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The Effect of Copper Nanoparticle Extract of *Myrtus communis* and *Nerium oleander* Plants on Biofilm Formation in *Staphylococcus aureus*.

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Abstract

This study examined the *fnbA* gene, which plays a role in biofilm formation, in *Staphylococcus aureus* bacteria that were exposed to copper nanoparticles (CuNPs) produced using an aqueous extract from the leaves of *Myrtus communis* and *Nerium oleander* plants. A total of 54 *S. aureus* bacteria were isolated from wastewater samples. Gene expression analysis was conducted using a quantitative RT-PCR readyMix apparatus with a classical conventional PCR model of BIOER – Germany. The cDNA samples from *S. aureus* bacteria and *fnbA* gene were quantified using quantitative real-time PCR by applying SYBR green using a two-step RT-PCR method to determine the effect of CuNPs on biofilm formation gene. Characterization of CuNPs was performed using UV-light Visible spectroscopy, Atomic Force Microscope (AFM), and X-ray diffractometer (XRD). The obtained results of gene expression level in *S. aureus* isolates showed an increase in the level of expression of *fnbA* genes of bacteria isolates subjected to the CuNPs biosynthesized using *M. communis, N. oleander* aqueous extracts in addition to the mix of CuNPs, where the mean *fnbA* level of control bacteria were 26.525. In contrast, the mean level was increased significantly to 34.0, 34.15, and 35.025 for CuNPs biosynthesized using *M. communis, N. oleander,* and CuNPs mix respectively. In summary, all extracts effected on gene expression of *fnbA* gene but the mix extract of CuNPs had the highest effect.

Keywords: CuNPs, gene expression, *M. communis*, *N. oleander*, *S. aureus*

تأثير مستخلص جسيمات النحاس النانوية لنباتي *Myrtus communis* و *Nerium oleander* في تكوين الأغشية الحيوية في بكتريا المكورات العنقودية

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الخلاصه

تناولت هذه الدراسة فحص جين *fnbA*، الذي يلعب دورًا في تكوين الأغشية الحيوية، في بكتيريا المكورات العنقودية الذهبية التي تعرضت لجسيمات النحاس النانوية (CuNPs) والتي تم إنتاجها باستخدام مستخلص مائي من أوراق نبات الآس*Myrtus communis* الشائع ونبات الدفلى*Nerium oleander*. تم

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عزل ما مجموعه 54 بكتيريا من المكورات العنقودية الذهبية من عينات مياه الصرف الصحي. إجري فحص التعبير الجيني باستخدام جهاز RT–PCR readyMix الكمي مع نموذج PCR التقليدي الكلاسيكي من BIOER – ألمانيا. حددت كمية عينات cDNA من بكتيريا المكورات العنقودية الذهبية وجين *fnbA* باستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الحقيقي من خلال تطبيق SYBR green باستخدام طريقة RT–PCR المكونة من خطوتين لتحديد تأثير جسيمات النحاس النانوية على جين تكوين الأغشية الحيوية. إجري توصيف لجسيمات النحاس النانوية باستخدام مطيافية الأشعة فوق البنفسجية المرئية، ومجهر القوة الذرية (AFM)، ومقياس حيود الأشعة السينية (XRD). أظهرت نتائج مستوى التعبير الجيني في عزلات *S. aureus* زيادة في مستوى التعبير لجينات *fnbA* لعزلات البكتيريا الخاضعة لـ CuNPs المحضر حيويا باستخدام المستخلصين المائيين *M. communis* و *N. oleander* بالإضافة إلى المزيج من CuNPs. حيث كان متوسط مستوى *fnbA* للعينة الكنترول في البكتيريا 26.525. في المقابل، ازدادت النتائج معنويا إلى 34.0، 34.15، و35.025 لـ CuNPs التي تم تحضيرها حيويًا باستخدام *M. communis*، *N. oleander*، وCuNPs mix على التوالي. باختصار، جميع المستخلصات أثرت على التعبير الجيني لجين *fnbA* لكن مستخلص مزيج CuNPs كان له التأثير الأعلى.

Introduction

Staphylococcus aureus bacteria are well known as hostile pathogenic bacteria causing a vast range of human morbidity and mortality, such as pneumonia affecting the lungs [1], meningitis targeting brain and spinal cord [2], heart valves via endocarditis [3], bone inflammatory [4], skin problems [5] and other clinical disorders [6].

Similar to other biofilm-forming bacteria, *Staphylococcus aureus* produces a biofilm that acts as a barrier, hindering the penetration of antimicrobial agents and thereby protecting the cells within the biofilm from the effects of these medications [7]. The phenotypic characteristics of *Staphylococcus aureus* cells in their biofilm-encased and protected state differ from those of the cells in their planktonic form. Associated with biofilms compared to their free-living counterparts, *S. aureus* cells display variations in gene expression, protein synthesis, and cell size and growth as well as increased antibiotic resistance [8]. Numerous studies have examined genes of *S. aureus*, such as *fnBPa* & *fnBPB* [9] [10], *icaC* & *icaD* [11], *SpA* and *Rsp* [12], *icaA* [13], *icaR*, *sarA*, *katA* and *sigB* [14] and finally *fnbA* [12,15]. Among these, the most examined biofilm formation gene was Fibronectin-binding protein-A (*fnbA*), adherence and secretory protein crucial in biofilm formation [10]. However, the expression of these genes in *S. aureus* bacteria can be impacted by several environmental variables such as starvation [16], cold stress [17], temperature, pH [18], metal ion pollution [19], disinfectants [20], antibiotic medicines [21] and various vaccines [22].

Bacterial biofilms employ numerous mechanisms to propagate their pathogenicity, such as solid surface, evading host defenses, producing high concentrations of toxins, resisting anti-microbial agents, exchanging genes leading to more virulent strains, and dispersing microbial aggregates. Consequently, the microbes are spread to other locations [23]. Treatment for *S. aureus* infections is challenging since the bacteria are resistant to several medications known as beta-lactams, such as amoxicillin, penicillin, and oxacillin.

Nanotechnology is opening the door to more sensitive and successful ways of diagnosing and treating bacterial illnesses as a result of the rise in antimicrobial resistance. Researchers can synthesis nanoparticles particles smaller than 100 nm in at least one dimension-and employ them for a range of purposes thanks to nanotechnology. Furthermore, in comparison to traditional micron-sized particles, nanoparticles have larger surface areas and, as a result, interact with biological targets like bacteria more often. Moreover, nanoparticles are easier to infiltrate cells than microparticles [24]. In addition, there was little information in the litreture

on the possible effects of CuNPs on the *fnbA* gene in *S. aureus* bacteria that governs biofilm formation. Suood *et al*., 2021, investigated the production of CuNPs utilizing *Aspergillus niger* supernatant, their characteristics, and their antibacterial action either in combination with Ciprofloxacin or alone against *S. aureus isolates* [25]. This study aimed to determine the effect of CuNPs extract of *M. communis* and *N. oleander* extracts on gene expression of *S. aureus.*

Materials and Methods

Bacterial Isolation and Identification

Fifty-four *S. aureus* bacteria were isolated from wastewater collected at Al-Madaen public hospital in Baghdad-Iraq. The samples were stored in closed glass bottles after being washed with Distilled water (DW) and sterile by placing the samples in the oven at 200°C for 4hrs. After that, the specimen was saved in the cool box and taken to the lab for examination. The conventional detection of *S. aureus* depended on the cultural properties of blood agar and mannitol salt agar, followed by microbiological tests, including gram stain morphology, coagulase reaction, catalase reaction, and other standard routine tests for detection**.** Finally, the confirmed diagnosis for *S. aureus* isolates was performed using the Vitek2 system compact.

Plant Collection and Preparation of the extract

Nerium oleander and M. communis fresh leaves were collected from the general garden of Al-Madaen. According to Flaih and Al-Saadi **(**2020), a slight modification to the synthesis leaves extract of the *M. communis* and *Nerium oleander* plant was carried out by taking 100 g of air-dried *M. communis* leaves and *Nerium oleander,* placed in a glass electric mixer and 700 ml of distilled water was added and left for 15 minutes at laboratory temperature. Next, the sample was filtered through a cloth to remove plant residues. It filtered through Whatman No. 1 filter paper to remove the remaining plant wastes and then centrifuged at 8000 rpm for 30 minutes to obtain a clear plant extract from the supernatant [26].

Synthesis of CuNPs and Characterization of CuNPs nanoparticles

CuNPs were prepared according to Daylee (2022) [27]. Eco-friendly synthesis of copper nanoparticles (CuNPs), prepared from *M. communis* and *Nerium oleander,* primarily were characterized by UV-light Visible spectroscopy, Atomic Force Microscope (AFM), and finally, X-ray diffractometer (XRD).

Biofilm Formation Assay

Biofilm formation of *S. aureus* was performed following the method suggested by an earlier study [28], where the isolated bacteria were grown in Brain-Heart Infusion broth (BHI) for gene expression test. Sterile 96-well polystyrene microplates were used, and 4 wells were selected to represent aqueous extract CuNPs (*M. communis*, *N. oleander*, and mix *M. communis and N. oleander*) aqueous extract and Brain-Heart Infusion broth (BHI) as a control, forming 16 wells. One hundred microliters (up to 1×10^9 CFU) from *S. aureus* bacteria culture (equivalent to McFarland standard no.0.5) was added to each microplate well. Each of the first 4 wells received 100 µl from the examined extract with a concentration of 50.0 mg per ml and mixed thoroughly. Then, 100 µl from these mixed wells were transferred to the next set of wells, where another 100 µl from these extracts were added, giving a concentration of 25.0 mg/ml and again remixed carefully. The same process was repeated in a similar sequence for the remaining wells to prepare concentrations of 6.25, 3.1, 1.5, 0.75, and 0.9 mg/ml, respectively.

The microplate was covered and incubated overnight at 37°C, and each isolate biofilm was assayed three times. Following incubation, the plankton cell was rinsed twice with distilled water (D.W) to remove the unattached bacteria and shake out the excessive water by tapping the microplate on paper towels, the wells were dried in an inverted position at room temperature. In each well, the adhering bacterial cells were fixed for 15 minutes using 200 µl of methanol at room temperature. The microplate was allowed to air-drying. Subsequently, 200 µl of 0.1% crystal violet were added for 5 minutes under room temperature and rewashed. The plate was incubated at 37°C for nearly 30 minutes to have perfect dryness. Finally, 200 µl of ethanol were added for around 10 minutes, then put into a different microplate. The optical density of each well was measured using a microplate at 600 nm.

Gene Expression

In this investigation, gene expression for six bacterial isolates was conducted; these isolates represented strong biofilm producers. Only one single gene (fnbA) was selected for this test. 16 SrRNA gene served as the reference gene.

1. *RNA extraction*

RNA was extracted from the biofilm cells of *S. aureus* isolates grown using the method recommended by previous work [29]. By using an RNA extraction kit and FavorPrep genomic RNA/ Mini Kit. The bacterial cells (up to 1×10^9 CFU) were poured into a 1.5 ml tube glass and then centrifuged at 14000 rpm for one min. The supernatants were discarded, and approximately 100µl of bacteria lysis buffer containing lysozyme was added to the bacterial cell pellet. The mixture was then incubated for five min at room temperature.

RNA binding was performed by clarifying that the supernatant from the collection tube was moved to a microcentrifuge tube, where the volume was measured, and one volume of 70 % RNase-absolute ethanol was added and mixed well by vortexing. A FARB Mini Column for a Collection tube was used, and ethanol was added. The mix was moved into the FARB Mini Column and centrifuged at 14000 rpm for one minute. The flow-through was discarded, and the FARB Mini Column was placed back into the collection tube. Then, 350 µl of FARB Buffer and 3.5 µl of ß-Mercaptoethanol were added, and the specimen was homogenized using a rotor-stator and then incubated for five min at room temperature. RNA was washed with 500 µl wash buffer, re-centrifuged14000 rpm for 30 sec., and returned to the FARB Mini Column after discarding flow-through. Then, 750 µl of Wash Buffer 2 was added to the FARB Mini Column and centrifuged at 14000 rpm for 30 sec. After discarding the flow-through, the column was returned to the collection tube. For RNA elution, the FARB Mini Column was washed by an Elution tube and placed in a 1.5 ml microcentrifuge tube (RNase-free) where 40 -100 µl of RNase-free water was added to the center of the column matrix, center and left to stand for three minutes, then centrifuged at 14000 rpm for one minute to remove the purified RNA.

2. *cDNA Synthesis*

LunaScript Reverse Transcriptase from England Biolabs in England was utilized to synthesize cDNA. The reaction mixture was prepared to carry out the cDNA synthesis by adding 5 µl of RNA to the RT super mix tube. Afterward, the volume was completed with nuclease free water up to 3 µl. Then reaction mixture was incubated under 25°C, 2 minutes.

3. *Quantitative real-time PCR*

In order to assess the gene expression of the target gene, the results were normalized using 16SrRNA as the house-keeping gene. Primer's sequence of the gene was used forward: CCGCTATTTTGGCCACCTTCA and reverse primer CAAGCACAAGGGCCAATCGAG for *FnbA* target gene, as well as Primer's sequence of housekeeping gene were forward:

CAGCTCGTGTCGTGAGATGT and reverse: G CGTAAGGGCCATGATGACTT, were provided in a lyophilized form and were dissolved in sterile nuclease-free water to give a final concentration of 100 pmol /µl and stored in the deep freezer until use. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed. This procedure was performed using the Luna Universal qPCR Master Mix kit and SYBR green fluorescent dye. The reaction mixture is summarized in a total volume of 25 µL, comprising 10 µL of Luna Universal qPCR Master Mix, 1 µL of each primer forward and reverse, 8 µL of cDNA. And 5 µL of Nuclease-free water.

After multiple trials, the thermocycler protocol was optimized. The protocol condition included an initial denaturation at 95°C for 60 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 55°C for 15 min for (*FnbA* and 16 sRNA, respectively (reverse transcriptase, cDNA synthesis). A final extension at 60 °C for 20 sec. The results of qRT-PCR were analyzed using the Livak and Schmittgen equation ($2^{-\Delta\Delta CT}$) [30].

Statistical analysis

The statistical package for Social Science (SPSS) 2018 was utilized to examine the biofilm-producing capacity of *S. aureus* isolates [31].

Results and Discussion

Synthesis of CuNPs

The CuNPs were successfully synthesized using *M. comminus* and *Oleander* plant leaves aqueous extract; Figure 1 illustrates a color changing to brownish black. The crude extract of *M. comminus* and *N. oleander* was considered a reducing agent for Cu and a stabilizer for CuNPs. The green synthesis of CuNPs was confirmed primarily based on the reaction mixture's color change within 24 hours [32].

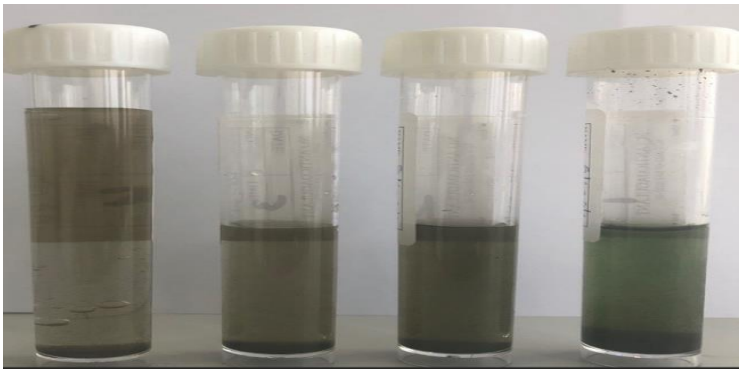

Figure 1: Photograph showing color changing of CuNPs

Characterization of Synthesized CuNPs

The ultraviolet-visible (UV-vis) spectroscopy method was utilized to determine the chemical characteristics of CuNPs biosynthesized by *M. communis, N. oleander*. The results indicated that the sample had formed CuNPs by *M. communis N. oleander*, as evidenced by the prominent peak of characteristic emission found at wavelengths 206 nm in the spectra of the Surface Plasmon Resonance (SPR) absorption band. These findings, presented in Figure .2, agreed with the previous study that reported 234 nm [33].

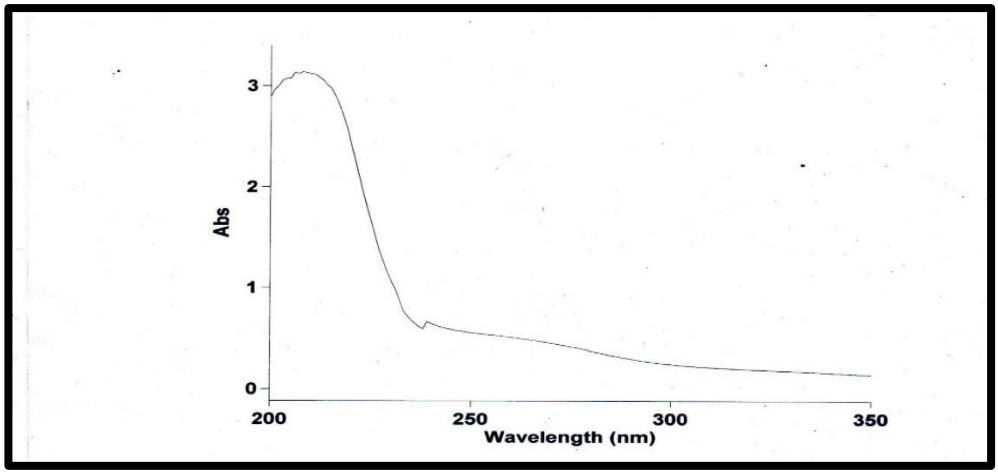

Figure 2: UV-Vis Spectroscopy of CuNPs biosynthesized by *M. communis* and *N. oleander*
Atomic Force Microscopy (AFM)

The average CuNPs diameter determined through AFM analysis utilizing *M. communis* and *N. oleander* extract is presented in Table 1.

Table 1: AFM shows the average diameter CuNPs synthesis by *M. communis* and *N. oleander*.

Extract		Dimeter
CuNPs biosynthesis using *M. communis aqueous extract*	Avg. Diameter:52.37 nm <=50% Diameter:40.00 nm	<=10% Diameter:15.00 nm <=90% Diameter:90.00 nm
CuNPs biosynthesis by *N. oleander*	Avg. Diameter:48.42 nm <=50% Diameter:40.00 nm	<=10% Diameter:20.00 nm <=90% Diameter:80.00 nm

The surface shape formation of the CuNPs from *M. communis* and *N. oleander* was examined by atomic force microscopy (AFM) and found to have an average diameter of 52.37 and 48.42 nm. Furthermore, AFM images have shown that the biosynthesized CuNPs synthesis using *M. communis* and *N. oleander aqueous extracts* was spherical (Figure 3(A) and 3(B)). A comparable study has reported a similar average diameter range from 17 nm to 55 nm [34].

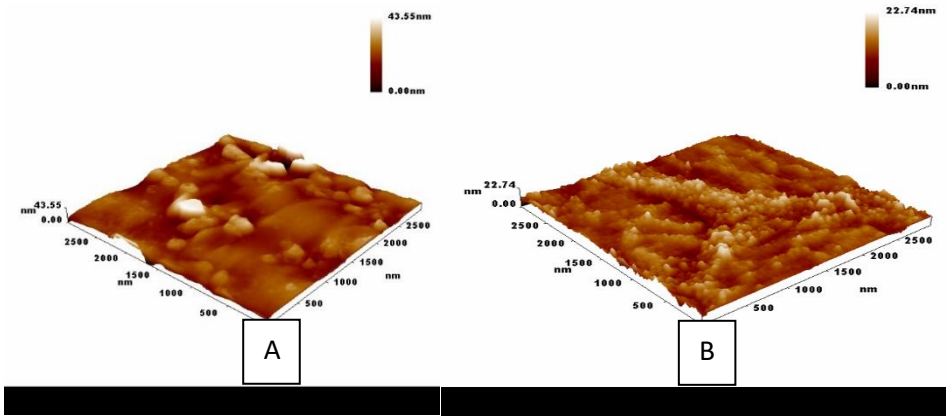

Figure 3: AFM shows the average diameter of both CuNPs synthesis by A) *M. communis* and B) *N. oleander* aqueous extracts.

X-Ray D diffraction (XRD)

The XRD analysis of eco-friendly synthesized copper nanoparticles (CuNPs) using *M. communis* and *N. oleander* leaves aqueous extract as a reducing and stabilizing agent was applied to determine crystal structure at the atomic level. Furthermore, the XRD plot of the bio-synthesized CuNPs is displayed in Figure (4). The pattern exhibits numerous sharp, intense peaks, indicating the successful formation of Cu-NPs and confirming their crystalline structure. In addition to these peaks, the XRD pattern also presents a number of other small peaks that were not specified. The XRD technique examined the structure of the synthesized CuNPs. As shown in Figure 4., the formed CuNPs had five distinguished peaks at 2 theta degrees, which were 38.9 °, 45.7 °, 64.49 °, 77.50 ° and 84.25°. XRD analysis specifies the size and shape of biosynthesized CuNPs formed in both *M. communis* and *N. oleander* plant leaves. However, similar XRD results of biosynthesized CuNPs were reported by previous studies [35].

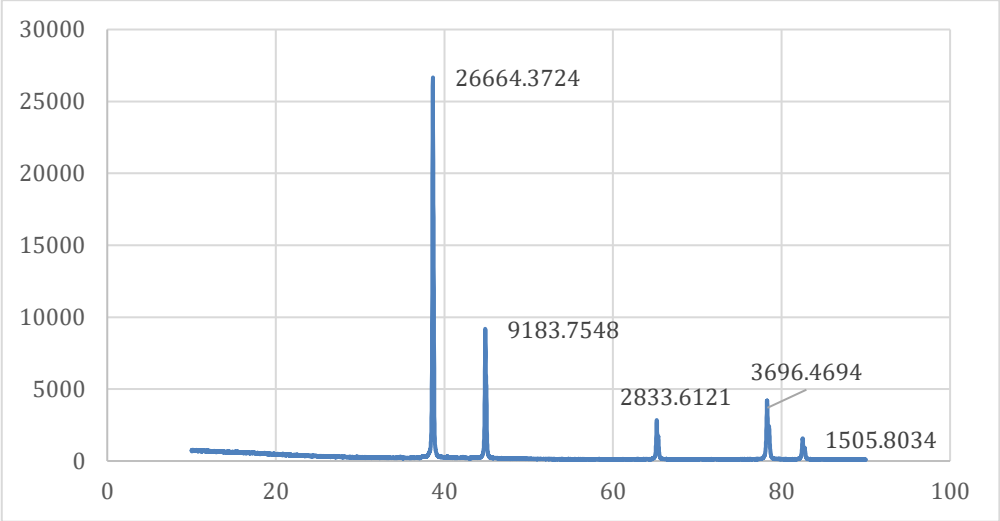

Figure 4: X RD Patterns of CuNPs bio-synthesized

Genes expression

The result of the expression levels of *fnbA* genes of *S. aureus* control and three other bacterial isolates, with four wells of each, are illustrated in Table 2. Additionally, Table 3 shows the number of wells, mean values of gene expression level, standard deviation, and standard error of all four examined *S. aureus* bacterial groups.

The gene expression levels in the control isolate ranged from 26.4 in wells 1 and 4 to 26.7 in well 3, resulting in a mean value of 26.525 ± 0.15. Whereas CuNPs biosynthesized using M. communis extract had values varying from 32.7 in well 3 to 35.9 in well 4, with a mean value of 34.0 ± 1.4989. These values in CuNPs biosynthesized using *N. oleander* extract range from 31.0 in well 4 to 36.5 in well 3 with a mean of 34.15 ± 2.3951. In the mix of CuNPs biosynthesized using both plants, the values varied from 32.8 in wells 2 and 3 to 35.9 in well 4, forming a mean value of 35.025 ± 1.5756, Tables 2 and 3, Figures 5 and 6.

Table 2: *fnbA* values of gene expression examined in *S. aureus* subjected to CuNPs biosynthesized using each of *M. communis* and *N. oleander* in addition to a mix of CuNPs of both plants' extracts + control untreated bacteria

Group	Well no.	*fnbA*
Control	Well 1	26.4
	Well 2	26.6
	Well 3	26.7
	Well 4	26.4
CuNPs biosynthesized using *M. communis*	Well 1	34.5
	Well 2	32.8
	Well 3	32.7
	Well 4	35.9
CuNPs biosynthesized using *N. oleander*	Well 1	35.4
	Well 2	36.5
	Well 3	33.7
	Well 4	31.0
Mix of CuNPs biosynthesized using both plants	Well 1	35.1
	Well 2	34.2
	Well 3	33.6
	Well 4	37.2

Table 3: Number of wells, mean, standard deviation, and standard error of *fnbA* gene expression level in *S. aureus* bacteria control and treated isolated by CuNPs biosynthesized using *M. communis* and *N. oleander.*

***S. aureus* isolates**	**N**	**Mean**	**Std. Dev.**	**Std. Error**
Control	4	26.525	0.15	0.075
CuNPs biosynthesized using *M. communis*	4	34.0	1.4989	0.7494
CuNPs biosynthesized using *N. oleander*	4	34.15	2.3951	1.1976
Mix of CuNPs biosynthesized by both plants	4	35.025	1.5756	0.7878
Total	28			

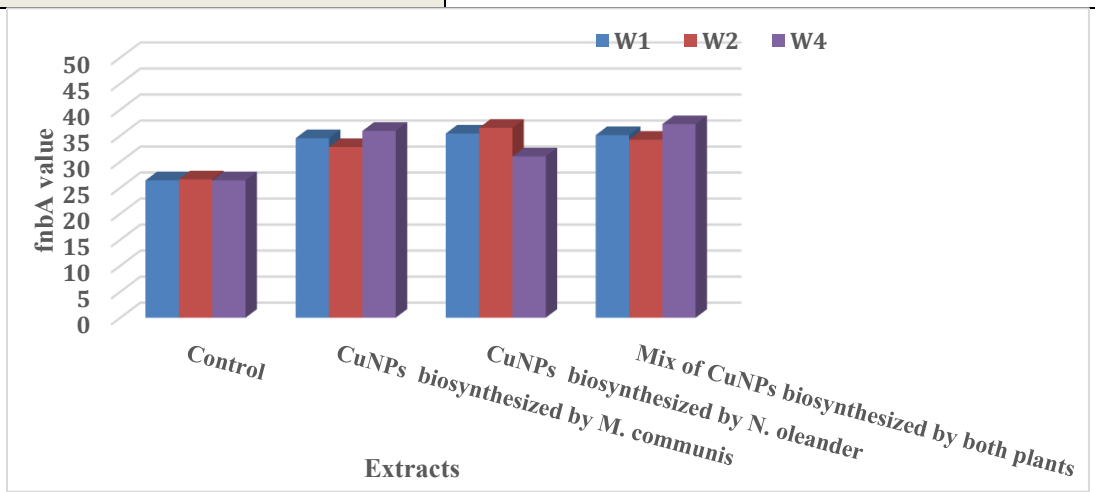

Figure 5: *fnbA* values in *S. aureus* of all examined bacterial groups

Regarding the mean values of expression levels of the four wells of each *S. aureus* isolate, the obtained results of gene expression in *S. aureus* isolates have revealed an increase in the expression levels of *fnbA* genes of bacterial isolates subjected to the CuNPs biosynthesized using *M. communis*, *N. oleander* aqueous and CuNPs mix extract respectively.

The lowest mean *fnbA* level in the control (untreated bacteria) was 26. 525. In contrast, the mean level increased to 34.0, 34.15, and 35.025 for CuNPs biosynthesized using *M. communis, N. oleander,* and CuNPs mix, respectively (Figure 6). Based on these results, the CuNPs mix shows the highest mean *fnbA* gene level, followed by CuNPs biosynthesized *N. oleander* plant and finally by CuNPs biosynthesized using *M. communis* plant.

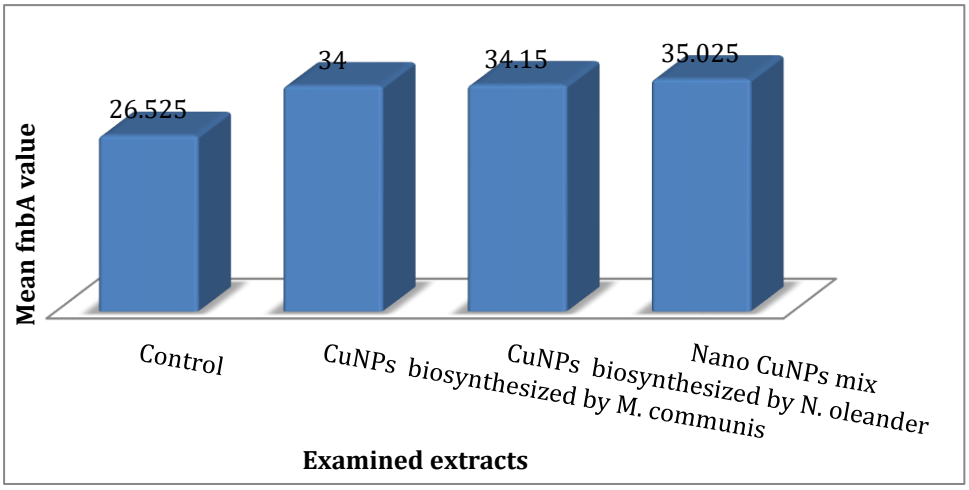

Figure 6: Mean *fnbA* gene level of 4 wells in *S. aureus* of all examined bacterial groups

The analysis of the variance presented in Table 4 indicates significant differences (P≥0.0001) between examined *S. aureus* bacteria groups, particularly between those of control and treated groups. These differences are further emphasized by the least significant differences (LSD) value was 3.498.

Table 4: Analysis of variance of examined *fnbA* gene in *S. aureus* bacteria groups subjected to various treatments.

SOURCE OF VARIATION	DF	SS	MS	F VALUE	PROBABILITY
BETWEEN GROUPS	3	188.105	62.7017	23.9132	0.0001
WITHIN GROUPS	12	31.4647	2.6221		
TOTAL	15	219.5697			
LSD	3.498				

The current results are consistent with findings from numerous previous studies about gene expression of various bacterial isolates affected by metal nanoparticles and other environmental stresses. Gene expression of *sdrC*, *fnbA*, *fhuD*, *sstD*, and *hla* in *S. aureus* bacteria regarding colonization and diseases [36]. Also, studied the effects of amino acid on the *Lactococcus lacti* bacterial *fnbA* gene and was found affected by amino acid [37]. In contrast, a study on the impact of starvation on biofilm formation in *P. aeruginosa* and *S, aureus* has pointed out the higher expression of both *fnbA* and *pslA* genes [38]. AgNPs on gene expression of *P. aeruginosa* and recorded that AgNPs was highly effective and has

reduced *lasI* and *rhlR* gene expression [39]. Also, gene expression for *pslA*, *pelA* and *algD* genes in *P. aeruginosa* isolates was found affected by environmental stresses like temperature, pH, and oxygen change [40,41].

Conclusion

The current results indicated increased expression levels of *fnbA* gene for CuNPs biosynthesized using *M. communis*, *N. oleander*, and nano CuNPs mix. In addition, nano CuNPs mix shows the highest gene expression for *fnbA* gene.

Conflict of Interest: The authors declare that they have no conflicts of interest.

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