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Green Synthesis of Silver Nanoparticles Using *Aloe vera* L. and Screening Its Activity Against Gram Negative Bacteria

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Received: 2 June 2022 **Accepted: 15 August 2022**

DOI: https://dx.doi.org/10.24237/djps.1804.602B

Abstract

The current research was done to produce silver nanoparticles by green synthesis from Aloe vera leaf extract to evaluate the antibacterial properties against gram-negative bacteria (Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis and Acinetobacter baumannii). Atomic Force Microscopy (AFM) was used to determine the average size and form of the nanoparticles which was 55 nm. Scanning electron microscopy (SEM) shows the Ag NPs have a spherical and smooth surface region. UV–visible spectroscopy (UV-Vis) was used to measure the wavelength range, which showed a sharp peak at 450 nm. X-ray Diffraction (XRD) which show the Ag NPs have three strongest peaks, the measured particle sizes were 15.4 and 18.3 and 22.1 nm. Different functional groups of biomolecules are responsible for the reduction and capping processes, as shown by Fourier Transform Infrared Spectroscopy (FTIR). Different concentrations of Ag NPs (12.5, 25, 50 and100) mg/ml were examined against multiple drug resistance (MDR) isolates, and the results showed the highest diameter of inhibition zone against *P. mirabilis*, *K. pneumoniae*, *E.coli*, *A. baumannii* were (23, 22, 21 and 20)mm

Diyala Journal for Pure Science

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respectively at concentration(100) mg/ml and the lowest zone at concentration (12.5)mg/ml at the same isolates were (13, 12, 10 and10) mm.

Keywords: Ag NPs, *Aloe vera*, AFM, XRD, FTIR, SEM, Antibacterial.

التخليق الأخضر لجسيمات الفضة النانوية باستخدام نبات الصبار Aloe vera والتحري عن فاعليتها ضد البكتريا السالبة لصبغة كرام

عباس ياسين حسن وابتهال محمد عبد

قسم علوم الحياة - كلية العلوم – جامعة ديالى

الخلاصة

أجـري البحث الحالي لإنتـاج جسيمات الفضة النانوية عن طريق التخليق الأخضر من مستخلص أوراق نبات الصبار لتقييم الخصائص المضادة للبكتريا السالبة لصبغة كرام (*Klebsiella pneumoniae*، *E. coli*، *Proteus mirabilis*، *Acinetobacter baumannii*) أستخدم مجهر القوة الذرية (AFM) لتحديد متوسط حجم وشكل الجسيمات النانوية التي كانت 55 نانومتر. يظهر المجهر الإلكتروني الماسح (SEM) أن Ag NPs لها سطح كروي أملس. واستخدم التحليل الطيفي المرئي للأشعة فوق البنفسجية (UV-Vis) لقياس نطاق الطول الموجي، والذي أظهر قمة حادة عند 450 نانومتر. وبين حيود الأشعة السينية (XRD) أن Ag NPs لها ثلاث قمم قوية، وكانت أحجام الجسيمات المقاسة 15.4، 18.3 و 22.1 نانومتر. المجموعات الوظيفية المختلفة من الجزيئات الحيوية هي المسؤولة عن عمليات الاختزال والتحميل، كما هو موضح في التحليل الطيفي بالأشعة تحت الحمراء (FTIR). فحصت تراكيز مختلفة من Ag NPs (12.5، 25، 50 و 100) ملغم / مل ضد العزلات متعددة المقاومة للمضادات الحيوية (MDR)، فأظهرت النتائج أن أعلى قطر لمنطقة التثبيط ضد P. mirabilis، K. pneumoniae، E.coli و A. baumannii كان (23، 22، 21 و 20) ملم على الترتيب عند التركيز (100) ملغم / مل وأدنى منطقة تثبيط عند التركيز (12.5) ملغم / مل لنفس العزلات فقد كان (13، 12، 10 و10) ملم .

الكلمات المفتاحية: جسيمات الفضة النانوية، نبات الصبار، اختبار AFM، XRD، FTIR، SEM، مضاد البكتريا.

Diyala Journal for Pure Science

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Abbas Yaseen Hasan and Ebtihal Mohmmed Abd

Introduction

Burns and wounds are the most common public health issues around the world [1].Many factors such as heat, chemical agents and electricity may increase the issue [2]. Despite the presence of bacteria, most burns and wounds cure through natural repair mechanisms [3]. Therefore, the healing process can be slowed down or stopped in some cases, not only due to the influence of the microbial flora, but also due to the interference of many other autoimmune causes. The presence of dead tissue, as well as uncontrolled and ineffective inflammatory responses, have a major impact on the normal healing process of burns and wounds [4].

Nanomaterials synthesis is currently one of the most prominent research topics. Nanoscience has a lot of active regions. Their enhancement of the human quality have received special attention. A way of life, silver nanoparticles are an excellent example (Ag NPs) which are known for their bactericidal and inhibitory effects. Depending on the fabrication process (physical, chemical and biological, or hybrid), Ag NPs may be made in a variety of sizes and shapes. Toxic chemicals are unsuitable for biomedical applications because they are not environmentally friendly, especially, chemical reduction approaches that are commonly used [5].

Typically, poisonous and dangerous materials are used, posing a range of biological threats. Physical approaches, but in the other hand, are prohibitively expensive and incompatible with large-scale manufacturing. As a result, to prevent toxic and unsafe situations, green synthesis methods have been advanced for chemicals. Since they are eco-friendly, fast, easy, and energy efficient, they are generating a lot of interest. [6].

The uses of massive biological molecules derived from plant extracts [7] is possible due to the infinite number of biological molecules derived from plant extracts. Metal nanoparticles' size and morphology can be regulated more easily.

Earlier studies have used *Aloe vera* extracts for the synthesis of stable silver nanoparticles to investigate their antibacterial, antifungal activity [8]. *Aloe vera* extracts have substances that prevent nanoparticles from aggregating by causing steric repulsion between individuals [9]. **The current study aimed to:** using the *Aloe vera* extract for preparing the Ag NPs, and determine their antibacterial activity against gram negative bacteria which cause wound and burn infections (Multidrug resistance).

Materials And Methods

Samples Collection

The study included collecting 150 samples from clinical sources (wounds and burns) and from various ages of patients in Baqubah Teaching Hospital in Diyala province during the period from the beginning of November 2020 to the end of January 2021 that included taking direct swabs from patients. Blood agar, MacConkey agar and EMB were used to culture the samples by streaking method. The agar plates were incubated for 24 h. at 37°C [10]. Colony characteristics, biochemical tests, Gram-stain were used to identify the isolates and used Vitek 2 system to confirm it.

Antibiotic Susceptibility test

The sensitivity test was conducted according to the CLSI guidelines [11]. Mueller-Hinton agar plates were used in the Kirby- Bauer process for the use of rapidly developing bacteria. In the plates, the solvent was sterile and had a depth of about 4 mm. As an inoculum, pure culture was used; 2-4 associated colonies were chosen and transferred to about 5ml of normal sterile saline. The turbidity of the microbial suspension was compared to the turbidity of the McFarland Standard to obtain (1.5x 108) CFU/ml. The regular inoculum was soaked in a sterile cotton swab, and streaking was done three times on the entire agar surface of the plate. After that, the

plates were incubated at 37 ˚C for 18 to 24 h. before being analyzed. The diameters of inhibition zones were measured to the nearest millimeter and reported.

Collection of Plant Samples

The plants which were used in the study were collected from the local market of Baquba city, namely, *Aloe vera*. plant was diagnosed by Prof. Dr. Khazal D. Wadi / College of Sciences / University of Diyala.

Preparation of *Aloe vera*

Aloe vera was washed thoroughly to remove impurities, then dried for 4 days at 35˚C in an oven, then crushed with an electric grinder to produce a fine powder, which was stored at 4˚C in a sterile and sealed glass vial [12].

Biosynthesis of silver Nanoparticles from aqueous extract *Aloe vera*

The producer of biosynthesis of Ag nanoparticles was carried out according to Medda *et al*. [13]. In a flask holding 500 ml of distilled water (ddH2O), 1.576 g of silver nitrate was dissolved and mixed for ten minutes. Twenty gm of *Aloe vera* extract added to flask containing 200 ml of ddH2O with magnetic stirrer hot plate for 2 h. at 60˚C with 1200 rpm.

The extract was allowed to cool to room temperature before being separated using filter paper (Whatman No. 1) and 100 ml from extract mixed with 400 ml of silver nitrate. The solution was dried at 60 °C in a vacuum oven for 24h. to produce light brown an indication of the formation of Ag NPs and placed in the furnace, heated up to 300 °C and were kept at this temperature for 3 h. [14].

Characterizations of Silver Nanoparticles

To approximate the diameter and form of the Ag the greatest wavelength of UV–Vis (PD-303, Apel, Japan) absorption was ranged from 200–1200 nm. Fourier transform–infrared (FTIR)

Shimadzu (Germany) spectra was used to detect the functional group in the sample [15]. The shape and size of nanoparticles were studied using atomic force microscopy (AFM) (Park Systems Suwan South Korea). Using a Shimadzu 600 X-ray Diffractometer (XRD) to get details about crystal structures and crystallite size. The morphology, form, and particle size of the samples were examined using a scanning electron microscope (SEM) [16].

Determination the antibacterial activity of Ag NPs

The antibacterial activity of the Ag NPs was tested using the agar well diffusion process according to Obeidat *et al*. [17]. This suspended bacteria (*K. pneumoniae*, *E.coli*, *P. mirabilis* and *A. baumannii*) was spread by sterile swab on Muller Hinton agar plates after compared the suspension to the standard MacFarland solution (1.5 x 10^8) CFU/ml and then left the plate for a while to dry. A hole was made with a diameter of 5 mm in the culture media by using sterilized a cork borer. Gradient concentrations were done of the extracts (12.5, 25, 50, and 100) mg/ml and add 100 µl of the concentrations to each hole individually by micropipette and sterile distilled water was used as a positive control, then the dishes incubated at 37 ˚C for 24 h.

Results and Discussion

Bacterial isolation and identification

Table (1) shows that Gram negative bacteria were isolated from the total 150 wound and burn swabs, they included:

K. pneumoniae 35 isolates 38.8%, *E.coli* 23 isolates 25.5%, *P. mirabilis* 23 isolates 100% and *A. baumannii* 21isolates 70% which were isolated from wounds. While they were isolated from burns with *K. pneumoniae* 22 isolates 36.6%, *E.coli* 17 isolates 28.3%, *P. mirabilis* 0 isolates 0% and *A. baumannii* 9 isolates 30% .

Diyala Journal for Pure Science

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Table 1: Number and percentage of the bacterial isolates from wound and burns

BACTERIA	NO. (%) OF WOUNDS ISOLATES	NO. (%) OF BURNS ISOLATES	TOTAL OF ISOLATES
Klebsiella pneumoniae	35(38.8%)	22(36.6%)	57
Escherichia coli	23 (25.5%)	17(28.3%)	40
Proteus mirabilis	23 (100%)	0 (0%)	23
Acinetobacter baumannii	21 (70%)	9 (30 %)	30

Antimicrobial Susceptibility Test

Using the disk diffusion process, all different bacterial species were screened against 14 antimicrobial agents of each bacteria. The outcomes were show in the figures (1, 2, and 3) and interpreted in accordance with CLSI [11]. The excessive use and abuse of antibiotics, as well as improper dosage, are the main factors contributing to antibiotic resistance[18].This resistance is also due to the bacteria producing a large number of enzymes that destroy the antibiotics. Recommendations and revealed that there were many bacteria isolates with multiple drug resistance (MDR) from various types of bacteria.

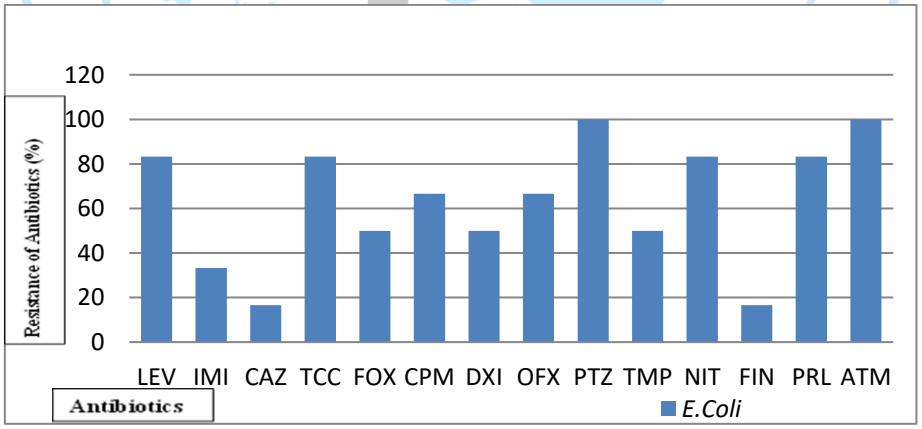

Figure 1: The percentages of antibiotic resistance of *E.coli*

(LEV: Levofloxacin, IMI:Imipenem, CAZ: Ceftazidime, TCC: Ticarcillin / clavulanate, FOX: Cefoxitin, CPM: Cefepime, DXI: Doxycycline, OFX: Ofloxacin, PTZ: Piperacillin Tazobactam, TMP: Trimethprim, NIT: Nirofuranation, FIN: Finafloxacin, PRL: Piperacillin, ATM: Aztronam)

Diyala Journal for Pure Science

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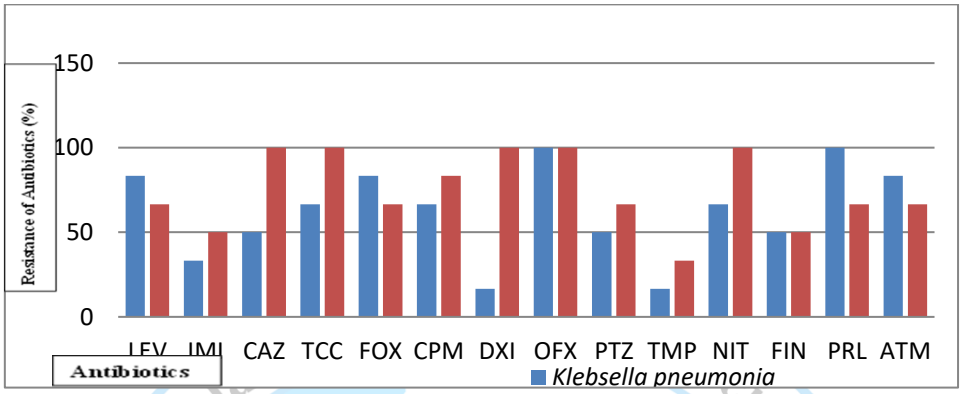

Figure 2: The percentages of antibiotic resistance of *K. pneumoniae* and *P. mirabilis*

(LEV:Levofloxacin, IMI:Imipenem, CAZ: Ceftazidime, TCC: Ticarcillin clavulanate FOX:Cefoxitin,CPM:Cefepime,DXI:Doxycycline,OFX:Ofloxacin,PTZ:Piperacillin Tazobactam,TMP:Trimethprim,NIT:Nitrofuranation,FIN:Finafloxacin,PRL:Piperacillin, ATM:Aztronam).

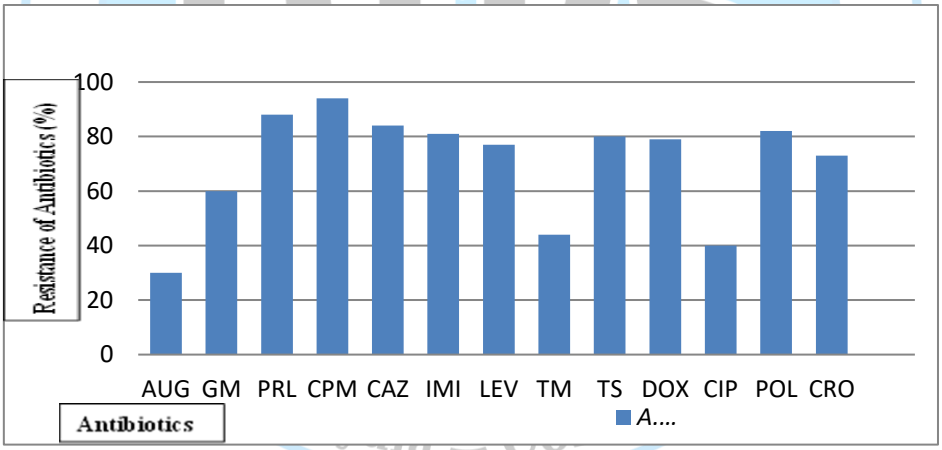

Figure 3: The resistance of antibiotics to *A. baumannii*

(AUG:Amoxicillin/Clavulanic acid, GM: Gentamicin, PRL: Piperacillin, CPM: CefepimeCAZ:Ceftazidime,IMI:Imipenem,LEV:Levofloxacin,TM:Trimethprim, TS:Trimethoprim/Sulfamethoxazole,DOX:Doxycycline,CIP:Cimprofloxacin, POL:Polymyxin, CRO: Ceftriaxone)

Diyala Journal for Pure Science

Biosynthesis of Ag NPs from *Aloe vera*

The silver nanoparticles were made according to Medda *et al*. [13]. Ag NPs were produced using an aqueous extract of *Aloe vera* leaves. The active compounds of the plant serve as reducing agents, converting silver ions to Ag NPS. Changes in color show the formation of nanoparticles, the color changed from light yellow to dark brown, figure (4) showing the formation of Ag NPs [19]. The most important advantages of silver nanoparticle biosynthesis are inexpensive, environmentally friendly, risk-free, simple to implement, and low in toxicity [20].

Figure 4: The biosynthesis of Ag NPs from *Aloe vera* leaves

Characterization of Silver Nanoparticles

Atomic Force Microscopy (AFM)

The surface form of silver nanoparticles biosynthesized by using aqueous extract of *Aloe vera* plant was validated using atomic force microscopy (AFM) the two-dimensional and three-dimensional images were calculated using AFM table (2), figure (5) there were variations in the phenotypic properties of silver nanoparticles indicating that Ag NPs were 55 nm in size.

Diyala Journal for Pure Science

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Abbas Yaseen Hasan and Ebtihal Mohmmed Abd

Table 2: The Cumulation size of Ag NPs biosynthesized by *Aloe vera*

Diameter (Nm)<	Volume (%)	Cumulation (%)	Diameter (Nm)<	Volume (%)	Cumulation (%)	Diameter (Nm)<	Volume (%)	Cumulation (%)
40.00	6.86	16.99	55.00	8.82	40.85	70.00	6.86	69.61
45.00	6.54	23.53	60.00	11.44	52.29			
50.00	8.50	32.03	65.00	10.46	62.75			

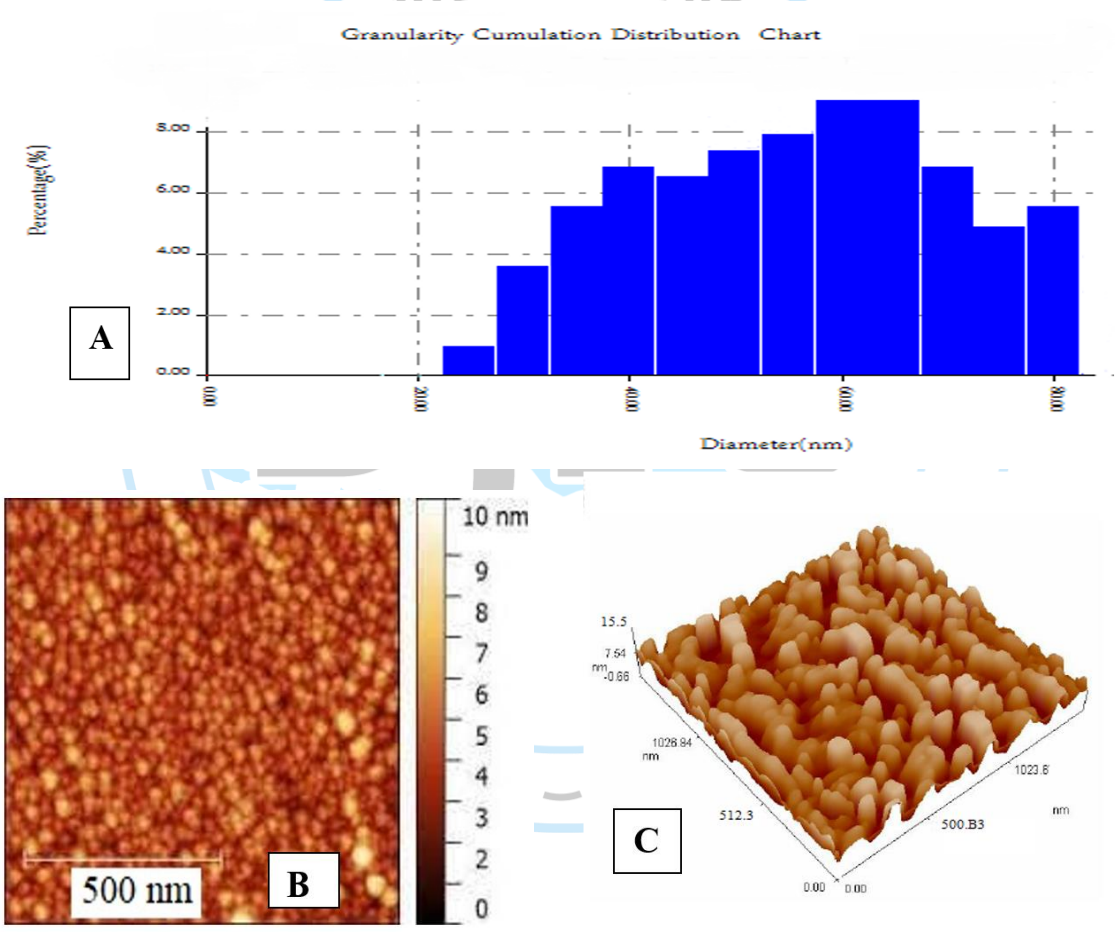

Figure 5: A- The range sizes of biosynthesized Ag NPs.
B- Topography of 2D Ag NPs.
C- Topography of 3D Ag NPs

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Abbas Yaseen Hasan and Ebtihal Mohmmed Abd

Ultraviolet-visible spectrum

Visible-UV Spectroscopy is used to determine the presence and or volume of a material that absorbs light within a sample by allowing visible light and/or ultraviolet rays to pass through the sample. The presence of Ag nanoparticles was detected using UV-vis spectra.

UV-visible spectroscopy (UV/VIS) in the range of 300 to 800 nm was used to stimulate the synthesis of Ag NPs from an aqueous extract of *Aloe vera* leaves, with the absorbance peak observed at 450 nm (figure 6). This result was similar to Tippayawat *et al*.[21] . Since the maximum absorption for bulk Ag NPs is around 350-550 nm, the highly maximum absorption around 450 nm confirms the formation of the Ag NPs portion [22].

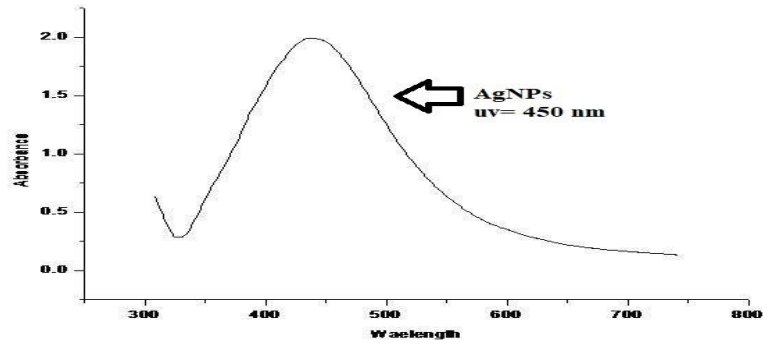

Figure 6: UV-V is spectrum of synthesized Ag NPs from *Aloe vera* extract

X-ray diffraction (XRD)

The crystallinity and average particle size of bio-synthesized Ag nanoparticles from *Aloe Vera* extract were determined using X-ray diffraction instruments figure (7). The prominent peaks corresponding to the diffraction levels found at 2 levels 38.44 ° (111), 44.37 ° (200), 64.66 ° (220), and 78.04 ° (311) are in good agreement with the JCPDS card No.4-783.

The Debye Scherer formulation was used to calculate the average particle size (D) of synthesized nanoparticles [23].

$D = 0.9 \lambda / \beta \cos \theta$

Where λ is the wavelength so X-ray sources (Cu Kα lines – 0.1541 nm), β is the full width as half maximums (FWHM) in radians and θ is Brag's diffraction angle. D was found to have a measured value of 55 nm.

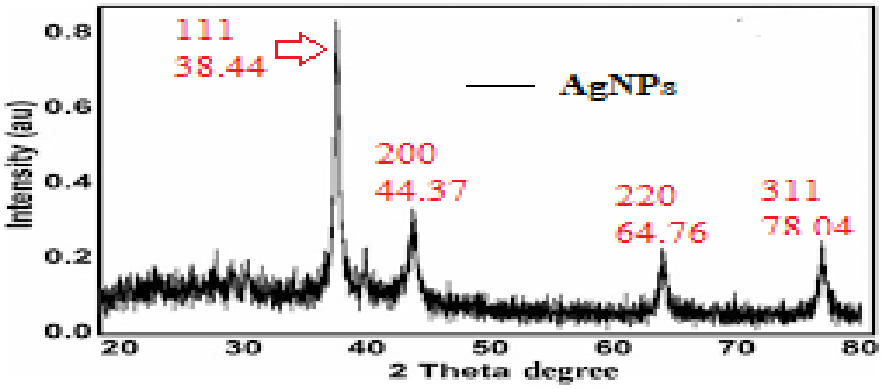

Figure 7: XR Diffraction spectra of Ag NPs

Fourier Transform Infrared Spectroscopy (FTIR)

The potential functional classes of biomolecules involved in silver ion reduction and stabilization of biosynthesized Ag NPs synthesized by *Aloe vera* aqueous extract were investigated using FTIR. The band intensities of the test sample were analyzed,figure (8).

The prominent peaks at 3773.5 and 3710.9 cm^{-1} are due to the N-H stretching amine vibration, while the observed peak at 3413.4 cm^{-1} is due to the – OH stretching vibration of flavonoidand, carboxyl and alcohol functional groups. The peaks of alkanes and alkynes at 2930 and 1608 cm^{-1} in the sennosides may be attributed to chelated carbonyl groups or –OH from carboxylic groups. Aromaticring is responsible for the prominent peak at 1510 cm^{-1}, while –C–O is responsible for the observed peak at 1037 cm^{-1}. Carboxylic acids, amides, alkanes,alcohol groups, alkenes, acids, and alkyl halides are the functional groups responsible for Ag NPs formation.

Diyala Journal for Pure Science

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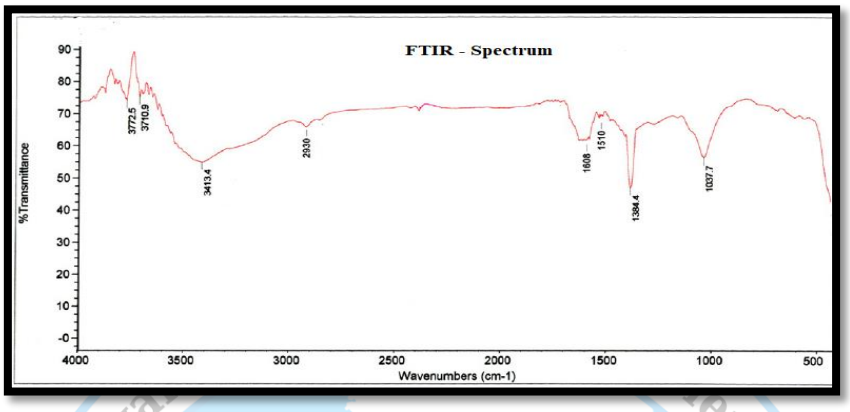

Figure 8: FTIR of active groups of the silver NPs synthesized

Scanning Electron Microscopy (SEM) analysis

The Scanning Electron Microscope (SEM) was used to determine the scale, shape, and distribution of green synthesized silver nanoparticles. Figure (9) shows that the particles were spherical with a smooth surface area with size of Ag NPs as following (33.21,35.1,40, 44.23,52.6 and 60.5) nm. The current research was effective in developing a limited range of silver nanoparticle sizes.

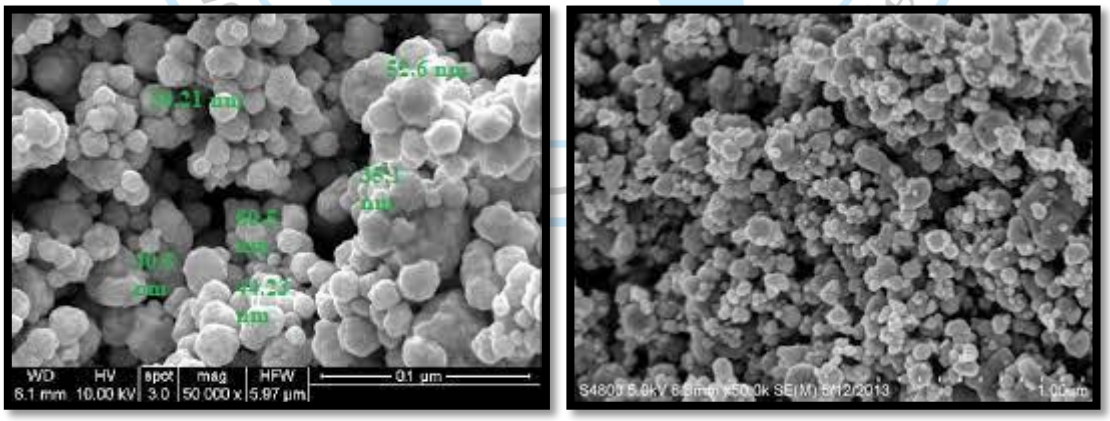

Figure 9: SEM image of Ag Nanoparticles

Determination the antibacterial activity of Ag NPs against bacteria

Ag NPs show the antibacterial activity against Multidrug resistance bacteria isolated from different sources. Aqueous extract of *Aloe vera* leaves which using to biosynthesized Ag NPs and the results shown in table (3), figure (10).

The Ag NPs had the largest diameter of inhibition zone against *K. pneumoniae, E.coli, P. mirabilis* and *A. baumannii* at concentrations of (100) mg/ml, reaching (22, 21, 23 and 20) mm, while the Ag NPs had the smallest diameter of inhibition zone against the same isolates at concentrations of (12.5) mg/ml, reaching (12,10,13 and 10) mm.

Table 3: Antibacterial activity of Ag NPs on bacterial growth (zones inhibition)

Bacterial isolates	Average of inhibition zone diameter (mm)			
	Concentraction 12.5mg/ml	Concentraction 25mg/ml	Concentraction 50mg/ml	Concentraction 100mg/ml
A. baumannii	10	13	16	20
K. pneumoniae	12	14	17	22
E. coli	10	13	16	21
P. mirabilis	13	16	19	23

Diyala Journal for Pure Science

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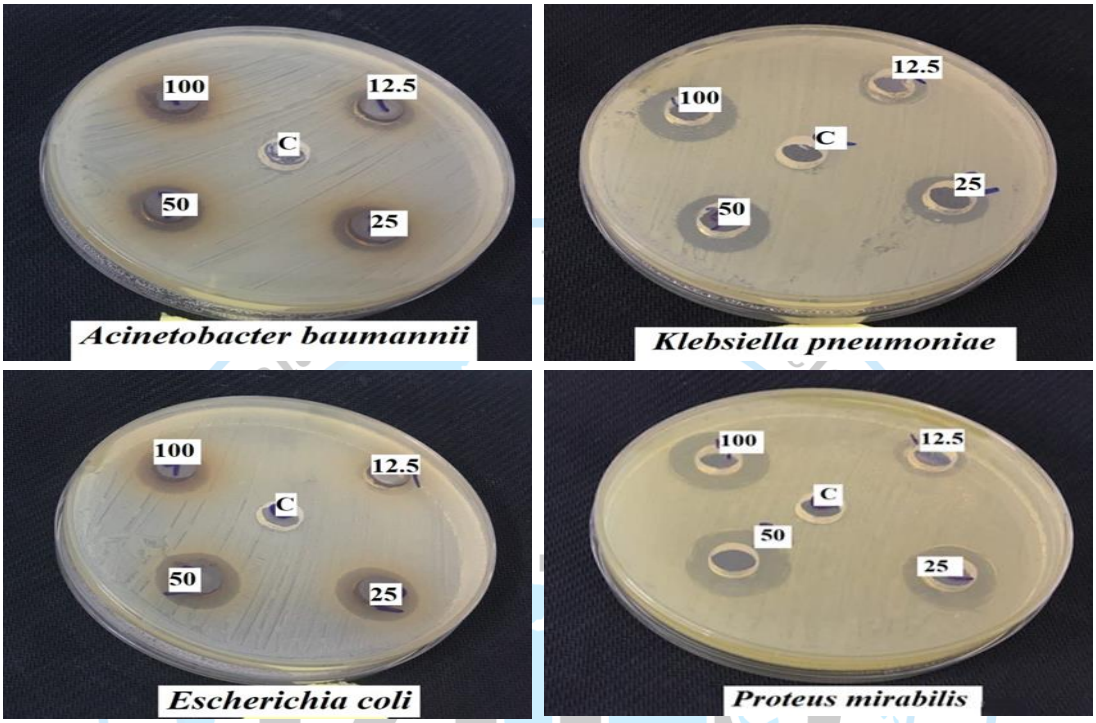

Figure 10: Antibacterial activity of Ag NPs against Pathogenic Bacteria C= Control (ddH2O)

The antimicrobial activity of Ag NPs against microbial pathogens was achieved by increasing reactive oxygen levels, including the development of free radicals [24], which enables them to penetrate the bacterial cell walls due to their small size [25].

Silver nanoparticles have ability to release Ag^{+} ions, which interact with the thiol group in bacterial proteins, influencing the position of DNA, and killing bacteria. Silver nanoparticles can also penetrate the bacterial cell wall, inactivating certain bacterial cell enzymes and producing hydrogen peroxide H_2O_2 [26,27]. Since silver nanoparticles attach to the cell wall and penetrate the bacterial cell wall negatively to the gram stain, they have antimicrobial properties. This increases cell permeability and contributes to uncontrolled cell permeability. Furthermore, extracellular DNA (eDNA) is found in the biological membrane matrix of

bacteria, and there is a connection between the positively charged silver nanoparticles Ag NPs and the negatively charged eDNA, which aids in the removal of microbes [28].

Conclusion

Antibiotic resistance was found in most bacteria isolated from wounds and burns (resistance to more than one antibiotic), so this paper presented simple and environment friendly way to produce Ag NPs. As a stabilizing agent, *Aloe vera* extract solutions were used. The nanoparticles were finely spherically frmed. XRD, AFM, FTIR, SEM, and UV-vis were used to investigate Ag NPs. Silver nanoparticles have antibacterial properties suggested that they could be used as a highly effective antibacterial agent with low cytotoxicity in humans.

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