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Antimicrobial Activity of Biosynthesized Selenium Nanoparticles from *Staphylococcus warneri* and its Impact on the *PhzM* Gene Expression in Clinical *Pseudomonas aeruginosa* Isolates

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Abstract: Antibiotic-resistant bacterial infections have been identified as a worldwide public health problem. In order to battle bacteria resistant to antibiotics, new strategies are required. As a subset of antimicrobial peptides, bacteriocin-selenium nanoparticles (NPs) can be used in place of or in addition to recognized antibiotics. Se NPs were characterized by different techniques following their biosynthesis by *Staphylococcus warneri* bacteriocin like inhibitory substances. The inhibitory effect of synergetic consortia was examined using a broad panel of Gram-positive and Gram-negative bacteria. Ultimately, Conventional PCR was employed to identify the pyocyanin *PhzM* gene and RT-PCR to determine the impact of Se NPs on its gene expression. The results showed considerable antibacterial activity against most isolates of *staphylococcus* spp. as well as *Candida albicans*, *Candida guilliermondii*, and *Candida ciferrii*, along with low toxicity and strong antioxidant action. The results of the real-time PCR approach showed that the expression of *PhzM* was either considerably up- or down-regulated in the isolates treated with SeNPs compared to the untreated isolates. It was concluded that Se NPs have antimicrobial properties that make them a promising alternative to antibiotics.

Key word: *Candida guilliermondii*, warnericin, *PhzM* gene, BLIS.

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Introduction

As a member of the coagulase-negative Staphylococci group, *Staphylococcus warneri* is nonmotile, facultatively anaerobic, catalase-positive, and oxidase negative, additionally, it is also a normal component of the skin's flora on the head, legs, arms, and nares. is found in over 50% of healthy persons and makes up 1%–7% of all cutaneous *staphylococci* in adults in good health (1). Furthermore, it is thought to be an opportunistic etiological agent that causes serious infections in humans, including blood sepsis in neonates and patients with indwelling catheters and artificial medical devices, as well as severe infections like bacteremia, endocarditis, vertebral osteomyelitis, discitis, subdural empyema, and meningitis associated with ventriculoperitoneal shunts. Bacteriocins, which are produced by bacteria belonging to the Gram-positive and Gram-negative classes, Archaea, and Eukaryotes, have been extensively studied as possible antimicrobials with various uses and demonstrated their potential as a promising alternative

therapy for the treatment of pathogens resistant to antibiotics (2). The use of natural or bioengineered bacteriocins, in addition to preparations of bacteriocins and conventional antibiotics that work synergistically, can be the next step in the fight against drug-resistant pathogens, most likely to address issues related to the growing number of bacteria that are resistant to antibiotics(3). The benefits of selenium nanoparticles (SeNPs) include their high rate of absorption, high biological activity, and low toxicity. The human body can absorb selenium nanoparticles directly and transform them into organic selenium. The traditional selenium supplement has been gradually replaced by selenium nanoparticles, which have many applications in the food and medical industries (4). *P. aeruginosa* is classified as an opportunistic pathogen that causes a wide range of infections in humans, involving nearly all body systems, that vary from local to systemic and from self-limiting to life-threatening(5). Multidrug resistance in *P. aeruginosa*, as well as in all other bacterial pathogens, is a growing concern. Aminoglycoside resistance, in particular, is a major concern in *P. aeruginosa* infections and must be better understood in order to maintain effective clinical treatment (6). The genes *phzM* and *phzS* are the most important as they encode the twostep conversion of Phenazine 1 caroylic acid (PCA) to pyocyanin, enzyme *PhzM* converts PCA to 5- methylphenazine-1-carboxylic acid betaine and the enzyme *PhzS* catalyzes the hydroxylative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin (7). This study aimed to investigate the effect of selenium nanoparticles biosynthesis on the growth rate of some bacteria and fungi, and gene expression of pyocyanin *PhzM* gene in *Pseudomonas aeruginosa* isolated from burn infection.

Materials and methods

Staphylococcus spp isolation and identification

In this study, one hundred and twenty specimens of urine from (Urinary tract infection) patients were collected during September 2023 to December 2023 from urinary tract infection patients in Iraqi hospitals in Baghdad, Iraq. Each isolate was inoculated onto Mannitol Salt Agar plates and incubated at 37°C for 18-24h. Isolates were subsequently confirmed as *Staphylococcus spp* by using the Vitek 2 compact system (BioMe`rieux/France), following the instructions provided by the manufacturer (8).

Indicator strains

Ten isolates of *Pseudomonas aeruginosa* bacteria were isolated and identified from patients with wound infections in Iraqi hospitals. Samples were cultivated in sterile conditions on Cetrimide agar and MacConkey agar. Additionally, the pathogenic fungal isolates from skin infection (*Candida albicans, Candida guilliermondii, Candida ciferrii)* that were used were obtained, confirmed the identification and isolated, from the Iraqi University, College of Medicine, Ibn Sina.

Biosynthesis of bacteriocin-like inhibitory substance (BLIS)

All isolates of *Staphylococcus* spp was screened for their inhibitory activity towards an indicator strains using by Agar well diffusion assay (AWD) method and Filter paper disc (FPD) method to select the higher bacteriocin-like inhibitory substance producing isolate. After inoculum of

each isolate with 10ml of (Brain heart infusion broth) and 24h of incubation at 37^0C and under the suitable condition for each isolate, the (bacteriocin-like inhibitory substance) were recovered by centrifuging (6,000 rpm, 15 min).

Agar well diffusion method (AWD)

The AWD method was used to evaluate the production of bacteriocin-like inhibitory substances of isolates as follows: Tubes contained 10 ml of brain heart infusion broth were inoculated with Bacteria of the staphylococci producer isolates each tube with 0.1µl from glucose, peptone water, Yeast extract. After incubation for 24 hr and 37°C under aerobic conditions, the cultured broth was centrifuged at 6000 rpm for 15 min and the bacteriocin-like inhibitory substances (BLIS) was collected. Indicator bacteria were spread on the surface of Muller Hinton agar plates and wells cut into the pour plates with 5 mm diameter by sterile cork-borer. Pours were filled with 80µl of staphylococci bacteriocin-like inhibitory substances (BLIS) and the plates were kept at room temperature for 2 hr. then incubated for 18-24 hr. After incubation, measuring the zones of Inhibition in cm to determine the best bacteriocin-like inhibitory substances antimicrobial activity (9).

Filter paper disc method (FPD)

Staphylococcus spp were inoculated in brain heart infusion broth in the same procedure of agar well diffusion assay and incubated aerobically at 37°C for 18-24 hr., then bacteriocin-like inhibitory substances were obtained by centrifuging at 6000 rpm for 15 min. Indicator bacteria were spread on the surface of Muller Hinton agar plates. 5 mm diameter sterile filter paper discs saturated with 100µl of suspensions were placed on the seeded Muller Hinton agar plates. The plates were kept at room temperature for 2hr before being incubated aerobically at 37°C for 18-24 hrs. The inhibition zones that formed around the paper discs were measured in cm and recorded (10).

Partial purification of like inhibitory substances

Partial purification of bacteriocin like inhibitory substances was prepared via precipitation with ammonium sulfate at different Concentrations. Ammonium sulfate was precipitated by gradually adding it to the crude enzyme while swirling it continuously on ice at various saturation levels. then centrifuged for 20 minutes at 4°C at 6,000 rpm. While the precipitate in each concentration was dissolved in the appropriate volumes of phosphate buffer solution, the supernatant was discarded. The optimal saturation ratio was ascertained by measuring the protein activity. Following a 24-hour chilling period, the ammonium sulfate precipitate was dialyzed. Concentration was determined according to (11).

Biosynthesis of selenium nanoparticles SeNPs

For the green synthesis of Se NPs a concentration of 1.5g sodium selenite was added to 20 ml BLIS solution produced from *Staphylococcus warneri* after centrifugation and removal of the bacterial cells precipitate and partial purification. Then the suspension was used to synthesize Selenium nanoparticles in 80 ml Heatproof Glass Conical Erlenmeyer Flask with Screw Cap Lid. The flask were incubated at 37°C -38°C in the dark for two days, and any color change was recorded. After incubation, the reaction mixture was centrifuged at 6000 rpm for 30 min, to remove the supernatant. The pellet-

shaped collection of nanoparticles they were transferred into a hot air oven set at 120°C to evaporate off all the liquid content. The dried powder was carefully gathered and kept in storage for additional analysis (12).

Characterization of biosynthesized selenium nanoparticles

By characterization of selenium nanoparticles, the morphology, component, functional groups, optical properties and size of the Se NPs were investigated, such as UV-Visible (UV-VIS) Spectroscopy, Atomic force microscopy (AFM), Energy dispersive X-ray (EDX), Xray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) and Field Emission Scanning Electron Microscope (FESEM) (13).

Testing the antimicrobial activity of selenium nanoparticles

The MIC values of selenium nanoparticles were examined against *Staphylococcus* species and *candida* species via the use of the broth microdilution method. Sterilized inoculating loop or swab were used to spread the test isolates on Mueller-Hinton agar plates and Agar Well Diffusion assay was used. A sterile cork-borer was used to create 5 wells 5 mm, which were then filled with 80 µl of varied concentrations of selenium nanoparticles (1000, 500, 250, 125, and 62.5µg/mL). Then subsequently incubated for 24 hours at 37 ° C, after which the zones of inhibition were determined the following day (14).

Determination of minimum inhibition concentration (MIC) of selenium nanoparticles

The broth microdilution method was also used to assess the minimum inhibitory concentrations (MIC) of Se NPs against strains of *Pseudomonas aeruginosa* that were resistant to multiple drugs in same range of concentrations were prepared. Sterile microtiter plate wells were filled with 100 µL of Mueller Hinton (MH) broth and then filled with test samples (Se NPs) (100 µL) at different concentrations. Bacterial cell suspension (10 µL) was added to all but the negative control well. Bacterial solution was added to positive control wells in order to determine whether MH broth could sustain bacterial growth. A 96-well microtiter plate was incubated at 37 degrees for 18 to 24 hours. The obtained area of inhibition was recorded using a scale (15).

Molecular detection

The tested gene was amplified by conventional PCR using primers obtained from (16). PCR amplifications were carried out in 20 µl volumes containing 10 µl of GoTaq Green Master Mix (2X), 1µl of primer (10 pmol), 6 µl of nuclease-free water, and 2 µl of template DNA. The PCR was cycled using Thermo Fisher Scientific, USA's PCR Express (Thermal Cycler) in accordance with the subsequent temperature program: At 95°C, denaturation was initiated for five minutes. Subsequently, there were thirty cycles of denaturation for thirty seconds at 95°C, annealing for thirty seconds at 50, 55, and 60°C, and extension for thirty seconds at 72°C. The final extension step involved running the reactions for seven minutes at 72°C and stopping them with a ten-minute incubation at 10°C. The PhzM primer was used, as (Table 1) shows (17).

Table (1): Primer used in PCR and real-time PCR.

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)
phzM	F-ACGGCTGTGGCGGTTTA	60
	R-CCGTGACCGTCGCATT	
fbp	F-CCTACCTGTTGGTCTTCGACCCG	58
	R-GCTGATGTTGTCGTGGGTGAGG	

RT-qPCR protocol

Real-time quantification of cDNA was carried out on the GoTaq® 1-Step RT-qPCR System (Promega, USA) using the qPCR master mix. Real-time PCR was used to investigate the expression levels of the *phzM* and *fbp* genes. In order to assess the gene expression of the *phzM* gene, the results were normalized using the *fbp* gene, which is considered a housekeeping gene. Primers of these genes (table 1) were provided in a lyophilized form and dissolved in sterile nuclease-free water to give a final concentration of 100 pmol/µl. Afterwards, they were stored in a deep freezer until used in qPCR. The reaction mixture was summarized in (Table 2) and qRT-PCR program in (Table 3) (18).

Table (2): The components qRT-PCR mixture.

Master mix components	Stock	Unit	Final	Unit	Volume
					1sample
qPCR Master Mix	2	X	1	X	5
RT mix	50	x	1	x	0.25
MgCl2					0.25
Forward primer	10	µM	0.5	µM	0.5
Reverse primer	10	µM	0.5	µM	0.5
Nuclease Free Water					2.5
RNA		ng/µl		ng/µl	1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

Table (3): Real-Time PCR Program.

Steps	°C	m: s	Cycle
RT. Enzyme Activation	37	15:00	1
Initial Denaturation	95	05:00	
Denaturation	95	00:20	40
Annealing	58, 60	00:20	
Extension	72	00:20	

Results and discussion

Isolation of *Staphylococcus spp* and confirmation of identification *Staphylococcus species* using vitek 2 system

All *Staphylococcus spp* isolates obtained were cultured on Mannitol Salt Agar (MSA). The appearance of *staphylococcus* colonies was shiny, mucoid, smooth, spherical, and elevated. *Staphylococcus aureus* was identified by large golden colonies encircled by wide yellow zones brought on by the medium changing from pink

to yellow. The ID-Gram Positive Cocci cards (ID-GPC cards; bioMeʿrieux) were used for identification and provide confirmation of positive results for all strains with probability (94%–99%), as shown in (Figure 1).

The *Staphylococcus* species isolates that obtained were 81 isolates, most of the isolates were **(***Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*) respectively, while the least abundant isolates were (*Staphylococcus lentus*, *Staphylococcus warneri***)** respectively, as shown in (Table 4).

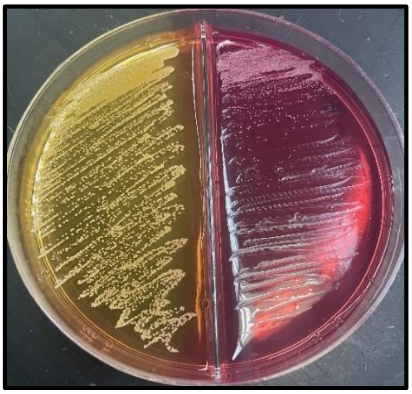

Figure (2): *Staphylococcus warneri* isolate on mannitol salt agar at 37°C for 24 hrs.

Table (4): Prevalence of *Staphylococcus* species isolates.

***Staphylococcus* species**	**No. Isolation (Percentage)**
S. aureus	23 (19.1%)
S. haemolyticus	22 (18,3 %)
S. epidermidis	19 (15.8%)
S. lentus	9 (7.5%)
S. warneri	8 (6.6 %)
Total	**81**

Screening for best crude bacteriocin production isolate

BLIS, also known as bacteriocin-like inhibitory substance, produced by these strains showed moderate antibacterial activity according to their ranges of inhibition zone which reached 24mm on the basic indicator isolates among the *Staphylococcus spp* isolates from urine sample.

These methods revealed that only 5% less activity of the *Staphylococcus spp.* The bacterial productivity of (BLIS) was investigated using the filter paper disc method (FPD) and the well diffusion method (AWD) to discriminate the isolates possessing the capacity of producing inhibitory substance (BLIS) revealed that about seven isolates of *Staphylococcus* of production inhibitory substance as primary screening and it recorded inhibition zones between (12-22) mm and after secondary screening was the best inhibition diameters of isolate shown in figure 3. Based on the 81 isolates, they were generally screened based on BLIS production utilizing the Filter Paper Disk and Agar Well Diffusion methods, and it turned out that the isolate whose name *Staphylococcus warneri* (F7) that shown in figure 2, was the most productive isolate and the result was

best shown in Filter Paper Disk method, as shown in figure (3).

In this work, bacteriocins generated by *Staphylococcus warneri* were partially purified using a step-gradient elution assay with ammonium sulfate at 60% isopropanol in 100 mM phosphate buffer (pH 6.5). The experimental model must be followed to the letter when applying the bacteriocins as a crude extract, partially purified preparations, or pure (homogeneous) protein. Purification is an expensive process, and pure bacteriocins are typically used in diagnostic or therapeutic settings. Primarily, a crude extract or partially purified bacteriocins are used for food-related experiments and/or sanitization(19).

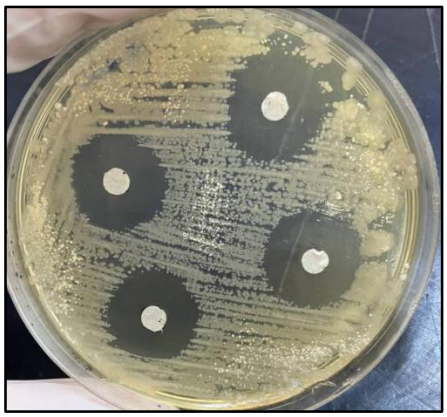

Figure (3): Screening of BLIS from *Staphylococcus species* on MHA at 37C for 24hrs shown by FPD method.

Biosynthesis of selenium nanoparticles SeNPs

Selenium nanoparticles were biosynthesized from warnericin from *S. warneri* F7. The result of nanoparticles formation indicated by the change of color from white to orange after incubation. The precipitate was brown after centrifugation, and a dark red powder was produced after microwave drying, as shown in (Figure 4). Biological molecules function as capping and reductants and are present in plant extracts or microbial as well as in substances released by bacteria and fungi. These materials include sugar, carbohydrates, proteins, and enzymes that change metallic ions from (M+) to (M0) through an oxidation/reduction process. By aggregating and forming clusters of nanomaterials, reduced metallic form can be validated by a color change in the reaction mixture (20).

Figure (4): Synthesized selenium nanoparticles by biological method.

Characterization of biosynthesized SeNPs

UV-visible spectroscopy is a highly used technique to distinguish the optical properties of the nanoparticles. This test is carried out in the range of 270–400 nm of the selenium nanoparticles solution. As presence in (Figure 5), an absorption peak observed at 293 nm indicates the successful biosynthesis of selenium nanoparticles. The characteristic of synthesis nanoparticles agrees with (21) the absorbance peak for *L. paracasei* HM1 was observed at 300 nm, while the absorption peak for LAB-SeNPs was found to be between 200 and 1000 nm in length.

In addition, the atomic force microscopy was used as a confirmatory technique to characterize the biosynthesis of selenium nanoparticles by detecting their average diameter in addition to the morphology in both two-dimension and three- dimension. The result diameter obtained in this study showed that the biosynthesized Se NPs by *S. warneri* 48.59 nm, as shown in (Figure 6). This finding disagrees with the following research (22) reported a size range of SeNPs between 100 and 550 nm, with a mean size of 245 nm.

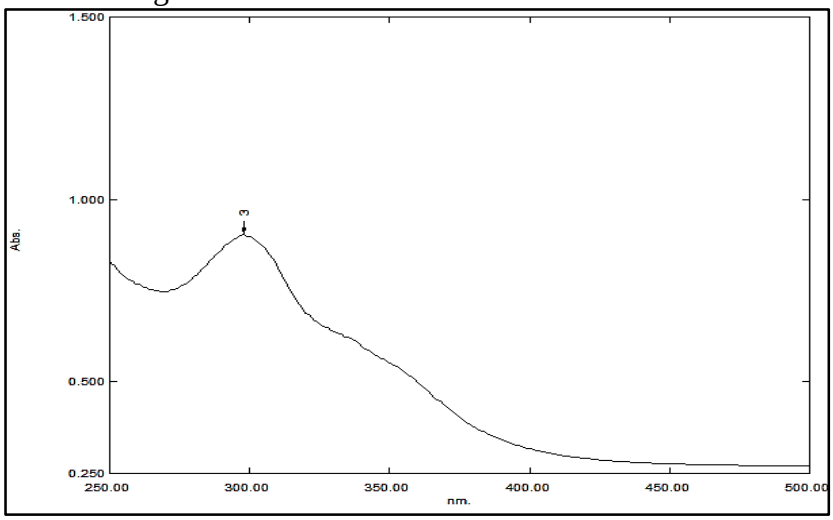

Figure (5): A: UV–vis spectroscopy analysis of Selenium nanoparticle.

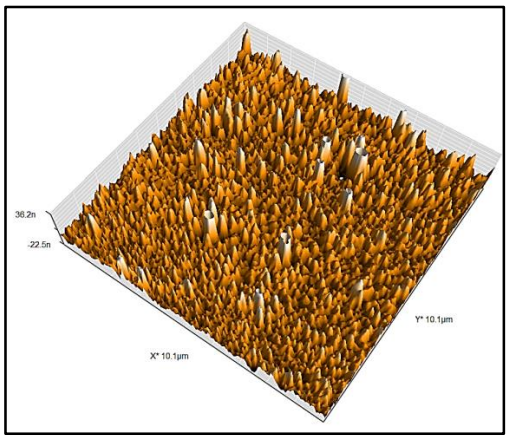

Figure (6): The 2 Dimension image of biosynthesized selenium nanoparticles under AFM.

Furthermore, the Se NPs components were examined using Energy Dispersive Xray Analysis (EDX). The results showed the EDX spectra together with the major elemental peak at 11 keV that is unique to the Se metal, as shown in (Figure 7). Other results by (23) the EDX spectrum elucidated strong absorption peaks of metallic selenium ions at 1.35 KeV, 11.20 KeV, and 12.40 KeV. The EDX analysis revealed that selenium also exists in elemental form along with other elements in the form of peaks.

Finally, SEM analysis was carried out to understand the morphology, size, and elemental and structural composition of the NPs samples. Images at 3000 kv in 500x magnification power demonstrate the spherical shape of Se NPs, as shown in (Figure 8). In other researge, the Se NPs display long rod-shaped particles along with an amorphous spherical aggregate (24).

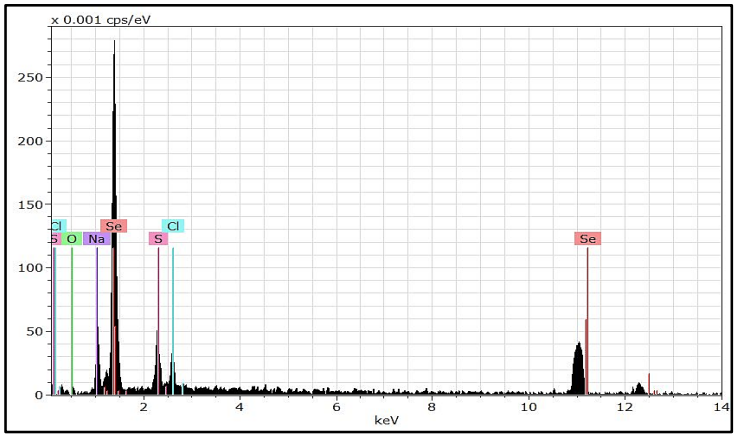

Figure (7): The images of EDX analysis shown pure selenium nanoparticles present.

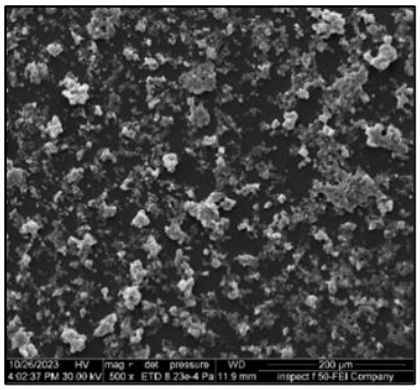

Figure (8): Determine the morphology of Se NPs by FESEM analyze that particles at 3000 kv and 500x magnification power.

Antibacterial activity of SeNPs

According to the MIC method and the diameters of zones of inhibition observed in (Figure 9) and (Table 5), it can be stated that the Selenium nanoparticles had significant antimicrobial activity against Staphylococcus species bacteria isolated from urine. The results of other study showed that Se nanoparticles exhibited dose-dependent antibacterial activity against all the four bacterial strains tested. Noticeably, PVA-SeNPs exhibited significant effect against *S. epidermidis* (MIC 125 ppm) and *S. aureus* (MIC 125 ppm), so the results of this study indicate that Se nanoparticles can be potentially used as antimicrobial and antioxidant agents (25).

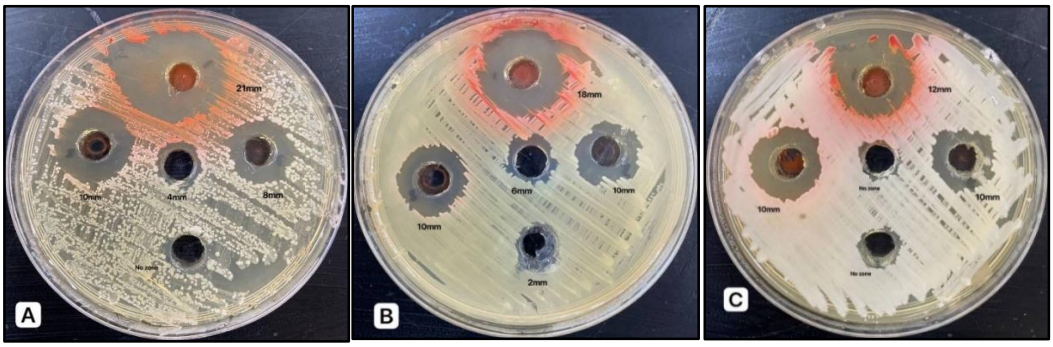

Figure (9): Showed inhibition zones of *Staphylococcus spp* (A: *S. epidermidis,* B: *S. haemolyticus,* C: *S. aureus*) on Muller Hinton Agar for 24h at 37C^0 treated with SeNPs by AWD assay.

Table (5): The range of inhibition zones of *Staphylococcus spp* treated with SeNPs in mm.

Staph isolates	1000 con.	500 con.	250 con.	125 con.	62,5 con.
S. haemolyticus	18 mm	10 mm	10 mm	6 mm	2 mm
S. epidermidis	21 mm	10 mm	8 mm	4 mm	No zone
S. warneri	18 mm	10 mm	14 mm	8 mm	4 mm
S. aureus	12 mm	10 mm	10 mm	No zone	No zone
S. lentus	20 mm	14 mm	14 mm	No zone	No zone

Antifungal activity of SeNPs

According to the MIC method the diameters of zones of inhibition observed in (Figure 10), it is possible to conclude that the selenium nanoparticles significantly inhibited the growth of *Candida species*. Generally speaking, as displayed in (Table 6),

SeNPs' antimicrobial activity against *Candida guilliermondii* was greater than that of *staphylococcus spp* in (Table 5) when compared to *Candida ciferrii and Candida albicans.* Results by (26) illustrated that Se-NPs have potential antimicrobial activity against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), fungi (*Candida albicans, Aspergillus niger* and *Aspergillus fumigatus*), and antioxidant activity.

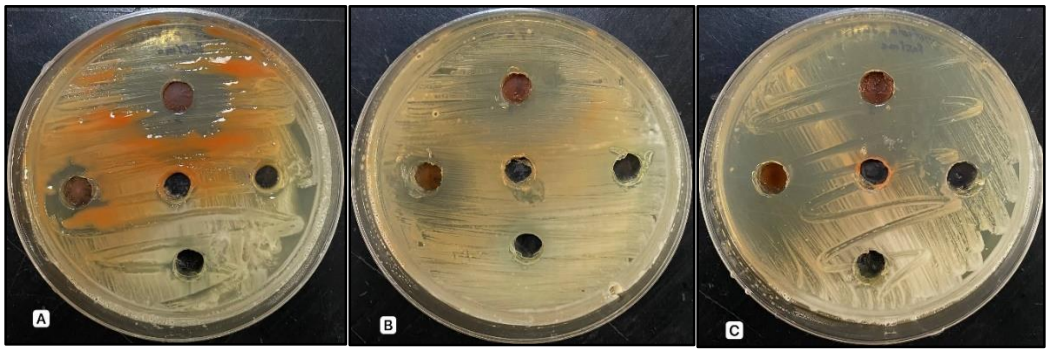

Figure (10): Showed inhibition zones of *Candida spp* (A: *Candida albicans,* B: *Candida guilliermondii,* C: *Candida ciferrii*) on Muller Hinton Agar for 24h at 37C^0 treated with SeNPs by AWD assay.

Table (6): The range of inhibition zones of *Candida spp* treated with SeNPs in mm.

Candida spp	1000 con.	500 con.	250 con.	125 con.	62,5 con.
Candida albicans	20 mm	8 mm	No zone	No zone	No zone
Candida guilliermondii	28 mm	16 mm	No zone	No zone	No zone
Candida ciferrii	16 mm	6 mm	No zone	No zone	No zone

Polymerase Chain Reaction (PCR) Technique

The PCR results identified ten isolates of *Pseudomonas aeruginosa* with the *phzM* gene, selected based on their multidrug resistance (MDR). The positive gene result was subsequently confirmed through electrophoresis on a 2% agarose gel stained with ethidium bromide, electrophoresed at 100 volts for 60 minutes, and visualized under an ultraviolet (UV) transilluminator. The present study revealed the presence of a sharp, singular, and non-dispersed 180 bp *phzM* gene band, which was clearly distinguished from the DNA ladder, as demonstrated in (Figure 11). Notably, there was no evidence of DNA degradation, as indicated by the absence of any smearing of the gene band. The results by (27) indicates that pyocyanin and its intermediate 5-methyl phenazine-1-carboxylic acid (5-Me-PCA) play important roles in *P. aeruginosa* resistance to cold atmospheric plasma (CAP) treatment. The unique enzymes, such as *PhzM* in the pyocyanin biosynthetic pathway, could be novel targets for the therapeutic strategy design to control the growing *P. aeruginosa* infections.

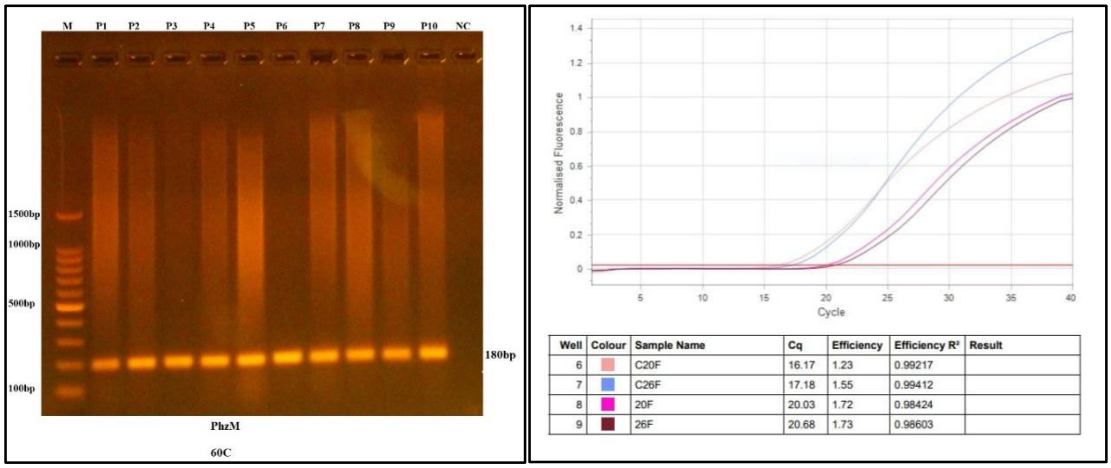

Figure (11): Outcomes of bacterial species' *PhzM* gene amplification and PhzM gene cycling.

To estimate the effect of biosynthesized selenium nanoparticles, two *P. aeruginosa* isolates were studied using the RT-qPCR technique. RT-PCR reveals in one isolate an important decrease in *PhzM* expression following the exposure to SeNPs compared to normal gene expression in bacteria, while the other when exposed to SeNPs, *PhzM* expression is significantly upregulated. According to (Table 7), fold change in gene expression indicates that in one isolate of *P. aeruginosa*, *PhzM* was up-regulated and in another isolate, it was down-regulated in response to SeNPs.

Table (7): *PhzM* gene expression yield before and after treatment with Se NPs.

Sample	(fbp) housekeeping gene	phzM	DCT	DDCT	Folding
(20F) before treatment of SeNPs	20.46	16.17	-4.30	0.00	1.00
(20F) after treatment of SeNPs	25.27	20.03	-5.24	-0.95	1.93
(26F) before treatment of SeNPs	21.20	17.18	-4.03	0.00	1.00
(26F) after treatment of SeNPs	24.03	20.68	-3.36	0.67	0.63

The results indicated a major up and down-regulation in *phzM* expression after exposure to selenium nanoparticles. These findings suggest that the SeNPs can have an inhibitory effect on *phzM* gene expression, which may lead to the loss of functional genes involved in pyocyanin biosynthesis.

Conclusion

The use of biosynthesized selenium nanoparticles as an alternative to antibiotics can reduce the use of antibiotics and is important in the context of the global problem of antibiotic resistance. Selenium nanoparticles synthesized by skin's flora *Staphylococcus warneri* have antimicrobial properties that make them a promising alternative to antibiotics and have been shown to be effective in inhibiting the expression of important virulence factors in *P*seudomonas *aeruginosa phzM* gene expression for pyocyanin. This inhibitory effect on pyocyanin gene expression may represent a potential strategy for controlling *P. aeruginosa* infections.

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