inhibitory diameter was 23.66±1.54 mm and 15.66±0.60mm with the concentration of 20 μg mL^{-1}, which agrees with [48,49].

Silver nanoparticles' great efficiency as shown in Figure (7), is owing to their small size and large surface area, which allows them to reach and contact the DNA of the bacterial cell [50,51]. According to a previous study, silver nanoparticles have a strong affinity for sulfur or phosphorous that contains soft bases like RS, R-SH, or RS-RPR3, sulfur-containing proteins in the cell membrane or inside cells, as well as phosphorous-containing elements like DNA, are likely to be preferred sites for silver nanoparticle binding [52].

Silver nanoparticles bind to the cell membrane and release silver ions, which increases the permeability of the cell membrane and causes the cell to die. Furthermore, the increased surface area of these nanoparticles allows for improved contact and interaction with the microbial cell. The release of silver ions from silver nanoparticles, causes the cell membrane to change structurally and then become more permeable. This process continues until the cell dies. These results support a previous study's finding that AgNPs are more effective against Gram-positive bacteria than Gram-negative bacteria in antibacterial activity. [53,49]. While other studies reported, that AgNPs have more antibacterial activity against Gram-negative bacteria than Gram-positive bacteria [54]. This is due to the fact that the cell walls of Gram-positive bacteria bind larger quantities of metals than do Gram-negative bacteria, due to changes in the molecular composition of both types of bacteria's cell walls. Although Gram-positive bacteria have a stiff and thick peptidoglycan layer that is absent in Gram-negative bacteria, Gram-negative bacteria have a 10 nm thick lipopolysaccharide (LPS) barrier covering the peptidoglycan outer layer, making it more difficult for AgNPs to access [55].

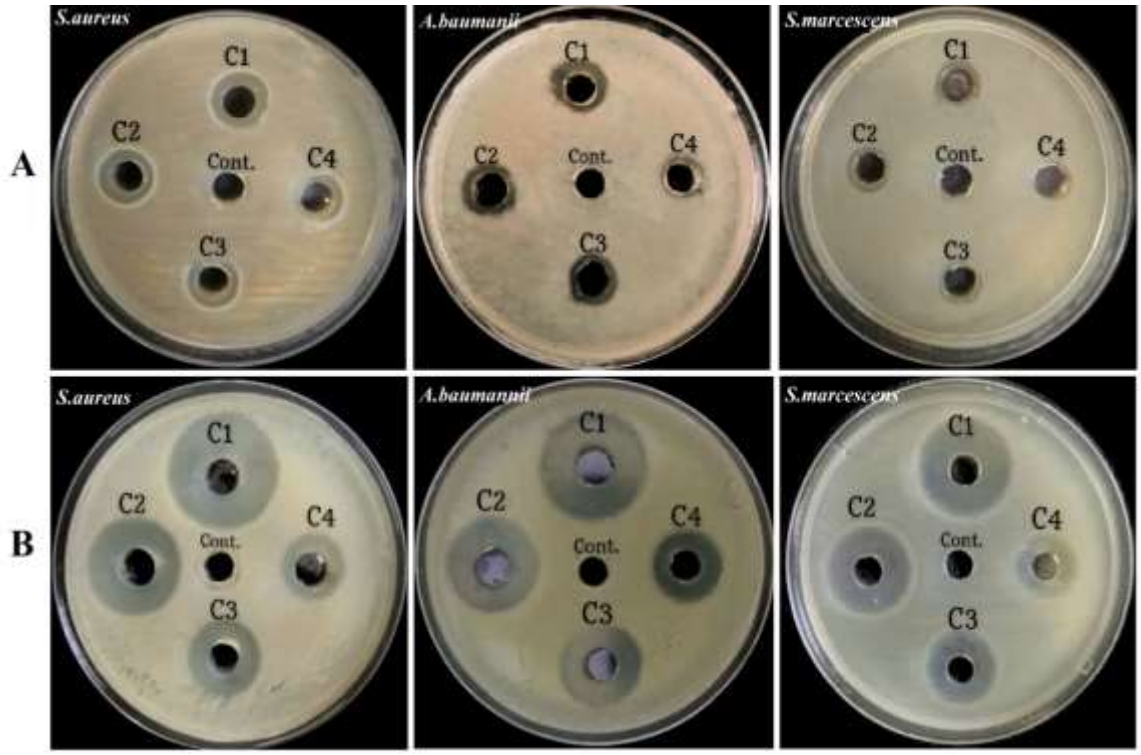

Figure 7: The inhibitory effect of Silver nitrate (A), and silver nanoparticles (B). At different concentrations against DF bacterial isolates. (C1) refers to 100 μg mL^{-1}, (C2) 80 μg mL^{-1}, (C3) 40 μg mL^{-1} , and (C4) 20 μg mL^{-1}.

Determination of MIC and MBC

Through the use of a turbidity essay, the influence of silver nanoparticles on the growth of bacterial isolates employed in this study was determined using liquid culture media. The MIC AgNPs for *S. aureus* was 40 μg mL^{-1} and for *A. baumannii* was 80 μg mL^{-1}. While for *S. marcescens* the value was 80 μg mL^{-1}. These results indicated that the MIC for *S. aureus* was less than other bacteria, which was more sensitive to AgNPs, as shown in Figure (8). The presence or absence of bacterial growth on solid culture media was used to calculate the MBC [45]. According to the result, the MBC of AgNPs for *A. baumannii* and *S. marcescens* bacteria was 100 μg mL^{-1} and 80 μg mL^{-1} for *S. aureus.*

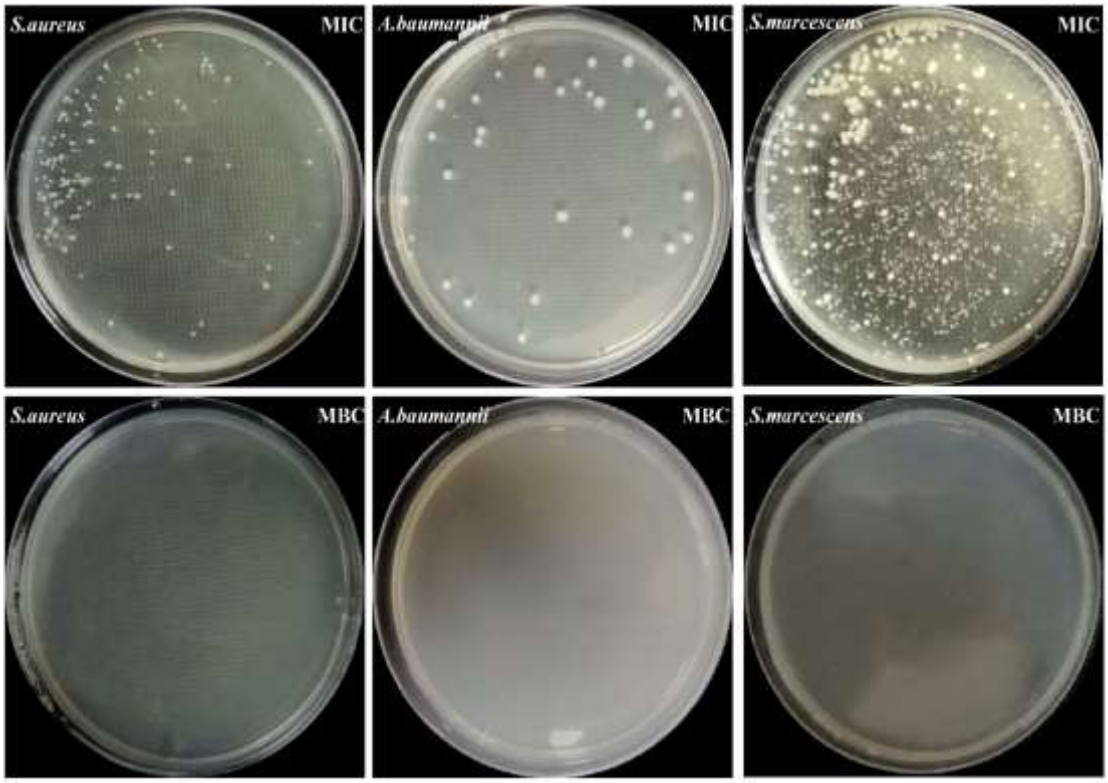

Figure 8: Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations for a bacterial isolate.

Determination of growth curve

The effect of silver nanoparticles on the growth of bacterial isolates was evaluated based on their interaction with bacterial culture, through observing the growth curve on the MHA medium and the results were interpreted in graphical form to give the growth kinetics. The results showed that the absorbance of growth of bacterial isolates in nutrient broth medium at 540 nm decreased with time, due to reducing the turbidity of bacterial growth. This is might be due to the antibacterial activity of AgNPs, the bacterial culture in the MHA medium decreases according to the period of incubation of bacteria with silver nanoparticles until the growth almost disappeared when it reaches 90min in interaction with AgNPs. The *S. aureus* growth showed to be reduced at 30 min culture and inhibited at 60 min. But in the case of *A. baumannii* and *S. marcescens* growth appeared to be inhibited at the culture of 90min, Figure (9).

Because the proteins of the cell membrane are the preferred places for the function of NPs, the contact between these particles and the groups of sulfur and phosphorus found in the bacterial cell membrane may be the cause of cell death and destruction [56, 28].

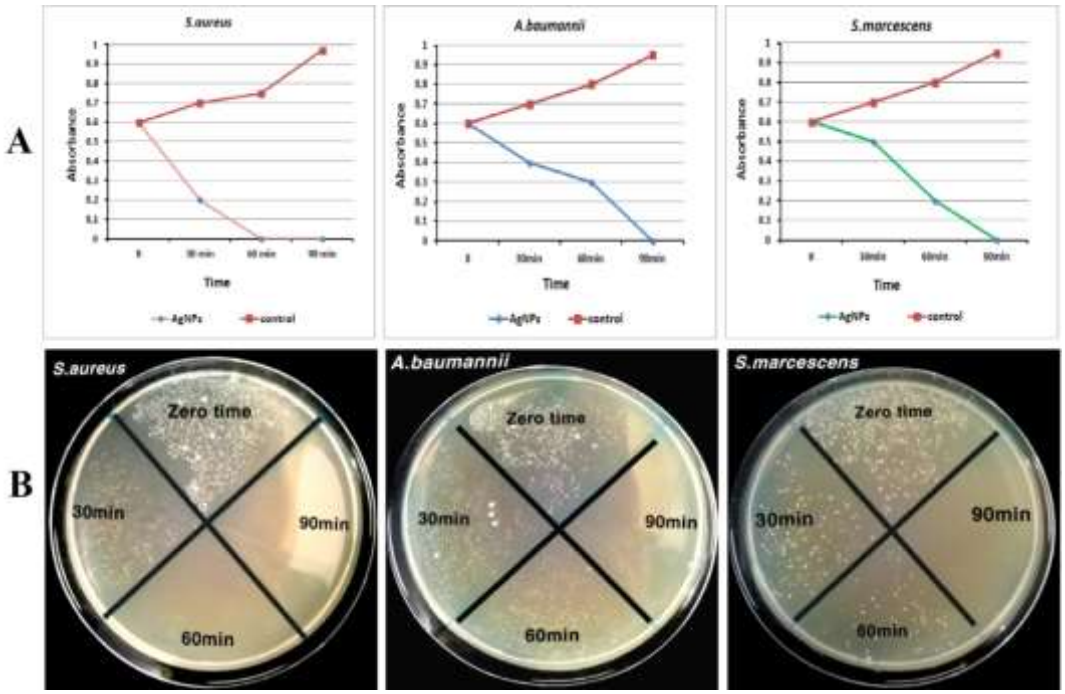

Figure 9: Effect of AgNPs on bacterial Growth Curve. A= Optical density of bacterial broth, B= bacterial growth on MHA medium.

Conclusions

DFI is considered one of the most dangerous diabetes mellitus complications. Different types of microorganisms contribute to the occurrence of foot ulcer infections, and bacteria stand in the way by having several virulence factors and antibiotic resistance. Gram-positive bacteria such as *S. aureus* are more predominantly in DF ulcers than gram-negative bacteria like *A. baumannii* and *S. marcescens*. Difficulty recovering due to bacterial resistance to most antibiotics due to unconsidered use of them. In this study, nanotechnology has been applied to find an alternative solution instead of antibiotics. Through the synthesis of silver nanoparticles by chemical reduction method. And characterized by using FE-SEM and TEM. The study finds that AgNPs had a high antibacterial effect against isolated bacteria more $AgNO_3$, primarily due to their nano-size. And both of them had more effectiveness against *S. aureus* than *A. baumannii* and *S. marcescens*. According to previous studies, this variation is due to the cell wall structure of bacteria.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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