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ANTIMICROBIAL SUSCEPTIBILITY OF FRUCTOPHILIC LACTIC ACID BACTERIA ON PHZM GENE OF PSEUDOMONAS AERUGINOSA ISOLATES FROM WOUNDS INFECTED

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Abstract. Introduction *The study was to isolate and characterize fructophilic lactic acid bacteria (FLAB) species from the honey bee gut. Based on the results of this study, it was found that the FLAB species obtained from honey were gram-positive and catalase-negative, and this identification was confirmed through 16S rRNA gene sequencing.* ***Materials and methods:*** *The results indicated that yeast extract was the most effective nitrogen source, while glucose was the preferred carbon source for cell-free supernatant (CFS) production. The optimal pH for CFS production was found to be 5, and the incubation period of 72 hours was determined to be the most suitable for obtaining a high yield of CFS. Another aspect of the study aimed to identify multidrug-resistant P. aeruginosa isolates from burn wound infections. The isolates were identified using the VITEK 2 system, and the phzM gene was detected in all nine strains. Furthermore, the study evaluated the effect of the CFS of the selected strain (E5) on the expression of the phzM gene.* ***Results:*** *The study showed a significant down-regulation of phzM gene expression in multidrug-resistant P. aeruginosa isolates following exposure to the CFS, indicating the potential of E. faecium as an effective antimicrobial agent against P. aeruginosa infections that are resistant to multiple drugs. We performed a primary screening to evaluate the effect of the CFS obtained from E. faecium (E5), and it was observed that the CFS showed a high inhibition zone of 23 mm against multidrug-resistant P. aeruginosa, as determined by the agar well diffusion assay. The study also investigated further to determine the optimal conditions for producing CFS.* ***Conclusion:*** *Down-regulation and up-regulation in the expression of the genes following exposure to CFS indicate the potential of E. faecium as an effective antimicrobial agent against multi-drug resistant (MDR) P. aeruginosa infections.*

Key words: *P. aeruginosa, phzM gene, bee gut, MDR*

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INTRODUCTION

Enterococcus faecium, found in the gut flora of both humans and animals, is also an opportunistic pathogen. It is mainly associated with hospital-acquired infections in humans as well as many in animals, such as mastitis in cattle, diarrhea in swine and cattle, and septicemic diseases in poultry [1]. Certain virulence factors can be produced by *E. faecium* that potentially increase their pathogenicity, thereby contributing to the development of diseases [2]. The present study aimed to analyze the antago-

nistic activity of lactic acid bacteria (LAB), which are present in the honey bee environment, against both known honey bee pathogens and opportunistic pathogens. The LAB strains used in the experiments were previously isolated and characterized from the honey bee environment [3]. This study examined the postbiotic properties of lactic acid bacteria (LAB) metabolites in inhibiting the growth of honeybee pathogens at both physiological and neutralized pH levels. The authors believe that the topic of LAB's antagonistic activity against honey bee pathogens has not been extensively studied, noting that no research has been conducted to assess the postbiotic impact, like cell-free supernatants (CFS), on the growth inhibition of pathogenic microorganisms [4]. *Pseudomonas aeruginosa* is most frequently found in patients with compromised immune systems who had an extensive medical operation or those with diseases such as diabetes. It is a non-fermenting, Gram-negative pathogen and is considered to cause one of the most hospital-associated infections [5]. *P. aeruginosa* infections usually develop in connection with medical devices such as central lines, ventilators, and urine catheters, and more recently, coinfection in patients hospitalized with COVID-19 has been discovered as prevalent with *P. aeruginosa* [6]. Without the existence of new therapeutic agents, the problem of drug resistance may worsen. Recently, targeting virulence factors has appeared to be a new line of action against multi-drug resistant (MDR) *P. aeruginosa* strains [7]. *P. aeruginosa* has the ability to synthesize several compounds, including pyocyanin. It stimulates the production of reactive oxygen compounds (ROS) by host cells and inhibits the expression of catalase, an enzyme that neutralizes ROS by oxidizing NADPH [8]. In addition, a decrease in lung function and a contribution to the dominance of *P. aeruginosa* in the cystic fibrosis lung is related to pyocyanin [9]. Alternative antibiotic treatment, such as probiotic Nisin A Nisin and a nisin-producing *Lactococcus lactis* probiotic, significantly decreased the levels of several periodontal pathogens, alveolar bone loss, and the oral and systemic inflammatory host response [10]. The aim of this research is to detect the effect of FLAB suspension in the upregulation or downregulation of the pyocyanin gene.

MATERIALS AND METHODS

Sample collection

During the summer between September and October (2022), twenty-five adult worker bees were collected and transported to the laboratory for analysis. To remove any external microbes, the bees were disinfected with 94% ethanol for 60 seconds. To isolate bacterial cultures from the nectar stomach of each bee [11]. The pure cultures obtained were regrown in MRS broth and after incubation.

Macroscopic Examination of FLAB

The isolation and identification of FLAB were carried out using MRS broth. The MRS plates were incubated anaerobically for 24-48 hrs at 28-30°C, and well-isolated colonies with pure white, small and complete borders were selected and transferred to new MRS plates for final purification.

Microscopic Examination

All bacterial isolates were subjected to microscopic examination, including Gram staining, to observe the reaction to the stain, cell shape, and arrangement under a compound microscope [12].

Cultural Examination and Colony Selection

The bacteria were first identified based on the morphological properties of the colonies, which included colony form, colony texture, color, and edges, as well as clearance zone formed from hydrolysis of the CaCO3 by lactic acid [13].

16S rRNA gene sequencing

Characterization of unidentified bacteria using 16S rRNA gene sequencing was carried out according to previous work [14] with some modifications briefly described here. Colonies of each unidentified bacteria were re-cultured for 24-48 hours, depending on their growth conditions. The genomic DNA of bacteria was extracted using (ABIOpureTM Total DNA kit/USA) before PCR amplification of 16S rRNA genes. Amplification of isolates was performed using universal primers (5'-AGAGTTTGATCCTGGCTCAG-3', and 5'-TACGGTTACCTTGTTACGACTT-3') [15]. PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation – Korea. The results were received by email and then analyzed using Geneious software.

Screening for antibacterial activity of FLAB Suspension

All isolates of FLAB were screened for their inhibitory activity towards a number of the indicator strains, including Gram-negative bacteria MDR *P. aeruginosa* 3.2.4.2 by Agar Well Diffusion Assay (AWD) method and Filter Paper Disc (FPD) method to select the best isolates producing higher antimicrobial activity [16].

Preparation of Fructophilic Lactic Acid Bacteria (FLAB) Suspension

Fructophilic lactic acid bacteria suspension was obtained by growing FLAB in tubes with MRS broth which contains 0.1% cysteine and 2% fructose an-

aerobically in an anaerobic jar at 28-30 °C for 24-48 hrs, then centrifuged at 6000 rpm for 10 minutes, after being transferred to fresh tubes, filtered with sterile syringe filters with a pore size of 0.22 µm, and kept at 4°C in sterilized test tubes, and the free cell FLAB suspension was prepared for various tests of antibacterial activities and effects on gene expression on various genes of clinical isolates of *P. aeruginosa* [17].

Antimicrobial optimized numerous factors were examined to determine the ideal settings for high-level bacteriocin synthesis, and these are [18]:

Determination of optimum pH

The production and growth of CFS of various isolates were examined to determine the impact of pH. To achieve different pH values of 5, 6, 7, and 8, MRS broth was prepared in 10 ml tubes and adjusted with 0.5 N HCl or 0.5 N NaOH after autoclaving, and tubes were then inoculated with selected strains and incubated at 37 °C under anaerobic conditions for 24 hours.

Determination of optimum incubation

To investigate the impact of the incubation period, 10 ml of a selected strain (1.5 x 10^9 CFU/ml) was incubated in a MRS broth medium for different durations (18, 24, 48, and 72 hours) at 37ºC under anaerobic conditions.

Effect of Nitrogen Source & carbon source (sugars)

Different types of carbon sources (tryptone, yeast extract, sucrose, and glucose) with concentrations were used, and 1% of each was added to MRS broth.

P. aeruginosa isolation

Ten samples were collected from patients suffering from wound infections in an indoor setting after obtaining ethical approval from the Ethical Committee in the Department of Biology, College of Science, University of Baghdad. Prior to sample collection, signed consent was obtained from the patients. The samples were cultured under sterile conditions on nutrient agar, followed by cetrimide agar and MacConkey agar culturing. The VITEK 2 system was then used to confirm the identification of the bacterial isolates [19].

Detection of phzM gene

The tested gene was amplified by conventional PCR using primers obtained from [19]. PCR amplifications were carried out in 20 µl volumes containing 10 µl of GoTaq Green Master Mix (2X), 1 µl of primer (10 pmol), 6 µl of nuclease-free water, and 2 µl of template DNA. The PCR cycling was performed using a PCR Express (Thermal Cycler, Thermo Fisher Scientific, USA), following this temperature program: initial denaturation at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55, 58, 60, 63, or 65 °C for 30 seconds, and extension at 72 °C for 30 seconds [20]. The final extension step was carried out at 72°C for 7 minutes, followed by a 10-minute incubation at 4 °C to stop the reactions. The primer sequence for phzM used in this study is mentioned in Table 1.

RT-qPCR protocol

Real-time quantification of cDNA was carried out on GoTaq® 1-Step RT-qPCR System (Promega, USA) using the SYBR green PCR master mix. Real-time PCR was used to investigate the expression level of phzM and fbp genes. To assess the gene expression of the phzM gene, though, results were normalized using the fbp gene, which is considered a house keeping 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. Afterward, they were stored in a deep freezer until used in qPCR. The reaction mixture is summarized in Table 2.

Table 1. Primer used in PCR and real-time PCR

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)	Reference
phzM-F	ACGGCTGTGGCGGTTTA	60	~180	
phzM-R	CCGTGACCGTCGCATT			
fbp-F fbp-R	CCTACCTGTTGGTCTTCGACCCG GCTGATGTTGTCGTGGGTGAGG	53	53	[21, 22]

Table 2. The components of the master mix in qRT-PCR

Master mix components	Unit	Volume / 1 Sample µl
qPCR Master Mix	X	5
RT mix	x	0.25
$MgCl_2$		0.25
Forward primer	µM	0.5
Reverse primer	µM	0.5
Nuclease Free Water		2.5
RNA	ng/µl	1
Total volume	10	

RESULTS

Identification of Bacterial Isolates

The isolation FLAB species (E1-E10) characterization, such as Gram-positive and catalase-negative, appeared large, and white colonies were isolated on MRS. After sequencing the 16S rRNA gene, the isolate was identified, and the resulting sequence was submitted to the NCBI database, as depicted in Figure 1.

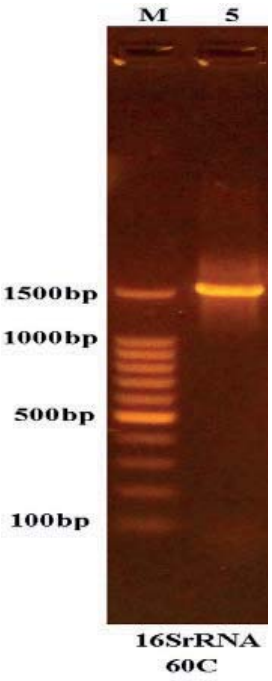

Fig. 1. Results of the amplification of 16SrRNA gene of bacterial samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100 bp ladder market. Lanes 5 resemble 1500 bp PCR

Samples were collected from patients with burn and wound infections, and several isolates of *P. aeruginosa* were identified. The colony shapes of these isolates were determined using selective media. On MacConkey agar, the isolates that were able to grow were observed as pale colonies, while on Cetrimide agar, *P. aeruginosa* isolates appear as mucoid, smooth colonies with flat edges and elevated centers exhibit the ability to produce a blue-green pigment after 72 hours of incubation. The confirmation of all isolates was done and diagnosed using the VITEK 2 From the 10 clinical isolates named (Z1, Z2, Z3, Z5, Z4, Z9, 72, 77, 79).

Screening of antimicrobial activity of LAB isolated from honey bees

E. faecium strain screening the best CFS crude antimicrobial activities' spectrum as each had activity against multi-drug resistant *P. aeruginosa*, as shown in Figure 2. The cell-free culture supernatant (CFS) antibacterial activities were assessed by agar well diffusion assay.

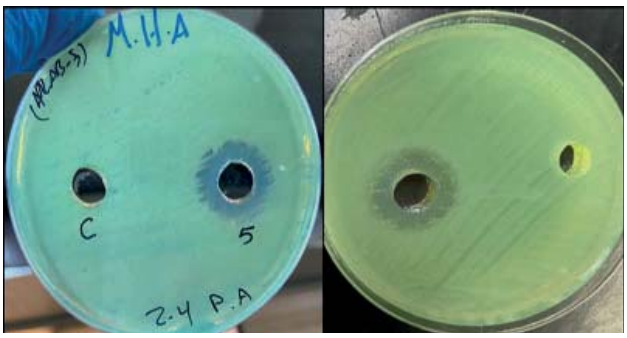

Fig. 2. Effect of antimicrobial activity of FLAB on *P. aeruginosa* on MHA anaerobic condition at 37ºC

Optimum conditions for bacteriocin production

Optimal pH

The results indicate that the diameter of the inhibition zone reached 22.5 mm, and pH 5 was the best value for production. The lower inhibition zone, 15.12 mm at pH 8, is shown in Figure 3.

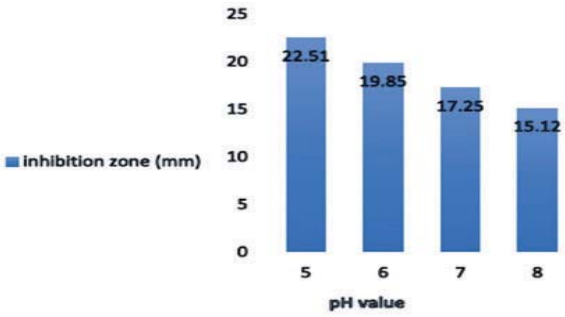

Fig. 3. Effect of pH on CFS production

Optimum incubation time

The production of CFS was observed at various incubation times, and it was noted that the highest production occurred after 72 hours of incubation, resulting in the largest inhibition zone, measuring 24 mm. However, the activity of CFS decreased after 48 and 18 hours, with the lowest inhibition zone measuring 10 mm (as illustrated in Figure 4).

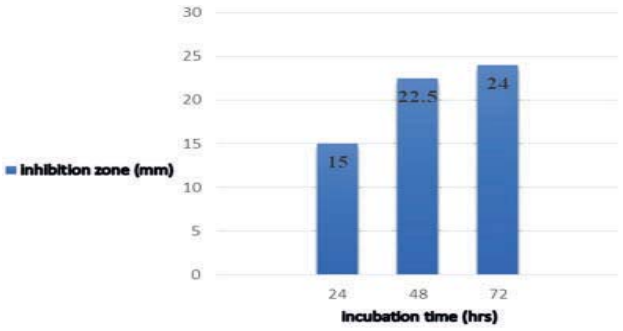

Fig. 4. Effect of incubation time on CFS production

Nitrogen and carbon source

Fructophilic lactic acid bacteria (FLAB) found in D-fructose-rich niches prefer D-fructose over D-glucose as a growth substrate. They need electron acceptors for growth on glucose, the organisms share carbohydrate metabolic properties. The different carbon sources of sugars related to their consumption by bacteria during metabolic processes growth of *E. faecium* in the presence of 1% glucose that recorded optimum carbon source that gave the greatest inhibition zone about 24 mm for E5 isolate, compared with other carbon sources. Glucose was the best carbon source compared with sucrose, giving an inhibition zone of about 21 mm (Figure 5).

Polymerase Chain Reaction (PCR)Technique

The PCR results identified nine isolates of *P. aeruginosa* with the phzM gene, selected based on their multi-drug resistance (MDR). The positive gene result was subsequently confirmed through electrophoresis on a 1.5% agarose gel stained with ethidium bromide, electrophoresed at 75 volts for 50 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 6. Notably, there was no evidence of DNA degradation, as indicated by the absence of any smearing of the gene band.

To estimate the effect of CFS of *E. faecium* at concentrations of 8 µg/ml, two *P. aeruginosa* isolates were studied using the RT-qPCR technique. RT-PCR reveals a major downregulation in phzM expression after exposure to CFS suspension of *E. faecium* compared to normal gene expression in bacteria. Fold change in gene expression reveals that phzM was downregulated in response to CFS in all isolates of *P. aeruginosa*, as entailed in Table 3.

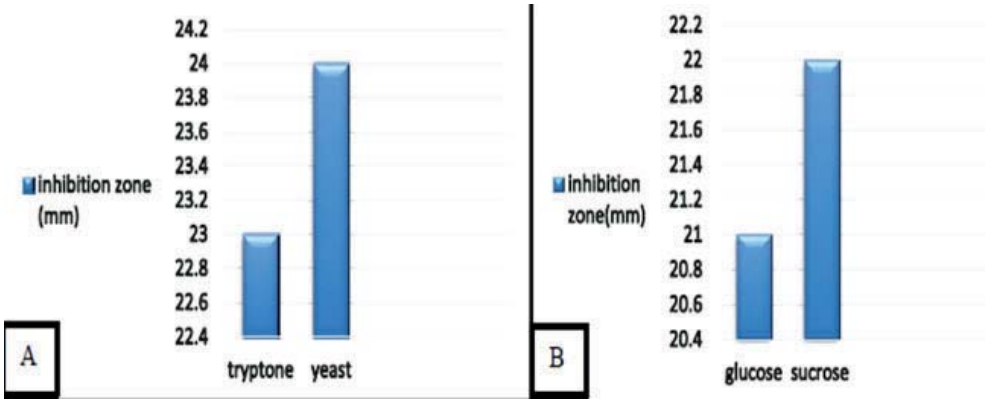

Fig. 5. A. Effect of Nitrogen source, **B.** Effect of carbon source (sugars) on CFS production

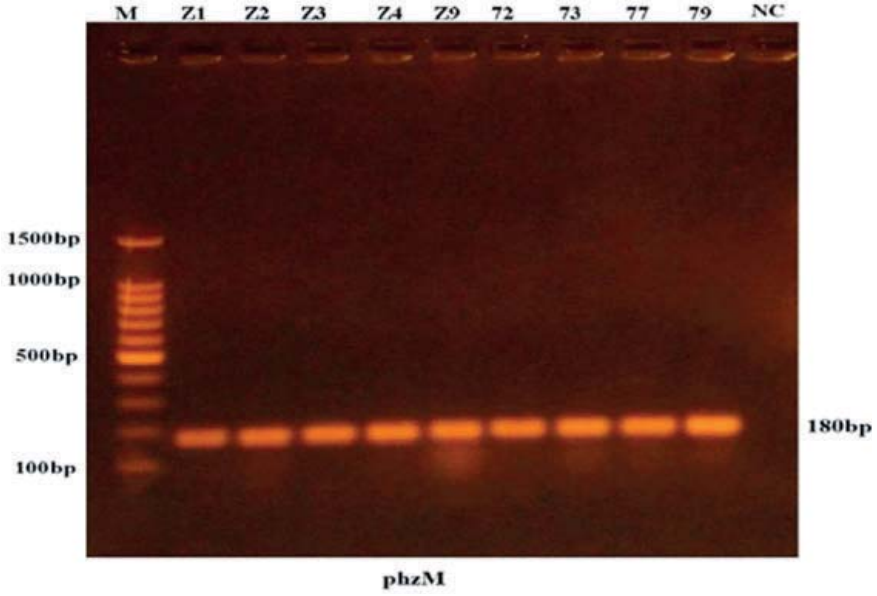

Fig. 6. Results of the amplification of the phzM gene of Pseudomonas aeruginosa bacterial samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth. Br. M: 100bp ladder marker. Lanes Z1-79 resemble 180bp PCR products

Table 3. Gene expression changes before and after treatment with CFS

Sample	Fbp	phzM	Dct	ddct	Folding
73 (before)	18.52	15.28	-3.25	0.00	1.00
73 (after)	19.00	16.08	-2.92	0.33	0.79
Z4 (before)	21.76	17.58	-4.17	0.00	1.00
Z4 (after)	19.76	16.17	-3.59	0.59	0.67

The results indicated a major down-regulation in phzM expression after exposure to CFS. The fold change in gene expression showed that phzM was down-regulated in response to CFS in all isolates of *P. aeruginosa*. These findings suggest that the CFS of *E. faecium* can have an inhibitory effect on phzM gene expression, which may lead to the loss of functional genes involved in pyocyanin biosynthesis.

DISCUSSION

The results agree with Elzeini et al. [23]. LAB was isolated from the gastrointestinal tract of the bee. The accession number showed a high level of similarity to *E. faecium*. This finding is consistent with the identification of enterococci isolates from both dairy and honey bee samples using 16S rRNA gene sequencing [24]. Extract fresh lactic acid bacteria from boza that produce bacteriocins, then contrast the bacteriocins with those that have already been reported for strains that have been separated from the same niche. From boza, five bacteriocin-producing lactic acid bacteria were identified, including Leuconostoc lactis ST63BZ, ST611BZ, and ST612BZ, Lactobacillus plantarum ST69BZ, and *E. faecium* ST62BZ [25]. To assess the frequency and expression of bacteriocin genetic determinants, a total of 424 Enterococcus spp. isolates from clinical samples (n = 398) and traditional Bulgarian artisanal cheeses (n = 26) were examined. Polymerase chain reaction (PCR) was used to identify the structural genes coding for enterocin A (entA), enterocin P (entP), enterocin B (entB), enterocin AS-48 (as-48), and enterolysin A (entlA). PCR was also used to determine the expression of these genes [26].

rsbA gene expression has been significantly downregulated, thereby preventing the formation of biofilms and swarming motility, as shown by this. Their significant downregulation of rsbA gene expression, which effectively encouraged swarm progress, provided further evidence for this [27]. The results of the Vitek 2 automated system, a probability of about 95-99%, belong to the genus Pseudomonas. The other study by Mais et al. [28] showed the bacteriocins secreted by LAB do not have activities against Gram-negative bacteria, including Salmonella spp and *P. aeruginosa* by using the Well diffusion assay method, such as LAB bacteriocins exhibit broad-spectrum activities against both Gram-positive and Gram-negative bacteria. To achieve different pH values of 5, 6, 7, and 8, MRS broth was prepared in 10 ml tubes and adjusted with 0.5N HCl or 0.5N NaOH after autoclaving, tubes after that were inoculated with selected strains and then incubated at 37°C under anaerobic conditions for 24-72 h [29]. The results show improvement in Bacillus subtilis culture conditions to maximize amylase output, and it was conducted in a shaking incubator. When starch that was soluble was used as the substrate, 350 Uml-1 was shown to be the optimal activity. The following parameters are taken into consideration to see whether they affect the best possible production of amylase: incubation temperature, duration, inoculum size, pH, carbon source, nitrogen source, and metallic ions [30, 31]. Antibiotic resistance and accompanying diseases brought on by harmful microorganisms are targets of numerous proposed techniques. These tactics try to reduce selection pressure and avoid future resistance risk by inhibiting biofilm formation and attenuating pathogenic bacteria's virulence factors, in addition to focusing on resistance enzyme manufacturing and efflux pump performance [32]. The phzM gene was found to be positive in nearly 78% of the examined isolates, which may indicate that pyocyanin is one of the most common virulent determinants of the strains isolated from bloodstream infections. It was also observed that the synthesis of pyocyanin decreased in a group of *P. aeruginosa* strains that were resistant to multiple drugs [33].

CONCLUSIONS

This research aimed to assess the anti-virulence activity of FLAB against *P. aeruginosa* infection *in vitro*. This study supports the further development of new potential anti-infective drugs. These findings suggest that E5 suspension has the potential to serve as an effective antimicrobial agent against *P. aeruginosa* infections that display resistance to multiple drugs and has shown effective in inhibiting the expression of important virulence factors of the *P. aeruginosa* phzM gene expression for pyocyanin. This inhibitory effect on pyocyanin gene expression may represent a potential strategy for controlling *P. aeruginosa* infections.

Disclosure Summary: *The author has nothing to disclose.*

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