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Antibacterial Activity of Silver Nanoparticles Synthesized by Aqueous Extract of *Carthamus oxycantha* M.Bieb. Against Antibiotics Resistant Bacteria

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Abstract:

Antibiotics resistant bacteria have become a global problem as a result of the unprogrammed use of antibiotics, resulting in bacterial strains resistant to many antibiotics, or to all available antibiotics. Plants are a good source of primary and secondary metabolites that have a major role in reducing silver nitrate to silver nanoparticles (AgNPs). The production of these nanoparticles were carried out by using aqueous extract of *Carthamus oxycantha* M.Bieb. This can be verified by color change of the reaction solution from yellow to dark brown because of the excitation of the surface plasmon resonance. AgNPs were characterized by UV-Vis spectroscopy, where they recorded the peak at 420 nm. Fourier Transformation-infrared (FTIR) was conducted to identify the effective plant group that contributes to the formation of AgNPs and it was found that proteins and phenols have the major role in the formation of those nanoparticles. Shapes and sizes of the synthesized AgNPs were characterized by Scanning Electron Microscope (SEM) with a range of 50-80nm in size and spherical in shapes. Antibacterial activity of AgNPs were tested against Multi-Drug Resistant bacteria (MDR), Extremely antibiotics Resistant (XDR), and Pan drug-resistant (PAN) bacteria, was done in concentrations ranging from 1000-63 µg/ml. The results showed that there were significant variations between the concentrations, the tested bacteria also showed significant differences in its sensitivity to AgNPs. The results recorded a proportional relation between the type of bacterial resistance to antibiotics and it's resistant to AgNPs, therefore the most resistant bacteria to AgNPs in this study *Enterobacter cloacae* EN2 was resistant to all antibiotics (PAN), while *Escherichia coli* E11 recorded was the most sensitive bacteria to AgNPs and its resistant only to 3 antibiotics**.**

Keywords: Antibiotics resistance bacteria, Antibacterial activity, Carthamus oxycantha, Silver nanoparticles.

Introduction:

There is a competition between human and pathogenic bacteria and as described by Baptista 2018 [1] "A battle of the Titans", when human discovered new antibiotics, the bacteria developed new mechanisms to fight these antibiotics until the limit is reached to obtain bacteria resistant to more than one antibiotics multidrug-resistant bacteria (MDR), and then resistant to most of the antibiotics extremely antibiotic resistant (XDR) bacteria, finally resistant to all types of antibiotics pan-drug-resistant bacteria (PAN). And if the situation remains as it is, it is expected that in 2050 there will be no antibiotic capable of eliminating these bacteria expect that humans discover new antimicrobial agents [2].

Green synthesis of metal nanoparticles using plant extracts had a universal interest due to their physiochemical and its implementation in different fields of biotechnology. These methods had attention in the last decade because these metal nanoparticles are mediated by eco-friendly plant extracts with low toxicity to human. Metal

nanoparticles mediated by plant extracts are characterized by high productivity in addition to its stability in size and shape and as well as having a good antimicrobial activity [3]. Plant extracts have an important role in reducing and stabilizing metal nanoparticles as they reduce the toxicity compared with using other methods in synthesizing nanoparticles. Plant extracts have secondary metabolites that play an important role in the manufacturing of metal nanoparticles such as polyphenols [4]. Silver nanoparticles show great attention because of their special characters like shapes and sizes. In the current years, green synthesis of silver nanoparticles by using plant extracts have been studied and invested for a wide range. Silver nanoparticles manufactured using plant extracts have numerous implantations as a result of their unique characters higher than in their bulk form. Silver nanoparticles have been used nearly 120 years and called colloidal silver [5].

Carthamus oxycantha M.Bieb.belonging to the family Compositae, spread as a wild plant in Iraq. According to [6, 7], it is present in dry and open places. It is distributed in Iraq and central Asia. The plant is used in the treatment of rheumatism, male infertility, chest disease, and high blood pressure [8].

The emergence of antibiotic- resistant bacteria is one of the biggest problems facing humanity. Antibiotic resistance bacteria first arise in hospitals and then spread everywhere, and this is because of the uncontrolled use of antibiotics, and this will lead to fewer antibiotics available to treat diseases 2. The current study aims to produce AgNPs composed by aqueous extract of *C. oxycantha* as antimicrobial agents since that is the first time that aqueous extract of this plant was tested as an antimicrobial agent to inhibit MDR,XDR, and PAN human pathogenic bacteria.

Materials and Methods:

Preparation of plant extracts

Carthamus oxycantha plant shoot was collected from Basrah University campus in flowering period and brought to a laboratory, washed with a much amount of tap water, then washed again with DW to remove dust, left to dry at room temperature, shoot was finely grounded to a fine powder using an electrical grinder. Refluxing extraction protocol was used to extract plant materials according to [9] with some modifications.10 g of plant powdered was added to 100 ml of DW supplemented to around, mixed well, refluxed for 5 hours. It was left to cool down filtered with no.1 Whatman filter paper. Filtrate was concentrated at room temperature and kept at 4 $^{\circ}$C until used.

Synthesis of silver nanoparticles

Green synthesis of silver nanoparticles was done according to [10]. Formation of silver nanoparticles was indicated by color change from pale green to dark brown. The formed AgNPs were centrifuged at 3000 rpm followed by washing with double distilled water.

Characterization of AgNPs

Synthesized AgNPs manufactured by using aqueous extract of *C.oxycantha* were characterized by using UV-Vis spectrophotometer, the range of absorbance was recorded from 350-700 nm. The dried AgNPs were documented by FTIR-84005-Shimadzu machine, Germany and in the region (500-4000cm-1) at room temperature; spectra were recorded at the University of Basrah / College of Education for Pure Sciences. SEM (Scanning Electron Microscope) analysis was done using Leo 1455vp (Germany) machine. AgNPs were coated with golds to conduct the samples.

In vitro antibacterial activity of silver nanoparticles

The antibacterial activity of AgNPs mediated by using aqueous extract of *C.oxyacantha* shoot was estimated against [9] isolates of MDR, XDR, and PAN Gram negative and Gram positive human pathogenic bacteria isolated from urine, stool and blood sample from out visit Al-Sadr Teaching Hospital, Central Laboratory, by agar well diffusion method according to Elbeshehy *et al*.2015[11] with some modifications. Briefly, tested bacterial suspension was swabbed with approximately $1X10^6$ CFU/ml on Muller-Hinton agar medium wells filled with synthesized AgNPs in concentrations ranging from 1000-63 µg/ml. Antibacterial activity was recorded by the diameter of inhibition zone in mm. Statical analysis was done by Chi-Square –Friedman test with probability value $P \leq 0.05$.

Results and Discussion:

Characterization of AgNPs

The color changing the reaction mixture from yellow to dark brown indicated the formation of AgNPs due to the reduction of silver ions to AgNPs, mediated by biomolecule founded in plant extracts [12] as shown in Fig. 1. The color changed due to the plasmon resonance surface of sedimented silver nanoparticles because of coherent and collective surface electron oscillation [13]. Color changed of solution due to the reduction of silver nitrate to silver nanoparticles as a result of the plant component extract, which acts as reducing, capping, and stabilizing of silver nanoparticles [14].

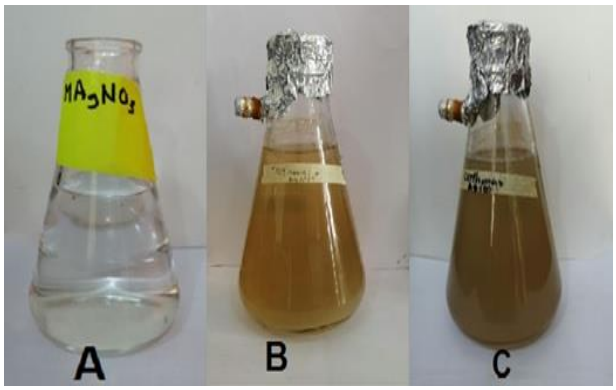

Figure 1. Color changed from yellow to dark brown indicating the formation of silver nanoparticles: A ($AgNO_3$) solution; B (*Carthamus oxycantha* aqueous extract); C (AgNPs).

Uv-Vis spectrophotometer analysis technique widely used in the characterization of AgNPs formation with range of absorbance ranging from 350-700nm AgNPs recorded absorbance peak at (420) nm showed in Fig. 2. The color changing the reaction mixture from yellow to dark brown indicated formation of AgNPs due to excitation of the surface Plasmon resonance [15]. This finding is also recorded by Aboutorabi *et al*.2018 [16] when they studied flower extract at *Carthamus tinctorius* to synthesized AgNPs, and they reported that Uv-Vis peak at (420)nm.

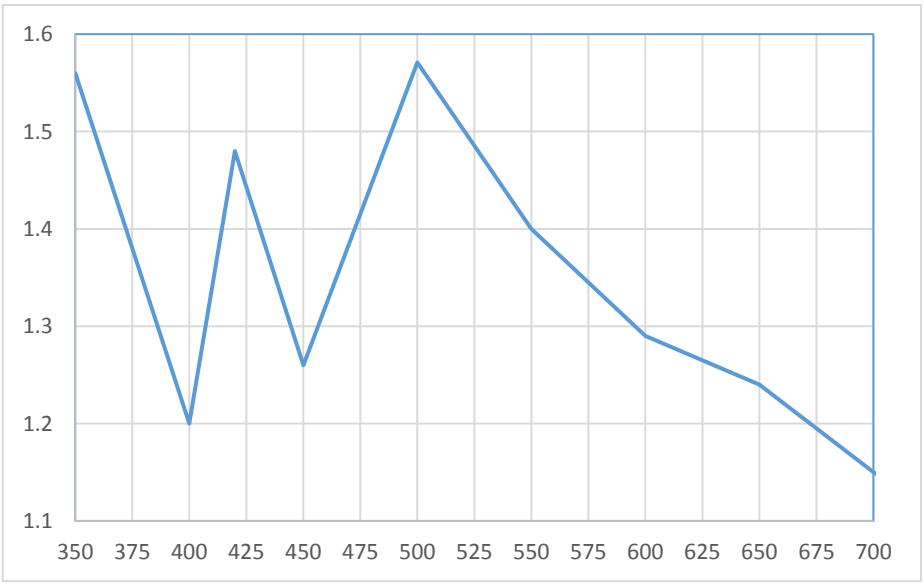

Figure 2. Results of UV-VIS analysis of AgNPs composed by aqueous extract of *Carthamus oxycantha*.

FTIR technique is used to characterize the formation of AgNPs by using aqueous extract of *Carthamus oxycantha*. AgNPs peaks are shown in Fig. 3 the spectra recorded at 3365, 2922, 2852, 1647, and 1519 cm-1 which represent the presence of alcohol/ phenols, secondary amide, primary amide. According to Mittal *et al.*2013 [17] peak at 3365indicated the presence of phenols, and peaks at1621 and 1535 cm-1 due to proteins which contain amine group that it is responsible for the reduction and capping agents of AgNPs. Also Masum *et al*.2018 [18] stated that plants extracts had flavonoids, polyphenol, amide groups of protein, act important role in AgNPs formation, and can be analyzed by FTIR. Ayad *et al*.2019 [19] declared that the compounds responsible for reducing silver nitrate to silver nanoparticles included free and bonded amide group of protein and polyphenols. The results above showed that protein and polyphenols may act as reducing, capping, and stabilizers of AgNPs.

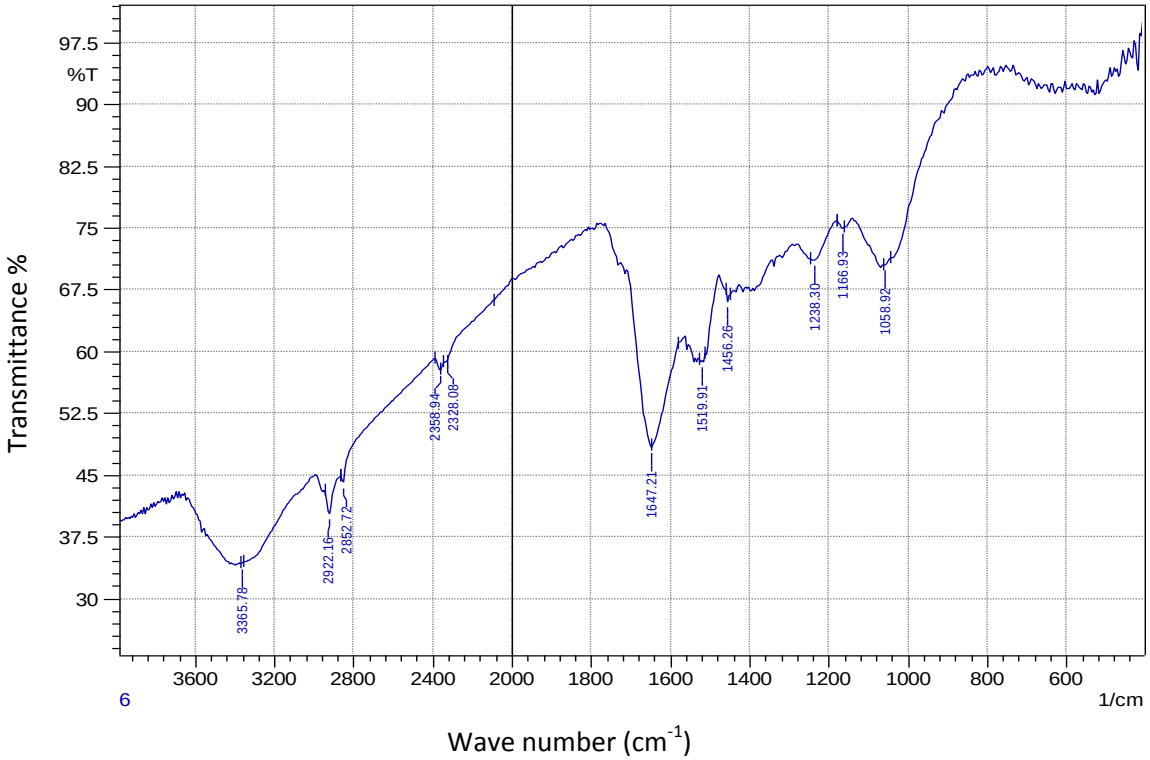

Figure 3. FTIR analysis of AgNPs composed by aqueous extract of *Carthamus oxycantha*.

Scanning Electron Microscope (SEM) images of AgNPs composed by aqueous extract of C. oxycantha are explained in Fig. 4A and B. These results characterized the shapes and sizes of Ag NPs synthesized by this method. SEM pictures showed the formation of AgNPs with range of 50-80 nm in size and spherical in shape. Aggregation of AgNPs resulted in agglomeration of these particles, which consist of large aggregates leading to the formation of large particles of AgNPs. This finding was also recorded by former researchers such as Kumar *et al.* 2013 [20] who founded that AgNPs size was 270nm, Shah et *al.*2015 [21] showed that AgNPS size ranges from 20-150nm, whereas Kumar *et al.*2017 [22] reported their size at 85-120nm. Formation of these aggregations can be attributed to the aggregation of nanoparticles during sample preparation [23, 19]. The formation of large-sized AgNPs was common when plant extracts were used in the green synthesis of these nanoparticles. It was found that the frequency of these large-sized nanoparticles was promoted by directly proportional to reaction temperature according to Ostwald ripening theory [24,16].

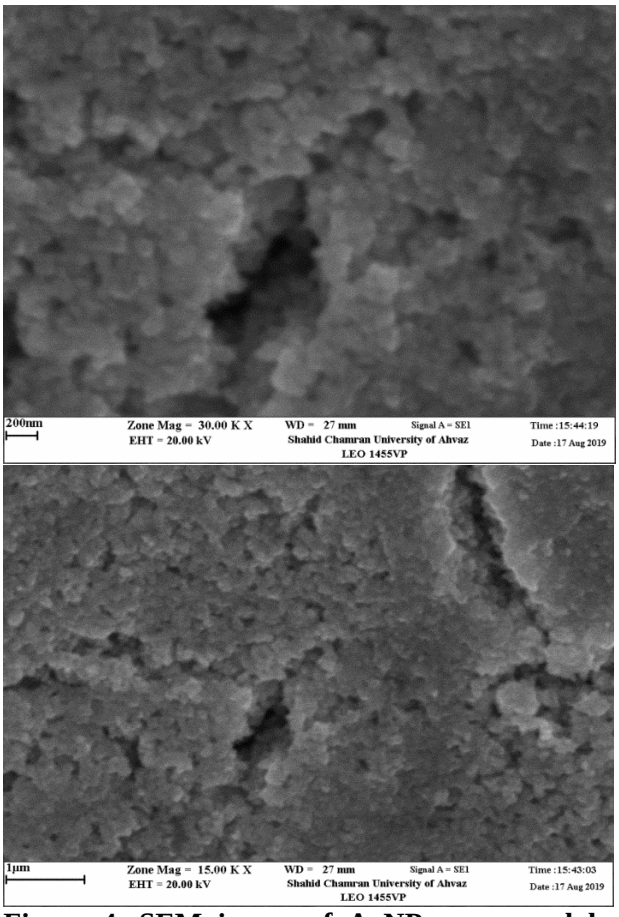

Figure 4. SEM image of AgNPs composed by aqueous extract of *Carthamus oxycantha*

The antibacterial activity of AgNPs synthesized by aqueous extracts of *C .oxycantha*. has been investigated against MDR, XDR, and PAN pathogenic bacteria showed the zone of inhibition produced. The results of the antibacterial activity of AgNPs showed that there were significant differences between the concentrations with Chi-Square 35.435, also the tested bacteria varied significantly in their sensitivity to AgNPs (Chi-Square 28.707) where *Escherichia coli* E1 was the most sensitive with mean rank value of 8.8, while the bacteria *Enterobacter cloacae* EN2 was the most resistant to AgNPs and the value of mean rank was 2 as shown in Table 1 and Fig. 5. The resistance of the bacteria AgNPs was directly proportional to its resistance to antibiotics, as the most resistant bacteria *Enterobacter cloacae* EN2, was recorded as resistant to all antibiotics, while *Escherichia coli* E11 bacteria was the most sensitive to AgNPs and was resistant to 3 antibiotics only Table 2. This can be explained as the targeted bacteria treated with AgNPs might change the permeability of the outer membrane and increased efflux pumps [25]. Here it can be concluded that the greater the resistance of bacteria to antibiotics, the greater their resistance to silver nanoparticles becomes. This can be explained by the fact that antibiotic-resistant bacteria have many mechanisms to resist anti-bacterial agents, including silver nanoparticles, and from those mechanisms is that bacteria can minimize the toxic impact of AgNPs because of reduced uptake of the plasma membrane and cell wall or augmented of nanoparticles [26]. Also, resistance to AgNPs can be assigned to the genetic changed in bacterial cells resulting in the quick emergence of resistance to AgNPs and induced transfers of managers antibiotics resistance genes [27,28] .*E. coli* treated with AgNPs, bacterial cells evolution of phenotypic changes may lead to the production of flagellin which weakened the antibacterial effect of AgNPs 25,29. Whereas *Enterobacter cloacae* EN2 in this study resistant to all antibiotics (PAN) was recorded sensitive to AgNPs, although it is the most resistant species studied. Here it can be concluded that the most resistant bacteria to antibiotics, can be inhibited with AgNPs. Furthermore, silver nanoparticles have an effect on the growth of the MDR mechanisms involved in the resistance to antimicrobial agents [30,] and these AgNPs can beat the bacterial resistant mechanisms in several ways such as Cracking the cell wall, production of reactive oxygen species (ROS) which results in inhibition of ATP production, DNA replication, cell wall shatter, increase of cell membranes permeability, inhibit RNA and proteins production, attack bacteria immune system, disarray of electron transport chain 1.

Table 1. Zone of inhibition of silver nanoparticles synthesized by aqueous extract of *C.oxycantha*

Bacteria	Mean rank	Type of resistance	AgNPs aqueous extract concentration (µg/ml)					Chi-Square
			1000 (9.94)*	500 (4.06)	250 (2.94)	125 (2)	63 (1.06)	35.435
***Escherichia coli* E11**	8.8	MDR	22 **	22	20	16	15	
***Escherichia coli* E12**	4	XDR	16	15	13	12	11	
***Escherichia coli* E13**	2.2	XDR	17	13	11	11	0.0	
***Enterobacter cloacae* EN1**	6.8	XDR	20	19	16	15	11	
***Enterobacter cloacae* EN2**	2	PAN	15	13	12	11	0.0	
***Pseudomonas aeruginosa* P1**	5.7	MDR	22	16	14	12	11	
***Pseudomonas stutzeri* P2**	5.3	PAN	19	18	16	14	0.0	
***Staphylococcus aureus* ST1**	3.6	MDR	20	17	13	0.0	0.0	
***Staphylococcus aureus* ST2**	6-6	MDR	22	20	18	13	0.0	
Chi-Square	28.707							

Use chi-square analysis for nonparametric data (Friedman Test – Chi-square) below the probability level P≤0.05

*: The numbers outside the parentheses represent the AgNPs concentration and inside the parentheses are Mean Rank

**: inhibition zone in (mm); MDR: multi-drug resistant bacteria / XDR; extremely antibiotics resistant bacteria/PAN: pan drug-resistant bacteria.

Table 2. The antibiotics profile of bacteria isolated in this study.

Antibiotics	***E.coli* strains**			***Enterobacter cloacae***		***Pseudomonas aeruginosa***	***Pseudomonas stutzeri***	***Staphylococcus aureus***	
	E11	E12	E13	EN1	EN2	P1	P2	ST1	**ST2**
Ampicillin	R				R	R	R		
Amoxilin-clavulanic acid					R	R			
Azithromycin						R			
aztreonam		R		R		R			
Piperacilin		R	R	R	R	R			
Piperacillin/Tazobactam		R		R		R	R		
Cefuroxime			R		R	R	R		
Cefuroxime Axetil					R	R	R		
Cefoxitin			R		R	R	R		
Cefotaxime			R			R	R		
Ofloxacin			R			R			
Cefixime			R		R	R			
Ceftazidime	R	R	R	R	R	R	R		
Ceftriaxone			R		R	R	R		
Cefepime		R	R	R		R			
Aztreonam /monobactam									
Gentamicin			R	R	R	R	R		
Ciprofloxacin	R	R	R		R	R	R		
Levofloxacin		R	R						
Tetracycline		R	R						
Nitrofurantoin					R	R	R		
Trimethoprime		R	R		R	R	R		**R**
ticaraillin			R						
Clindamycin								R	
Linezolid								R	
Benzylpenicillin								R	**R**
Fusic acid								R	
mupirocin								R	
Amikacin				R	R	R	R		
ertapenem					R				
Imipenem					R	R	R		
Meropenem					R				

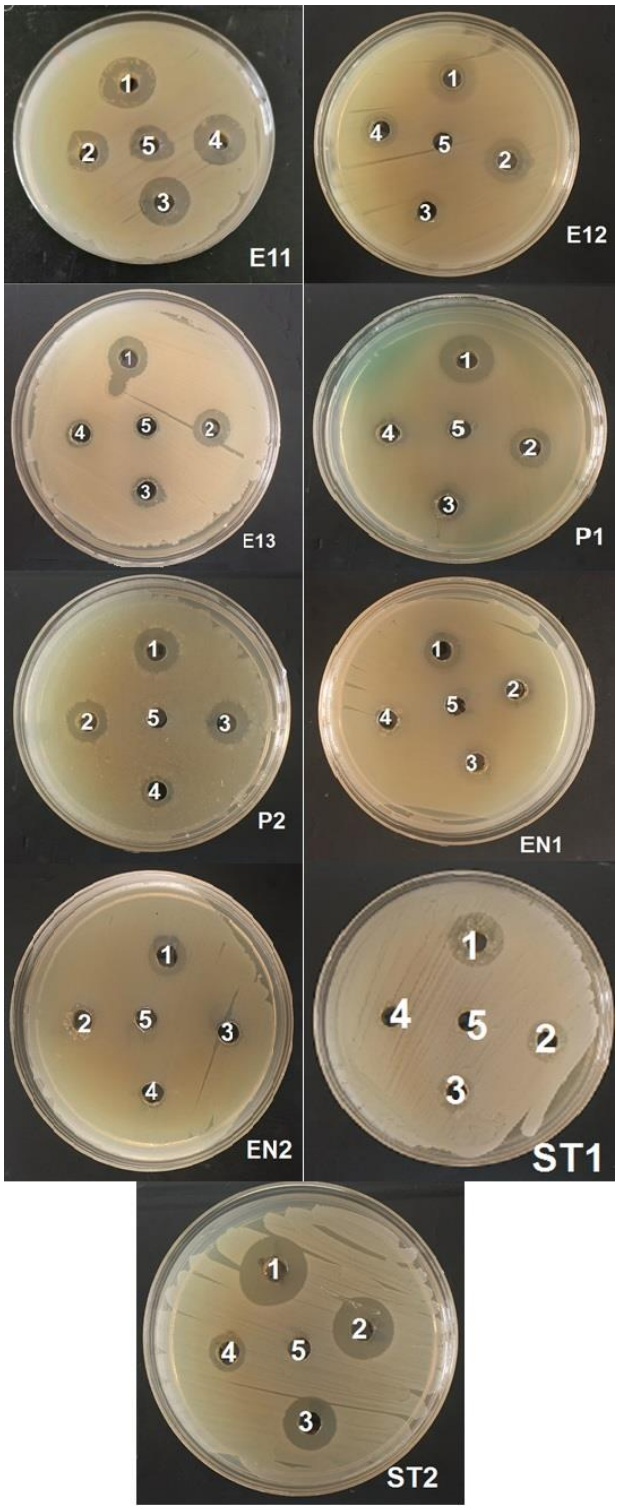

Figure 5. Antibacterial activity of AgNPs composed by aqueous extract of *Carthamus oxyacantha*.1:(1000 µg/ml);2(500 µg/ml) ;3:(250µg/ml); 4:(125 µg/ml) ; 5:(63 µg/ml), E11(*Escherichia coli* E11); E12(*Escherichia coli* E12);E13(*Escherichia coli* E13);P1(*Pseudomonas aeruginosa* P1);P2(*Pseudomonas aeruginosa* P2);EN1(*Enterobacter cloacae* EN1); EN2(*Enterobacter cloacae* EN2); ST1(*Staphylococcus aureus* ST1); ST2(*Staphylococcus aureus* ST2).

Conclusions:

Aqueous extract of *Carthamus oxycantha* M.Bieb was a good source to synthesize silver nanoparticles (AgNPs). These nanoparticles were characterized by UV-Vis (FTIR) and (SEM) spectroscopy, Antibacterial activity of AgNPs was tested against MDR,XDR, and PAN bacteria, in concentrations ranging from 1000-63 µg/ml. This study recorded a proportional relation between the type of bacterial resistance to antibiotics and it's resistance to AgNPs, The most resistant bacteria to AgNPs was *Enterobacter cloacae* EN2 was resistant to all antibiotics, while *Escherichia coli* E11was recorded the most sensitive bacteria to AgNPs and its resistant only to 3 antibiotics**.**

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basrah.

Authors' contributions statement:

Ali Aboud Shareef Conception, design., Zainab Alag Hassan analysis, interpretation, Majid Ahmed Kadhim acquisition of data and Abdulameer Abdullah Al-Mussawi revision and proofreading. All authors discussed the results and contributed to the final manuscript.

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الفعالية المضادة للبكتريا لدقائق الفضة النانوية المصنعة بواسطة المستخلص المائي لنبات القرطم حاد الشوك ضد البكتريا المقاومة للمضادات الحيوية

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[3] وزارة الصحة مستشفى الصدر التعليمي.

الخلاصة:

اصبحت البكتيريا المقاومة للمضادات الحيوية مشكلة عالمية نتيجة للاستخدام غير المبرمج للمضادات الحيوية مما أدى إلى ظهور سلالات بكتيرية مقاومة للعديد من المضادات الحيوية، أو لجميع المضادات الحيوية المتاحة. تعد النباتات مصدر جيد للأيضات الأولية والثانوية التي لها دور رئيسي في اختزال نترات الفضة إلى دقائق الفضة النانوية silver nanoparticles (AgNPs). تم في الدراسة الحالية إنتاج هذه الجسيمات النانوية باستخدام المستخلص المائي لنبات القرطم حاد الشوك *Carthamus oxycantha* M.Bieb كمواد مختزلة لنترات الفضة. وامكن التحقق من تكون دقائق الفضة النانوية بواسطة تغير لون مزيج التفاعل من الاصفر الى البني الغامق بسبب مايسمى بظاهرة surface plasmon resonance. ووصفت تلك الدقائق النانوية AgNPs بواسطة التحليل الطيفي للأشعة فوق البنفسجية Uv-Vis spectrophotometer، حيث سجلت قمة الطيف عند الطول الموجي 425 نانومتر. وفحصت هذه الدقائق ايضا باستخدام طيف الاشعة تحت الحمراء Fourier Transformation –infrared (FTIR) لغرض تحديد المجاميع الفعالة للمستخلص النباتي التي اسهمت في تكوين دقائق الفضة النانوية. ووجد أن البروتينات والفينولات لها دور رئيسي في تكوين تلك الدقائق النانوية. ومن اجل معرفة احجام واشكال تلك الدقائق النانوية فقد فحصت بالمجهر الالكتروني الماسح Scanning Electron Microscope (SEM) وسجلت الدراسة الحالية بان أحجام تلك الدقائق تراوحت بين 50-80 نانومتر وذات اشكال كروية. تم اختبار الفعالية المضادة للبكتيريا المدروسة المقاومة المعدودة لبعض المضادات الحيوية(MDR)، والمقاومة للعديد من المضادات الحيوية (XDR)، والبكتيريا المقاومة لجميع المضادات الحيوية المعروفة (PAN) وبتراكيز تراوحت بين 1000-63 ميكروغرام / مل. أظهرت النتائج وجود اختلافات معنوية مابين التراكيزفي تثبيطها للبكتريا المدروسة ، كما أظهرت البكتريا المختبرة اختلافات معنوية في حساسيتها لدقائق الفضة النانوية. وبينت النتائج إلى وجود علاقة طرديةبين نوع المقاومة البكتيرية للمضادات الحيوية ومقاومتها لـتلك الدقائق ، فالبكتريا *Enterobacter cloacae* EN2 كانت البكتيريا الأكثر مقاومة لـ AgNPs في هذه الدراسة وهي في نفس الوقت كانت مقاومة لجميع المضادات الحيوية (PAN) ، بينما سجلت *Escherichia coli* E11 أكثر أنواع البكتيريا حساسية لـ AgNPs ومقاومتها لـ 3 مضادات حيوية فقط.

الكلمات المفتاحية: البكتيريا المقاومة للمضادات الحيوية ، الفعالية المضاد للبكتيريا ، Carthamus oxycantha، جزيئات الفضة النانوية..