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Preparation and Diagnosis of Green Nanocomposite from Salvia Officinalis and Study of its Inhibitory Effectiveness on Resistant Bacteria Isolated from Different Clinical Cases

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A B S T R A C T

The current research aims to prepare the aqueous extract and nanocomposite of sage leaves using green synthesis method, and to identify the silver nanoparticles using FTIR and SEM analysis. The antibiotic ciprofloxacin was loaded on the nanocomposite and the inhibitory activity on the studied bacterial isolates G-ve Klebsiella pneumoniae and Pseudomonas aeruginosa was studied. The study groups were divided into two replicates and five groups with three concentrations. The first group was treated with the antibiotic ciprofloxacin, the second group was treated with the aqueous extract of sage, and the nanocomposite was applied to the third group. The extract loaded with the antibiotic was used to treat the fourth group, and the nanocomposite loaded with the antibiotic was used to treat the fifth group. The color change from yellow to dark brown is evidence of the formation of nanoparticles, and the wave shift in the nanocomposite loaded with the antibiotic towards the frequency 2156.93 cm^(-1) is evidence of the success of the antibiotic loading process on the nanocomposite. The resulting compounds were characterized using scanning electron microscopy, and its results showed that the extract particles were in the form of irregular aggregates with the presence of some small particles with a spherical shape and an average particle size of 85.49 nm, while the nanocomposite was cubic in shape and an average particle size of 35.42 nm. The inhibition zones were evident in the fourth treatment T4, the aqueous extract loaded with the antibiotic, and the fifth treatment T5, the nanocomposite loaded with the antibiotic. Silver nanoparticles showed the highest synergistic efficiency with the antibiotic ciprofloxacin against the multidrug-resistant. bacteria strain *K.pneumoniae*, recording a rate of 14.33±1.41, while against *P.auroginosa* bacteria, a rate of 13.44±88 was recorded.

1. INTRODUCTION

The increasing numbers of antibiotic-resistant microorganisms pose a threat to human health, especially among poor populations and in hospitals and intensive care units. This is due to the misuse of antibiotics, poor hygiene in society, unsafe food, inadequate infection control in medical facilities, accumulation of antibiotics in the environment and their use in the food and animal industries [1]. Bacteria rapidly develop new resistance systems that enable them to withstand the effects of antibiotics, through mutations that occur in some bacterial cells that make them resistant to the effects of antibiotics, and then this advantage is passed on to the next generation, which is generation with complete resistance to the antibiotic [2]. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *and Acinetobacter baumannii* have all been recognized as pathogens with mostly high rates of antibiotic resistance, resulting in a declining range of treatments available for these organisms [3].

To confront the problem of bacterial resistance to multiple drugs, scientists have invented nano-delivery systems that have demonstrated high efficiency in overcoming many anatomical and functional barriers to delivering the drug to the target site. Thus, they have been able to produce highly effective drugs with few side effects [4]. Green nanotechnology is a field that focuses on making products that are environmentally friendly, safer for all living organisms, less expensive and more stable than other manufacturing methods such as bacteria, fungi, yeasts and viruses or using different plant parts such as leaves, stems, fruits, peels, etc. The "green synthesis" of nanoparticles has attracted a lot of attention to the use of metal oxides, such as silver oxides ($Ag\text{-}NO_3$), because of their optical, chemical, electrical and optical properties [5]. It has been found that nanomaterials face the problem of increasing resistance to bacteria because of their unique properties whose dimensions fall within the nanoscale of (1-100) including increased solubility. Biocompatibility, ease of

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production, and stability. The nanoparticles' tiny size relative to the area of their surface is one of the most important characteristics that distinguishes these materials [6]. Many studies have proven that plants are considered a safe source for the pharmaceutical industry and a preventive source for many diseases, due to the medical effectiveness of plant compounds that contain antioxidant and antimicrobial properties [7]. Among the medicinal plants is the sage plant. It is part of the Lamiaceae family, which is often known as the "salvation plant", derived from the Latin word "salvarem". It means "preservation or treatment An evergreen perennial shrub, one of about 900 species of the genus Salvia, native to the Mediterranean and Middle East, Salvia grows as a subshrub up to 60 cm tall, with opposite and simple leaves, with white hairs on the lower surface of the leaf and a gray-green color on the upper surface of the leaf.".. Stems are erect with an abundance of dark green hairy branches. Leaves are elongated and petiolate with a serrated edge and a serrated surface, sometimes with basal lobes. The flowers are 2 to 4 mm long from their petioles. It is in the form of false upper flowers with 5 to 10 blue-violet colored, colorful flowers that form compound false spines. It blooms from March to July, depending on the habitat and climatic conditions [8]. It has been used for a variety of purposes, including reducing perspiration, treating sore throats (used as a gargle), regulating menstrual cycles, fighting infections, improving lipid status and liver function, improving appetite and digestion, and enhancing mental ability [9]. Thus, the goal of the present investigation was to prepare the aqueous extract and nanocomposite of the Salvia officinalis plant using the green synthesis method

2. MATERIALS AND METHODS

2.1. Isolation and Diagnosis of Bacteria

One hundred samples of both sexes, with ages ranging from (1 day to 70) years, were collected from Burn and wound patients and children in hospital. The number of burn isolates was 35 isolates, surgical isolates were 52 isolates, and children were 13 isolates. The isolates were transferred in swab form and placed in a sterile carrier medium. From hospitals in the Holy Governorate of Karbala, Al-Hussein General Hospital and Children's Teaching Hospital, all isolates were grown on MacConkey agar and Blood agar medium and incubated for 24 hours at 37 ℃ and the bacteria were diagnosed using the VITEK-2 Compact System device.

2.2. Preparation of the Aqueous Extract and Nanocomposite of Salvia Officinalis

Fifteen gram of dried *salvia* powder was weighed and 300 ml of boiled distilled water was added. After 30 minutes, it was left at room temperature. It was filtered using pieces of gauze and subsequently filtered twice utilizing filter papers (Whatman No. 1). The filtrate was then placed in a centrifuge and dried. 45°C was the temperature at which the filtrate was placed in an oven to obtain a dry extract in powder form. The powder was placed in a sealed, opaque tube and stored until it is used. Green Ag-NPs were manufactured by bio absorption of Ag+ in a clean solution of Salvia officinalis extract. By taking 1 mM of silver nitrate, the plant extract (1.5 gm of ready-made dry plant extract into 100 ml of non-ionic distilled water) was dropped onto the alkaline $AgNO_3$ solution with a ratio of (80% silver nitrate solution: 20% plant extract) and was done. Mixing for 30 minutes at 45-55 °C. Then the mixture is monitored for 3 hours. Then, it is observed that the color of the mixture changes from yellow to dark brown and this indicates the formation of Ag-NPs [10].

2.3. Diagnosis of Nanoparticles

FTIR analysis is used to determine the active groups responsible for reducing $AgNO_3$ to AgNPs, in addition to being responsible for the stability and coverage of the Ag NPs. SEM is employed to find out the shape and the nanoparticles' size and that all the prepared compounds are within the nanoscale limits [11].

2.4. Antibacterial Effectiveness

The minimum inhibitory concentration (MIC) was estimated utilizing the well diffusion method [12] for the antibiotic Ciprofloxacin, the aqueous extract of the free sage plant loaded with the antibiotic, and the nanocomposite before and after loading the antibiotic. The MIC for *K. pneumonia* bacteria, and *P. aeruginosa* is equal to 4 µg/ml of the VITEK and the MIC for extract 8 µg/ml and nanocomposite 8 µg/ml [13]. The extract is loaded with the antibody (2+4) µg/ml, and the nanocomposite is loaded with the antibody (2+4) µg/ml. The bacterial suspension was added to dishes containing Muller Hinton agar solid medium and was spread well on the surface of the dish using a diffuser. Figure - L sterile swab. Then, wells were made on the surface of the agar with equal dimensions for five groups. The first group was treated with the antibiotic ciprofloxacin, The Salvia officinalis plant's aqueous extract was used to treat the second group, The nanocomposite was applied to the third group, The antibiotic-loaded aqueous extract was given to the fourth group, and The Salvia officinalis plant's aqueous extract was given to the fifth group. It was treated with the nanocomposite loaded with the antibiotic, at three concentrations greater than the MIC, and at a concentration of MIC and less than the MIC, in a volume of 50 microlitres, using a micropipette. The concentrations were placed on the dishes containing the

bacterial culture, after which the plates were kept in the incubator for a full day at 37 °C.

3. RESULTS AND DISCUSSION

3. 1. Isolation and Diagnosis of Bacteria

The VITEK2 device was used to diagnose the identity of the bacterial isolates and the results were as shown in Figure 1.

klebsiella pneumonia 24%, *pseudomonas aeuroginosa* 21%, *staphylococcus aureus 7%, E. coli* 13% *coagulase negative staphylococcus 8%, Enterobacter aerogenes 5%, Staphylococcus Haemolyticus 4%, shigella 4%, Acinetobacter baumannii complex 4%, Enterococcus Avium %, Enterococcus Enterobacter 4% cloacae complex 1%, Serratia 1%, Enterococcus gallinarum 2%.*

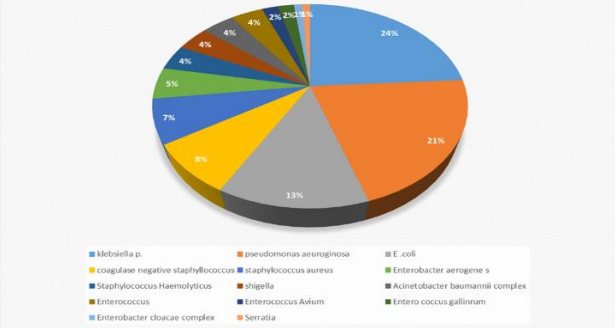


Figure 1. The percentages of bacterial isolates

3.2. Preparation of the Aqueous Extract and Nanocomposite of *Salvia Officinalis*

The change in the color of the reaction mixture resulting from (settling the aqueous extract of Salvia leaves at a rate of 20% on a solution of silver nitrate at a rate of 80%), after three hours from yellow to dark brown as in Figure 2 and 3, which represents the formation of silver nanoparticles, because of the reduction of Ag+ metal ions into particles. Nano silver Ag via active molecules display in S. officinalis extract such as violin compounds and organic acids [14].

Figure 2. Color change of the aqueous extract of Salvia officinalis from yellow to dark brown (A) solution of the aqueous extract of the Salvia plant. (B) solution of silver nitrate $AgNO_3$. (C) Aqueous extract of the plant Salvia officinalis after reacting with a 1 mM $AgNO_3$ solution after three hours.

3. 3. Diagnosis of Nanoparticles

The results of FTIR analysis, as shown in Figures 3 and 4, showed different extensions of the beams with various wave numbers. For the drug ciprofloxacin at a frequency of 3270.25 cm^{-1}, it was evidence of the stretching of the O-H bonds of hydroxyl groups present in alcohols and phenols. On the other hand, we notice a shift in examining the nanocomposite loaded with the drug at the frequency of 3276.57 cm^{-1}, and the drug shows an absorption peak at the frequency of 2153.22 cm^{-1} indicating an expansion of the $C \equiv C$ bond, the infrared spectrum of the nanocomposite loaded with the drug is between a shift towards the frequency. 2156.93 cm^{-1} stretching of the $C \equiv C$ bond, and the peak at 1636.05 cm^{-1} results from the expansion of the $C = C$ Alkene bond. In examining the nanocomposite loaded with the drug, it was noted that the peak remained at the same frequencies at 1636.36 cm^{-1}. SEM This technique is employed to ascertain nanoparticles' Shape and size. The examination results of the nanocomposite of salvia showed homogeneity, good distribution, and a dominance of the cubic shape, as the average sizes of the particles ranged between (100.5-270.8) nm, mean size (35.42) nm as in Figure 5, while the SEM examination results of the aqueous extract of the salvia plant were Irregular shapes with clusters and the presence of some small particles that have a spherical shape. The particle sizes ranged between (44.66-72.57) nm, mean size (85.49) nm. The most prominent results from earlier research confirm that hexagonal or rod-like nanoparticles are less effective than spherical nanoparticles and that the surface area and size of AgNPs primarily determine their biological activity as seen in Figure 6. The surface area of smaller nanoparticles is greater than the larger ones [15].

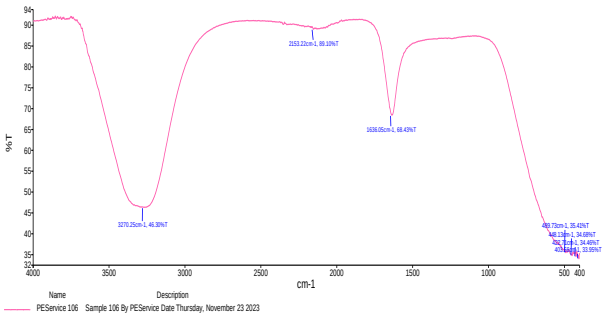


Figure 3. FT-IR Spectrum of Ciprofloxacin (CIP)

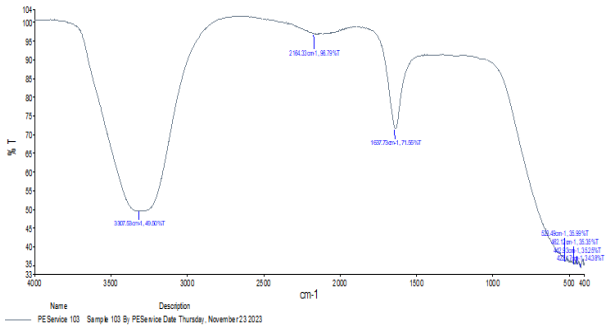


Figure 4. FT-IR Spectrum The Nanocomposite Loaded with the Drug Ciprofloxacin (CIP)

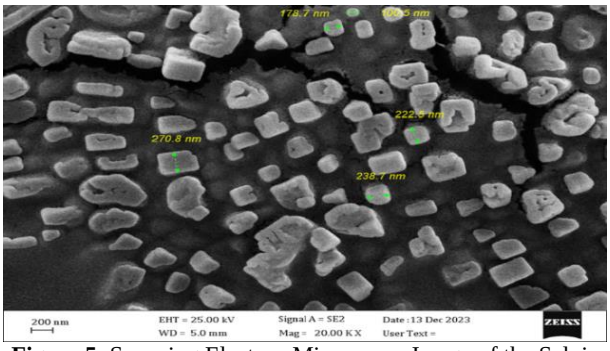


Figure 5. Scanning Electron Microscope Image of the Salvia Nanocomposite

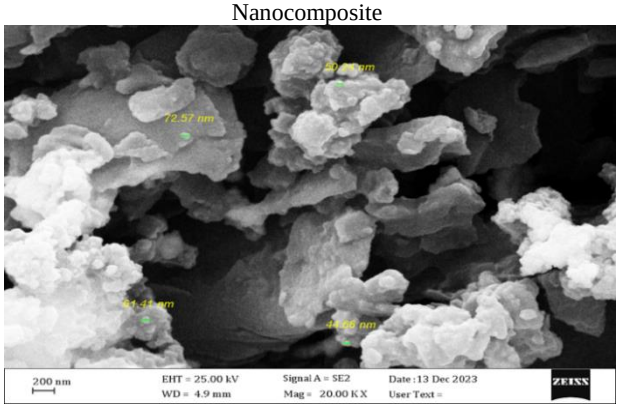


Figure 6. Scanning Electron Microscope Image of the Aqueous Extract of The Salvia Plant

3. 4. Estimation of Inhibitory Effectiveness

The culture medium for the *K.pneumonia* bacteria was exposed to the first treatment, T1, with the antibiotic CIP at a concentration of (8, 4, 2) µg/ml. Thus, the average diameters of inhibition were (.35 ± .83), no clear areas of inhibition of the antibiotic appeared at all concentrations because the *K.pneumonia* bacteria are resistant to the antibiotic CIP. The results are statistically significant: no significant difference exists (p>0.05), and that the K.pneumonia bacteria were treated with the second treatment, T2, the aqueous extract of the Salvia Sao plant at a concentration of (16.8.4) µg/ ml، . The average diameters of inhibition were(.22 ± .86). Analysing the results statistically indicated that there was no significant difference (p>0.05).

The biological activities and antimicrobial effect provided by *S. officinalis* are Because of the existence of many biologically active compounds in this plant, and the most significant are camphor (23.5 %), α-thujone (34.7 %), 1, 8-cineole (11.5 %). %), carvacrol (7.4 %) [16], phenols and flavonoids, and these compounds have the ability to eliminate free radicals. It has been proven that the extract of the S officinalis plant is an antioxidant and antibacterial due to its richness in flavonoids [17], and the treatment of bacteria with the third treatment T3 nanocomposite Sao/NPs at a concentration of (16, 8, 4) µg/ml, so the average diameters of inhibition were (.28±.83).Statistically, the results displayed that no significant difference exists (p>0.05).

Silver nanoparticles have several key mechanisms against microorganisms. When AgNPs contact the surface of bacteria that is negatively charged, they will change the chemical and structural characteristics of the cell wall and cell membranes and disrupt crucial roles including permeability, electron transfer, respiration, and osmosis [18]. The particles Ag NPs penetrate bacterial cell and bind to proteins, DNA and other cell elements that contain phosphorus and sulfur causing more bacterial cells damage [19], and Ag NPs release silver ions that generate a huge biocidal effect [20].

As for exposure to the fourth treatment, the aqueous extract of the Sao plant is loaded with the antibiotic CIP at a concentration (4+8, 2+4, 1+2) µg/ml, the average diameters of inhibition were (3.50 ± 14.44), the results showed statistically significant differences (p>0.05), and the fifth treatment the nanocomposite was loaded with the antibiotic SaoNPs/CIP at a concentration (4, 1+24+8, 2) µg/ml, the average damping diameters were (1.41 ± 14.33). The statistical results indicate the presence of a significant difference (p>0.05).

It is noted from Table 1 that there are differences between the treatments used, and the strongest treatment in terms of areas of inhibition that was T5, followed in strength by the treatment T4. As for within the treatments and the significance of the differences between the different concentrations of each treatment. The statistical analysis did not show any significant differences between the concentrations used for each treatment.

The culture medium for the T1 bacteria *P. auroginosa* was exposed to the first treatment with the antibiotic CIP at a concentration of (8, 4, 2) µg/ml, the average diameters of inhibition were (.49 ± .64), as no clear areas of inhibition for the antibiotic appeared at all

concentrations because the *P. auroginosa* bacteria are resistant to the antibiotic CIP . The results are statistically significant. We notice no significant difference (p >0.05) exist , and that treating the *P. auroginosa* bacteria with the second treatment T2, the Salvia Sao plant aqueous extract with (16, 8, 4) µg/ml concentration, average diameters of inhibition (.18 ± .89) and analysing the results statistically indicated that no significant difference exists (p>0.05) The bacteria were treated with the third treatment, T3, the nanocomposite Sao/NPs at a concentration of (16, 8, 4) µg/ml. modified The diameters of inhibition were (.43 ± .69). Statistically, the results presented no significant differences (p>0.05) exist.

If the culture medium for the *P.auroginosa* bacteria was exposed to the fourth treatment T4, the aqueous extract of the Sao plant loaded with the antibiotic at a concentration of (4+8, 2+4, 1+2) µg/ml, the average diameters of inhibition were (1.92 ± 12.78), the results were statistically clear, there were no significant differences (p>0.05), but at the fifth treatment T5 nanocomposite loaded with the antibiotic SaoNPs/CIP. Based on the statistical analysis, no significant differences were found (p>0.05).

The areas of inhibition appeared clearly in the treatments T4 and T5, the aqueous extract of the S officinalis plant loaded with the antibiotic, and the nanocomposite loaded with the antibiotic. The two treatments produced a synergistic effect against resistant pathogens. This is because when AgNPs bind to antibiotics, their stability, functions, and selectivity become stronger, and their transfer of the drug is specific. Significantly [21], silver nanoparticles conjugated with antibiotics showed greater inhibitory activity than when they acted alone, and the results showed that silver nanoparticles conjugated with antibiotics such as chloramphenicol, tetracycline, gentamicin, and ciprofloxacin targeted multiresistant bacteria such as K. pneumoniae. Several studies reported that nanocomposites that co-act with antibiotics have greater antibiotic activity compared to nanoparticles in their free form [22].

Gram-negative bacteria exhibit wider zones of inhibition when compared to gram-positive bacteria. The reason behind this is the variation in the constitution of the cell walls. Gram-positive bacteria's cell wall is made up of a thick layer of peptidoglycan, which possesses chains of linear polysaccharides that are linked to short peptides, thus creating a stiffer structure. A thin layer of peptidoglycan coats the cell wall of Gram-negative bacteria, preventing the strong penetration of silver nanoparticles [23]. Interestingly, When combined with ciprofloxacin, biotinylated silver nanoparticles had the greatest synergistic efficacy against the strain of bacteria that was resistant to multiple drugs *K.pneumonia*, where it was recorded. The ratio was 14.33±1.41, while for *P.auroginosa* bacteria, recording a synergistic ratio of .13.44±.88. This indicates that *P.aeruginosa* bacteria is less sensitive to the antibiotic ciprofloxacin.

Figure 7 shows the inhibitory effect on *K. Pneumonia* bacteria and on *p. Auroginosa* bacteria.

Table 1 shows the minimum inhibitory concentration (MIC) of the antibiotic ciprofloxacin, the free salvia extract, the free nanocomposite, the extract loaded with the antibiotic, and the nanocomposite loaded with the antibiotic on *K.Pneumonia* bacteria.

Table 2 shows the minimum inhibitory concentration (MIC) of the antibiotic ciprofloxacin, the free salvia extract, the antibiotic-loaded extract and the free nanocomposite, and the nanocomposite loaded with the antibiotic on p.auroginosa bacteria.

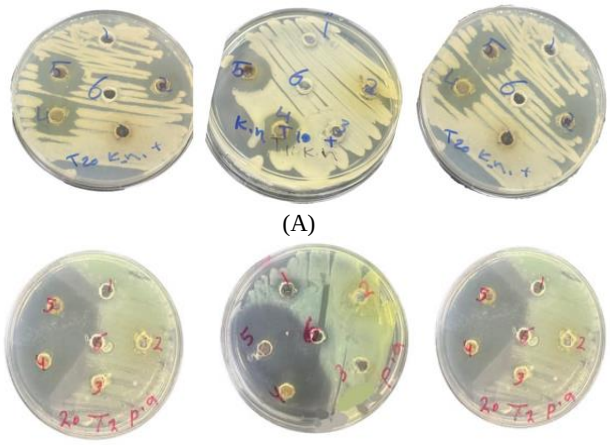
TABLE 1. The Inhibitory Effct on *K. Pnaumonia* Bacteria

Treatments	µg/ml Concentration	S.D ± Mean	LSD
T1	8 C1	.00 ± 1.00	n.s
	4 C2	.58 ± .67	
	2 C3	.29 ± 83	
	Total		
T2	16 C1	.29 ± .83	n.s
	8 C2	.29 ± .83	
	4 C3	.14 ± .92	
	Total		
T3	16 C1	.14 ± .92	n.s
	8 C2	.43 ± .75	
	4 C3	.29 ± .83	
	Total		
T4	4 +8 C1	4.04 ± 16.67	n.s
	2+ 4 C2	2.31 ± 15.67	
	1+2 C3	.00 ± 11.00	
	Total		
T5	4+8 C1	1.15 ± 15.67	1.21
	2+4 C2	1.15 ± 14.33	
	1+2 C3	.00 ± 13.00	
	Total		
Total	T1	.35 ± .83	1.20
	T2	.22 ± .86	
	T3	.28 ± .83	
	T4	3.50 ± 14.44	
	T5	1.41 ± 14.33	
	Total		
LSD Value		+.93	

TABLE 2. The Inhibitory Effct on *P. Aenuginosa* Bacteria

Treatments	µg/ml concentration	S.D ± Mean	LSD
T1	8 C1	.14 ± .92	n.s
	4 C2	.58 ± .33	
	2 C3	.58 ± .67	
	Total		
T2	16 C1	.14 ± .92	n.s
	8 C2	.29 ± .83	
	4 C3	.14 ± .92	
	Total		
T3	16 C1	.25 ± .75	n.s
	8 C2	.58 ± .67	
	4 C3	.58 ± .67	
	Total		
T4	4 +8 C1	4.04 ± 16.67	n.s
	2+ 4 C2	2.31 ± 15.67	
	1+2 C3	.00 ± 11.00	

		Total	
T5	4+8 C1	1.15 ± 14.33	n.s
	2+4 C2	2.52 ± 12.33	
	1+2 C3	1.15 ± 11.67	
		Total	
Total	T1	.49 ± .64	0.87
	T2	.18 ± .89	
	T3	.43 ± .69	
	T4	1.92 ± 12.78	
	T5	.88 ± 13.44	
		Total	
LSD value		n.s	



(A)

(B)

Figure 7. (A) The Inhibitory Effect On *K.Pneumonia* Bacteria, (B) Inhibitory Effect on *P.Auroginosa* Bacteria

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Arabic Abstract

يهدف البحث الحالي إلى تحضير المستخلص المائي والمركب النانوي لأوراق الميرمية باستخدام طريقة التخليق الأخضر، والتعرف على جسيمات الفضة النانوية باستخدام تحليل FTIR وSEM. تم تحميل المضاد الحيوي سيبروفلوكساسين على النانوكومبوسيت ودراسة النشاط المثبط على العزلات البكتيرية المدروسة G-ve Klebsiella pneumoniae وPseudomonas aeruginosa. تم تقسيم مجموعات الدراسة إلى مكررين وخمس مجموعات بثلاثة تراكيز. عولجت المجموعة الأولى بالمضاد الحيوي سيبروفلوكساسين، وعولجت المجموعة الثانية بالمستخلص المائي للميرمية، وتم تطبيق النانوكومبوسيت على المجموعة الثالثة. تم استخدام المستخلص المحمل بالمضاد الحيوي لعلاج المجموعة الرابعة، وتم استخدام النانوكومبوسيت المحمل بالمضاد الحيوي لعلاج المجموعة الخامسة. إن تغير اللون من الأصفر إلى البني الغامق دليل على تشكل الجسيمات النانوية، كما أن تحول الموجة في النانوكمبوسيت المحمل بالمضاد الحيوي نحو التردد 2156.93 سم^(-1) دليل على نجاح عملية تحميل المضاد الحيوي على النانوكمبوسيت. وقد تم توصيف المركبات الناتجة باستخدام المجهر الإلكتروني الماسح، وأظهرت نتائجه أن جزيئات المستخلص كانت على شكل تجمعات غير منتظمة مع وجود بعض الجسيمات الصغيرة ذات الشكل الكروي ومتوسط حجم الجسيمات 85.49 نانومتر، بينما كان النانوكمبوسيت مكعب الشكل ومتوسط حجم الجسيمات 35.42 نانومتر. وكانت مناطق التثبيط واضحة في المعاملة الرابعة T4 المستخلص المائي المحمل بالمضاد الحيوي، والمعاملة الخامسة T5 النانوكمبوسيت المحمل بالمضاد الحيوي. أظهرت الجسيمات النانوية الفضية أعلى كفاءة تآزرية مع المضاد الحيوي سيبروفلوكساسين ضد سلالة البكتيريا المقاومة للأدوية المتعددة K.pneumoniae، مسجلة معدل 14.33±1.41، بينما ضد بكتيريا P.auroginosa، تم تسجيل معدل 13.44±88.