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Comparison of the Effect Aqueous Extract and Nano-Sidr Synthetic of Leaves Ziziphus Spina-christi with Silver Nanoparticles the on Biofilm of Local Isolates of *Staphylococcus. Spp*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors FFN, SJA and QAK managed the research. Authors FFN, SJA and QAK done the research and wrote the main manuscript text. Authors FFN, SJA and QAK prepared tables and wrote a part of manuscript text. All authors read and approved the final

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ABSTRACT

Aims: This study aims to investigate the inhibitory susceptibility of extract Sidr leaf (Ziziphus spina-christi) aqueous, silver nanoparticles Ag NPs and Sidr nanoparticle "Nano-sidr" (Ziziphus spina-christi) against local isolates of *Staphylococcus.SPP ,*and their inhibition effect on biofilm formation.
Study Design: Initially diagnosed by The use of Mannitol salt agar medium, then 26 isolates were selected from them depending on the resistance to methicilln and Vancomycin that were conducted.
Place and Duration of Study: The samples were collected from AL-Najaf AL-Ashraf and Baghdad hospitals.
Methodology: These isolates were subjected to a VITEK-2 compact system "ID, AST", to ascertain the genus and type of Staphylococcus bacteria., morphological and biochemical tests were conducted on them to confirm them.
Results: The result of the diagnosis showed 10 isolates belonging to *Staphylococcus aureus,* and

a number of them were resistant to as MRSA-VRSA, and 7 were *Staphylococcus haemolyticus* and they were all resistant and known as MRSA-VRSA, and two isolates of *Staphylococcus sciuri* bacteria, one of them was resistant to VRSA and the other to MRSA-VRSA , two isolates of *Staphylococcus warneri* were both resistant to MRSA, two isolates of *Staphylococcus lugdunensis* were resistant to antibiotics, one of them was VRSA and the other was MRSA-VRSA, and one isolate of *Staphylococcus lentus* was also resistant to antibiotics MRSA-VRSA, and one isolate of *Staphylococcus warneri* was resistant to MRSA-VRSA, and one isolate of *Staphylococcus lugdunensis* was resistant to antibiotics.

Conclusion: Isolation of *Staphylococcus vitulinus.* 11 different isolates were selected from them according to their resistance to antibiotics, and after selecting the most efficient one by examining the inhibitory activity by diffusion method. The results showed the ability of both types of nanoparticles. Plant extracts prevent the formation of biofilms

Keywords: MRSA-VRSA; antibiotics; resistant bacteria; isolates.

1. INTRODUCTION

Excessive use of antibiotics contributed to the Appearance of and spread of antibiotic-resistant bacteria in different societies, so it was necessary to find successful solutions to this problem and in several ways, including returning to the use of herbs and plants that were considered medicines in Traditional medicine, as medicinal plants were approved as an antimicrobial agent to avoid the development of bacteria Multi-drug resistance. Despite the great development that has occurred in the manufacture of medicines and medical drugs, researchers have shown a remarkable interest in medicinal plants because they contain active substances that affect microorganisms [1]. With the successful discovery and development of antibiotics, infectious diseases remain the second leading source of death worldwide, while antibiotic resistance is among the important problems of the twenty-first century, and medicinal plants are rich in phytochemicals that can be structurally improved and converted into new drugs if treated. Plants since ancient times as an important source for the manufacture of natural medicinal plant compounds, and these compounds are a good source of anti-pathogens [2]. Among the most important medicinal plants, the Zizyphus Spina-Christi Sidr tree appears, as it contains active substances in all parts of the tree. If it produces secondary metabolic compounds, including alkaloids, flavonoids, saponin, anti-oxidants, anti-inflammatory, anti-diabetic, and other medically important secondary compounds, especially in the leaves of the tree [3]. Also, the synthetic silver nanoparticles AgNPs nanoparticles are considered a promising generation of antibiotics, as these particles have activity against cancer cells and bacteria, as they are used against Gram-positive and Gram-negative bacteria [4] [5]. These particles are considered to have an inhibitory effect on bacteria, especially those bacteria that are resistant to antibiotics, this resistance was considered a major health problem [6]. A biofilm is a group of microbial assemblies surrounded by extracellular materials that adhere to biological and non-biological surfaces [7] as it is a key factor for maintaining microbial organisms in diverse environments. Biofilm formation represents a good pattern to protect microbial organisms and allow them to grow. It allows cells to survive in harsh environments and also helps in the colonization and dissemination of microscopic microorganisms. Biofilm is one of the most important factors of virulence, thus contributing to the acquisition of microorganisms with high resistance to antibiotics and escaping from The mechanisms of host defense and that the biofilm has an important role in allowing pathogenic microorganisms to resist antibiotics, as it prevents or impedes their access to the inside of the microbial cell, in addition to the physiological changes and phenotypic patterns that bacteria possess within the biofilm, which makes them more resistant to antibiotics. Staphylococcus is one of the most important types of biofilm-forming bacteria [8] [9]. Staphylococcus represents a broad genus of Gram-positive bacteria that colonize the nose, mucous membranes and skin of humans and most mammals. *Staphylococcus aureus* is positive for coagulation, while the other types are negative for coagulation. Staphylococcus causes bacteremia, bloodstream inflammation, endocarditis, osteomyelitis, urinary tract infections, and wound infections [10] [11]. To find alternative treatment methods for antibiotics, so that they are effective and can

reduce the spread and increase the resistance of bacteria to those antibiotics, so Sidr leaves and their nanoparticles and silver nanoparticles AgNPs have been selected and hopefully they will be a suitable natural alternative to antibiotics.

2. MATERIALS AND METHODS

2.1 Leaf collection of Ziziphus Spina-christi

Young green Sidr leaves were collected from home gardens in Baghdad governorate and were cleaned of suspended dust and washed with water and salt for 3 minutes, then washed again with distilled water for a minute, spread on a clean sterile cloth, and left to dry at room temperature for 2 weeks, then ground by an electric grinder to Dry powder and kept in plastic bags and in a dry place until use.

2.2 Preparation of Aqueous Extract hot from Leaf Ziziphus Spina-christi

Takes 5, 10, 15, 20 g of dry leaf powder and mix it with 100 ml of hot distilled water separately and put in a water bath at a temperature of 45 ° C at 100 revolutions per minute for five hours and then filter the mixture using layers of sterile medical gauze to get rid of The leftover vegetable powder, the filtrate was taken and placed in a centrifuge at a speed of 3000 rpm for 10 minutes, then filtered the filtrate using the Milli Power Filter (0.45) micrometer , then kept in the refrigerator until use [12].

2.3 Preparation of Nanoparticles

2.3.1 Preparation of Sidr nanoparticles from Sidr leaf powder (*Ziziphus Spina-Christi*)

Preparation of Sidr nanoparticles from Sidr leaf powder (Ziziphus Spina-Christi) The nano solution was prepared in the Industrial Research and Development Authority - Chemical and Petrochemical Research Center at the Ministry of Industry and Minerals, and the method of [13] was adopted to prepare 100 gm of Sidr Nano - Sidr powder was added to 1000 ml of Distilled water, then mix and melt the mixture over low heat to dissolve all sediments, distribute the solution on dishes and leave to dry for two days. After two days, the powder was collected from the dishes, then weighed 20 gm of powdered Sidr powder, added to 800 ml of distilled water, and placed mixture in the Vibra-Cell Ultrasonic Liquid device for an hour and a half. This device worked for 10 seconds and stopped for 5 seconds. The process continued for ten consecutive days Then collect the mixture and store in the refrigerator at 4 ° C until use

2.3.2 Biosynthesis of silver nanoparticles using Aloe vera

Silver nanoparticles were prepared at the Industrial Research and Development Authority - Chemical and Petrochemical Research Center at the Ministry of Industry and Minerals, according to [14]. From silver nitrate in the presence of Aloe vera leaf extract, and the following steps were followed:

1- Aloe vera leaves were collected from the home garden, the leaves were washed with distilled water, dried ,and then crushed. 10 gm of leaves were mixed with 100 ml of distilled water and heated for 12 minutes. The extract was filtered using 0.45 µm filter paper, then kept in the refrigerator at 4°C

2- Dissolve 1.575 g of silver nitrate in 1000 ml of distilled water

3- 10% of the extract of the Aloe vera plant was mixed with a solution of silver nitrate at a ratio of 1:9 and kept at room temperature for 48 to 72 hours until the color change and the appearance of a reddish-brown color that indicated the formation of nanoparticles was observed.

2.4 Detection of Biofilm Formation by Microtiter Tissue Culture Plate

The method adopted by [15] was used and shown as follows

1) Bacterial isolates were cultured on a Nutrient agar medium and incubated at 37°C for 24 hours.
2) Some of the cultured colonies were transferred to tubes containing Brain Heart Infusion Broth, and the turbidity was measured with a spectrophotometer to ensure turbidity equal to the standard MacFarland tube at a concentration of 1.5 x 810 cells/ml.
3) 200 microliters of the prepared bacteria suspension were transferred and added to

the microtiter plates and incubated at 37°C for 24 hours.

4) After the incubation period, the contents of the pits were emptied and washed three times using a phosphate-brine buffer to remove bacteria cells that were not attached to the pits. Then 160 µl of 95% methanol was added and left for 10 minutes to fix the adherent bacterial cells.
5) 100 microliters of Crystal Violet dye were added at a concentration of 0.25 % to each hole of Microtiter plates and left for 15 minutes, then the plate was washed with distilled water to remove the crystal violet dye that is not attached to the bacterial cells.
6) After that, 160 µl was added at a concentration of 33% of glacial acetic acid, and the absorbance of the Microtiter plates was measured at a wavelength of 630 nm using a Micro ELISA reader. To find out the ability of bacteria to produce the membrane and the results were recorded according to the following Table 1.

3. RESULTS AND DISCUSSION

3.1 Production and Description of Sidr Nanoparticles from Ziziphus Spina-christi Leaves SID

The results showed the possibility of manufacturing nanoparticles from Sidr leaves according to the Fig. 1

Characterization of nano-synthetic Sidr. leaf particles The examination was carried out at Baghdad University of Jadriya, College of Science, Department of Chemistry. The results shown in Fig. 2 (A and B) where a scanning electron microscope was used in (A) The average diameters of nanoparticles for Sidr leaves were 69.34 nanometers, as there are 10% of nanoparticles With diameters of 45.00 nm, 50% of the nanoparticles are 70.00 nm in diameter, and 90% of the nanoparticles have diameters of 90.00 nm. As for (B) represents the diameters and size of nanoparticles, as the diameters ranged between (30-105) nanometers, as well as a description of the cumulativeness of nanoparticles in a graph representing the relationship between the diameter of the nanoparticle and the percentage represented by the cumulative distribution of nanoparticles by the device used Scanning probe microscope "SPM" is a tool used to study surfaces at the nanoscale. The SPM device contains a physical probe to scan and return to all surfaces of the sample and collect data by forming an image of the surfaces. Usually, an image of the surfaces is obtained in the form of a two-dimensional network of data points and displayed as an image on the computer during the scanning process, where the computer collects the data used to produce an image of the surface to appear properties of nano, SPM is used to distinguish between nanoparticles. The diagnostic technique using this microscope is a relatively recent diagnostic technique used to diagnose the surfaces of solid, soft, conductive and non-conductive materials and the surfaces of objects in the gaseous medium [16]. Fig. 3 shows the phenotypic morphology of Sidr leaf nanoparticles, the homogeneous structure of the nanoparticles, and how the nanoparticles are lined up in one line side by side in an orderly, visible and three-dimensional manner. Also, Fig. 4 in (A) shows the distribution and features of the surface of nanoparticles in two dimensions,and the color gradient (black to white) shows the height and low topography of the surface, where the white color represents the highest point and black the lowest point and(B) shows some information using a roughness analysis image Photographer surface , the value is (16.001 mm). CSPM

3.2 Production and Characterization of Silver AgNPs Nanoparticles

The ability of Aloe vera plant extract to form silver nanoparticles from silver nitrate was observed through the color change as shown in Fig. 5, where it is observed that after 72 hours of incubation at room temperature the appearance of reddish-brown color appears [14].

The results of the examination of silver nanoparticles using an SPM scanning electron microscope, shown in Fig. 6 (AB), showed that the average diameters of the nanoparticles were 4553. nm and that 10% of the particles had diameters of 40.00 nm, while 50% had diameters of 50.00 nm, while 90% Their diameters were 60.00 nm, and the same figure represents a miniature table of the diameters and size of those nanoparticles whose diameter ranged between (30-65) nm and a description of the accumulation of these nanoparticles, which were clarified in a graph representing the relationship between the diameter of the nanoparticle and the percentage represented by the cumulative distribution of the particles As shown in Figs. (1-7), the shape of

silver nanoparticles is shown, as the homogeneous structure of the nanoparticles is clear, and how the nanoparticles are lined up in one line side by side in an orderly, visible three-dimensional manner. As for Fig. 8, (A and B) shows the distribution and features of the surface of nanoparticles in two dimensions, and the color gradient (black to white) shows the height and low topography of the surface, where white represents the highest point and black is the lowest point and (B) shows some information using a roughness analysis image Surface with CSPM.

Table 1. The ability of bacteria to produce the membrane

(**non produce**)	(**weak**)	(**strong**)	**Biofilm production**
0.125 **>**	0.125- 0.240	0.240≤	Optical density

Fig. 1. The stages of manufacturing nano-Sidr

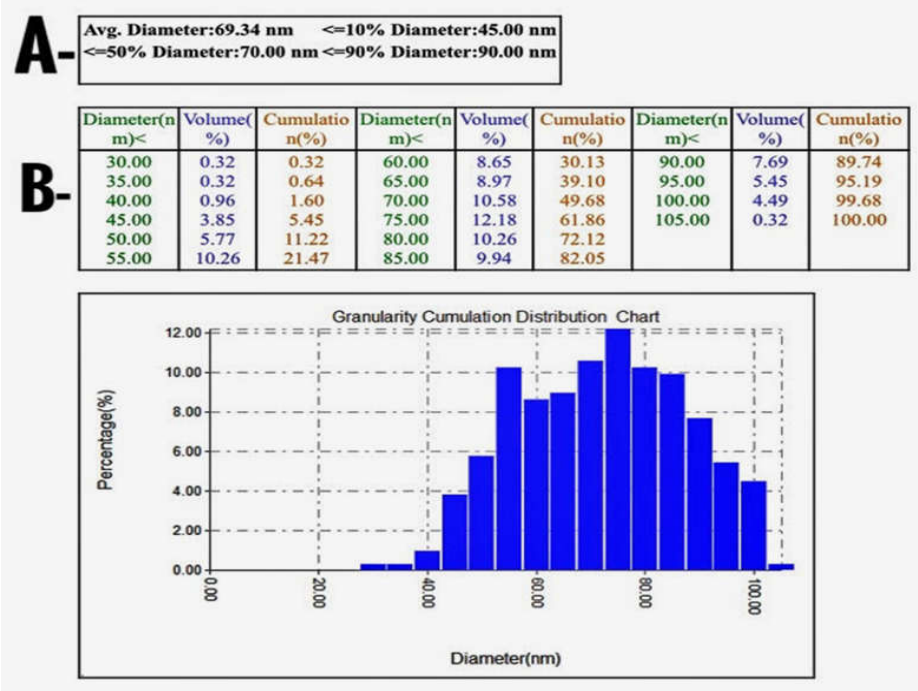


Fig. 2. A-Average diameters of nano-particles of Nano-Sidr leaves. B–Cumulative distribution and nanoparticle size

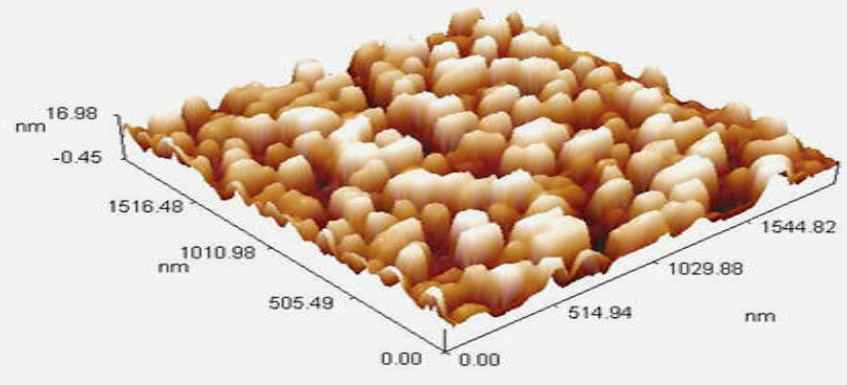


Fig. 3. The morphological morphology of the nanoparticles of Ziziphus spina-christi leaves and the homogeneous structure of the nanoparticles A three-dimensional image

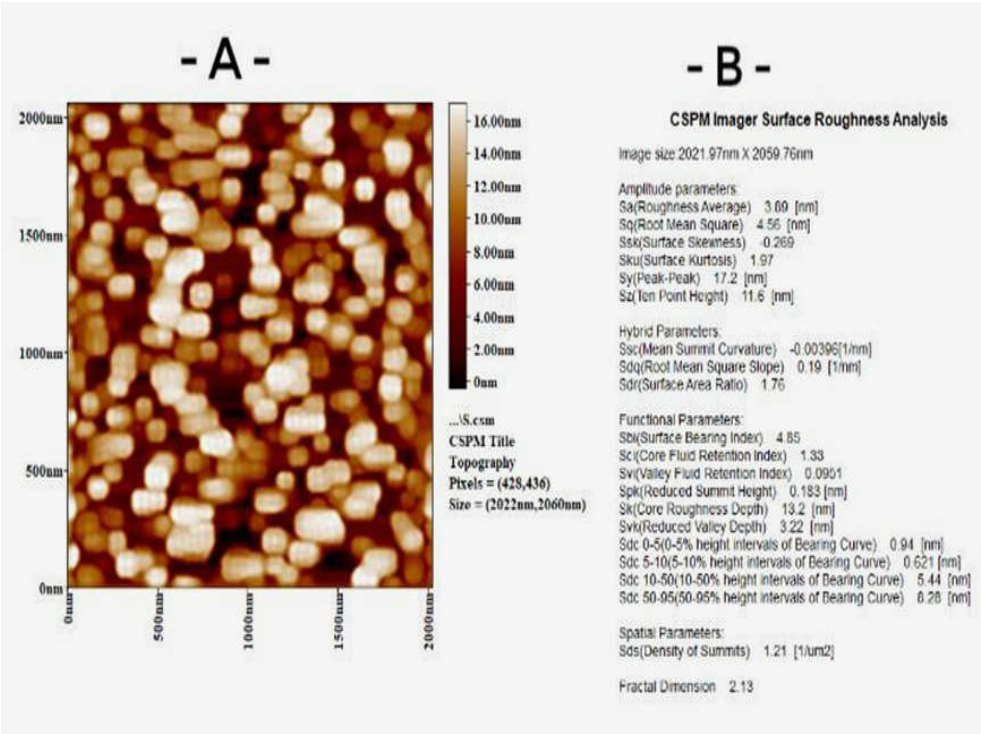
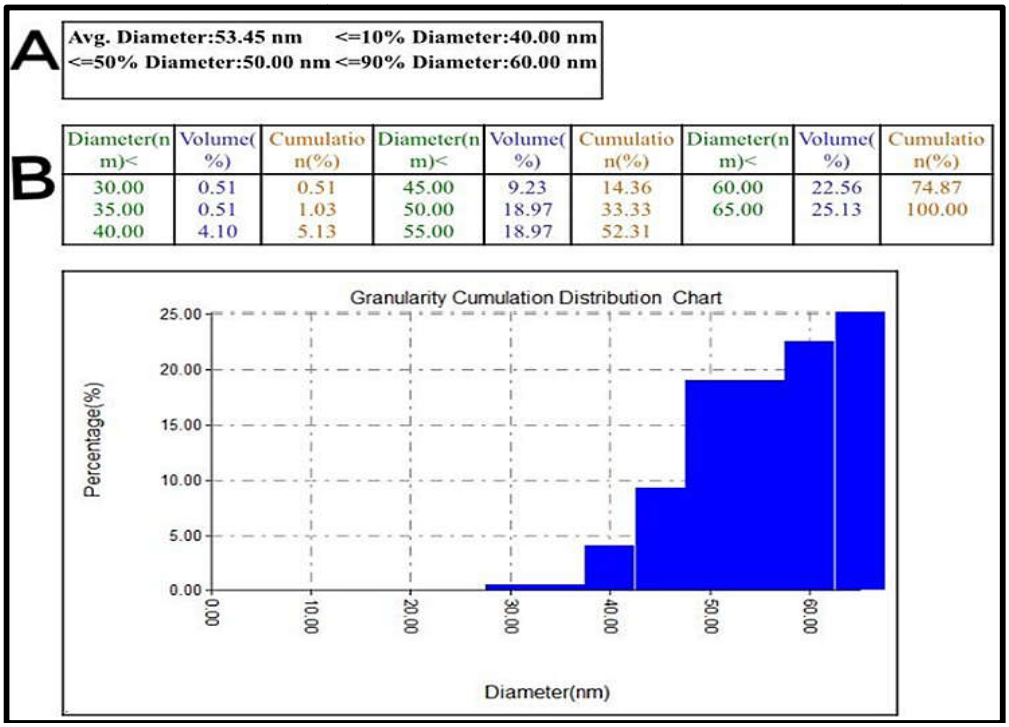


Fig. 4. A- Topography of nanoparticles in two dimensions and color gradient (black to white) showing the height and low topography of the surface. B- Some information by means of a photogrammetric surface roughness analysis. CSPM

The size of the nanoparticle, its surface formation, and the surface-to-particle ratio have a role in the interaction of the nanoparticle with the living cell. In this study, nanoparticles with sizes ranging from 30 nanometers to 105 nanometers were used for nanoparticles manufactured from Sidr leaves and 30 to 65 nanometers for silver nanoparticles. In general, small NPs nanoparticles show reactive oxidative activity due to the high surface-to-volume ratio compared to larger nanoparticles. The generation of reactive oxygen species (ROS) harmful to the cell can be observed as it disrupts essential biomolecules such as DNA, proteins, and lipids in the cell. Bacteria also act against the built-in antioxidant cell wall defense mechanisms. The nanoparticles interact with the bacterial cells directly resulting in damage to the biofilm of the bacterial cell.

Sometimes these particles penetrate the bacterial cell and some studies show that the adsorption by nanoparticles on the cell wall followed by its disintegration which is the primary mechanism of its toxicity [17-19]. The rate of nanoparticle adsorption depends on the cell type and varies according to the size, charge, and surface characteristics of the nanoparticle itself. A study conducted showed that nanoparticles with a size between 20 and 50 nanometers are absorbed more quickly than smaller or larger particles and that the positively charged particles attach easily to the cell surface. Negatively charged [20]. It is clear from the two-dimensional figure that Sidr nanoparticles and silver nanoparticles have a spherical shape, and that the multiple spherical shape of nanoparticles are well aligned, and it is one of the desirable properties as it improves the function of nanoparticles and facilitates their adhesion to the cell surface [21].

Fig. 5. The color change of the solution when forming silver AgNPs . particles



Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)	Diameter(nm)<	Volume(%)	Cumulation(%)
30.00	0.51	0.51	45.00	9.23	14.36	60.00	22.56	74.87
35.00	0.51	1.03	50.00	18.97	33.33	65.00	25.13	100.00
40.00	4.10	5.13	55.00	18.97	52.31			

Fig. 6. A-Average diameters of AgNPs nanoparticles. B- shows the distribution, size and accumulation of nanoparticles

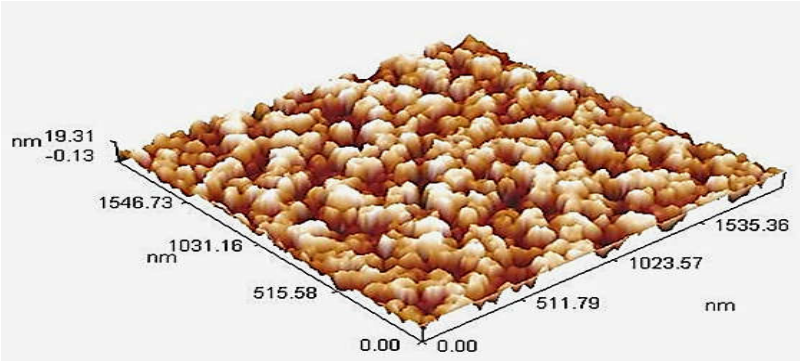


Fig. 7. Distribution and morphology of nanoparticles and the three-dimensional homogeneous structure of silver AgNPs nanoparticles

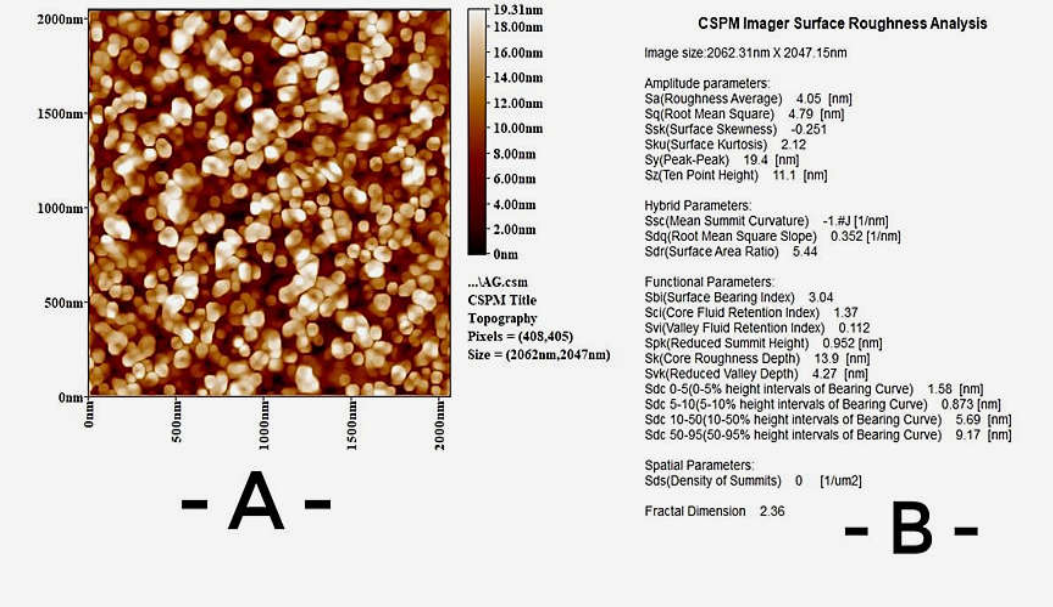


Fig. 8. A- Topography of nanoparticles in two dimensions and color gradient (black to white) showing the height and low topography of the surface. B- Some information by means of a photogrammetric surface roughness analysis. CSPM

3.3 The effect of hot aqueous of Sidr Leaves (Ziziphus spina-christi) on biofilm formation in *Staphylococcus spp*

The results of the investigation of the inhibitory susceptibility of the hot aqueous extract and alcoholic extract of Sidr leaves(Ziziphus spina-christi) showed the direction of the different stages of biofilm formation and for a group of staphylococcal bacteria isolates, namely, *S. aureus, S.sciuri, S.haemolyticus, S.lugdunensis., S.warner* and *S.Lentus* and *S. saprophyticus* and *S. vitulinus*, which were selected according to their resistance to antibiotics and after selecting the most efficient ones and investigating the inhibitory activity by digging diffusion method , using the "MTP" plate system. With three concentrations (20,15,10)% for the hot aqueous extract of Sidr leaves. It is noted from the statistical results shown in Table 2 and when compared with the control factor, the ability of the hot aqueous extract of Sidr leaves to inhibit biofilm formation and The aqueous extract of Sidr leaves is important in inhibiting the growth of bacteria because it contains inhibitory chemicals such as alkaloids, saponins, tannins, glycosides, flavonoids, and terpenoids. The aqueous extract

Table 2. Effect of Sidr leaves (Ziziphus spina- Christi) extract on the biofilm formation

Type of bacterial isolate	Biofilm composition (mean ± standard deviation)			
	Aqueous extract of Sidr leaves 15%	Aqueous extract of Sidr leaves 15%	Aqueous extract of Sidr leaves 10%	control
S.aureus(MRSA- VRSA) 1	0.006±0.001c	0.027±0.009c	0.029±0.006b	0.166±0.030
S.aureus(MRSA-VRSA) 2	0.011±0.001c	0.002±0.001c	0.023±0.003a	0.140±0.040
S.aureus(VRSA)3	0.008±0.001c	0.025±0.001c	0.026±0.001c	0.214±0.050
S.haemolyticus(MRSA-VRSA) 1	0.008±0.002a	0.023±0.007a	0.027±0.001c	0.127±0.039
S.haemolyticus(MRSA-VRSA) 2	0.009±0.001d	0.022±0.013d	0.028±0.004d	0.199±0.009
S.lentus(MRSA-VRSA)	0.004±0.001d	0.028±0.004d	0.028±0.001c	0.129±0.017
S.lugdunensis (MRSA)	0.019±0.005b	0.021±0.001b	0.023±0.001c	0.169±0.009
S.saprophyticus	0.015±0.003b	0.008±0.001b	0.011±0.001d	0.162±0.052
S.sciuri(MRSA)	0.007±0.001b	0.028±0.002b	0.023±0.007c	0.153±0.030
S.vitulinus	0.012±0.001b	0.003±0.001b	0.010±0.001e	0.175±0.080
S.warneri (MRSA)	0.003±0.001c	0.017±0.001c	0.019±0.002e	0.216±0.029

Similar English letters indicate that there are no significant differences at the level of 0.05>P

Table 3. The inhibitory susceptibility of nano-Sidr nanoparticles and silver AgNPs on The biofilm formation in Spp

Type of bacterial isolate	Biofilm composition (mean ± standard deviation)		
	Sidr particles Nanoparticles	Silver nanoparticles	control
S.aureus(MRSA- VRSA) 1	0.011±0.002c	0.008±0.001c	0.166±0.030
S.aureus(MRSA-VRSA) 2	0.017±0.001c	0.013±0.001a	0.140±0.040
S.aureus(VRSA)3	0.001±0.001d	0.012±0.001b	0.214±0.050
S.haemolyticus(MRSA-VRSA) 1	0.003±0.001b	0.001±0.001b	0.127±0.039
S.haemolyticus(MRSA-VRSA) 2	0.004±0.001c	0.003±0.001b	0.199±0.009
S.lentus(MRSA-VRSA)	0.002±0.001b	0.001±0.001c	0.129±0.017
S.lugdunensis (MRSA)	0.004±0.001b	0.017±0.003c	0.169±0.009
S.saprophyticus	0.021±0.001d	0.009±0.001b	0.162±0.052
S.sciuri(MRSA)	0.007±0.001c	0.013±0.002d	0.153±0.030
S.vitulinus	0.015±0.004e	0.009±0.001b	0.175±0.080
S.warneri (MRSA)	0.025±0.004a	0.003±0.001b	0.216±0.029

Similar English letters indicate that there are no significant differences at the level of 0.05>P

shows varying degrees of influence on negative bacteria and Gram-positive [22] and alkaloids have an anti-adhesion effect of bacterial cells to surfaces and biofilm formation [23] [24] It and the tannins also affect the phenomenon of quorum sensing, which is important in biofilm formation [25].

3.4 Effect of Nano-Sidr and Silver AgNPs on Biofilm Formation in *Staphylococcus .Spp*

The inhibitory ability of Nano-Sidr nanoparticles and silver AgNPs towards biofilm formation was investigated for a group of staphylococcal isolates namely, S. aureus, S.haemolyticus, lugdunensis.S, warneri.S, Lentus S., S. saprophyticus and S. The statistical results shown in Table 3 showed an effect of Sidr nano-particles and silver AgNPs when compared with the control coefficient. The biofilm measured the size of Sidr leaf nanoparticles, whose diameters ranged from 30 nanometers to 105 nanometers (Tuenter *et al.,* 2017), as Nano-Sidr was manufactured from the powder of Ziziphus spina-christi leaves by the Sol-Gel method, similar to the manufacture of Nano-curcumin As curcumin has anti-bacterial, anti-inflammatory, anti-cancer, and other properties, so researchers tried to improve the properties of curcumin by manufacturing nanocomposites to increase the effectiveness of this active compound containing functional groups [13] [26]. The same method has been used, and that Sidr plant possesses functional groups similar to curcumin. These functional groups are linked with other compounds through a unique hydrogen bond [27]. Sidr leaves contain secondary anti-bacterial and anti-inflammatory metabolic compounds such as alkaloids, flavonoids, sapogenins, saponins and tannins. By observing the chemical composition of Sidr, functional groups are noted as OH, CH, CO, CC, C = C [28] To improve the unique properties of Sidr, it was converted into nanoparticles, and manufacturing nanoparticles from natural ingredients to increase their efficiency is an environmentally friendly method, and a simple inexpensive method was used to manufacture nanoparticles. It can be seen that the efficiency of the nano-extract of Sidr leaves increased by comparing the concentration and effectiveness of the nano-solution. With the aqueous extract of Sidr leaves, it was observed that the low concentration of Sidr nano-solution 2.5% had an inhibitory activity higher or equal to the areas of inhibition of the aqueous extract represented (10, 15, 20)%. Thus, the results obtained in the current study are promising for other future studies because it is the first of its kind in Iraq and because the Sidr plant is available with its evergreen trees all year round and is easy to obtain.

The Silver nanoparticles AgNPs have a similar effect to that produced by nano-Sidr leaf, Silver AgNPs are good and promising alternatives against some types of pathogens and a good inhibitor of the biofilm formed in Staphylococcus

aureus bacteria [29]. A study conducted by (2020) et al., Solana showed that the biofilm formed is affected by silver nanoparticles, and the larger the size of the nanoparticle, the greater the effect on the biofilm, while the smaller nanoparticles were affected by the bacterial cell itself. Metal nanoparticles generally disrupt the quorum sensing system by inhibiting efflux pumps that play an important role in transmitting signals from one cell to another, thus preventing biofilm formation [30]. Silver nanoparticles bind with the extracellular materials that make up the biofilm, which leads to the inhibition of cell adhesion with each other. Silver nanoparticles can penetrate the bacterial cell and settle in the cytoplasmic substance, inhibiting the action of enzymes [31-32].

4. CONCLUSION

The Silver nanoparticles AgNPs have a similar effect to that produced by nano-Sidr leaf, Silver AgNPs are good and promising alternatives against some types of pathogens and a good inhibitor of the biofilm formed in Staphylococcus spp bacteria . Inhibitory ability of hot aqueous extract Sidr leaves (Ziziphus spina-christi), nano Sidr and silver nanoparticles towards biofilm formation in isolates of Staphylococcus S.pp resistant to methicin and vancomycin. The statistical results shown in Table 3 showed an effect of Sidr nano-particles and silver AgNPs when compared with the control coefficient.

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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