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Furobenzopyrans from *Ammi visnaga* suppress *Pseudomonas aeruginosa* virulence

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ABSTRACT

This study investigates the potential of Khellin (KH), a furobenzopyran derived from *Ammi visnaga*, to inhibit the virulence factors of *Pseudomonas aeruginosa*. KH was tested at concentrations ranging from 0 µg/ml to 900 µg/ml, with no visible growth inhibition observed at concentrations below 90 µg/ml (sub-minimum inhibitory concentration, MIC). The effects of KH (9 µg/ml), a positive control (ciprofloxacin, 0.1 µg/ml), and a control (0 µg/ml) on virulence factors, including pyocyanin and elastase production, N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12 HSL) secretion, and *lasR* gene expression, were assessed. KH treatment resulted in a significant reduction of 47% in pyocyanin levels compared to the control group ($p < 0.05$) and a significant decrease of 54.8% in elastase activity compared to the control group ($p < 0.05$). KH also significantly reduced (69%) the secretion of 3-oxo-C12 HSL, correlating with the 45% suppression of *lasR* gene expression ($p < 0.05$). These findings suggest that sub-MIC levels of KH reduce *P. aeruginosa* virulence by suppressing quorum-sensing genes, highlighting its potential for anti-virulence therapy against persistent infections and antibiotic resistance.

1. INTRODUCTION

Pseudomonas aeruginosa, a gram-negative bacteria and an opportunistic pathogen, is well-known for causing severe infections in immunocompromised individuals, such as those with cystic fibrosis, burns, or ventilator-associated pneumonia [1–3]. Its pathogenicity is driven by an arsenal of virulence factors, that includes quorum sensing (QS) regulated toxin secretion (e.g., elastase and pyocyanin), and biofilm formation [4–7]. Traditional antibiotics, which target bacterial growth or viability, impose strong selective pressures that drive resistance [8]. Due to its rapid development of multidrug resistance (MDR), the World Health Organization classifies *P. aeruginosa* as a "critical priority" pathogen, emphasizing the urgent need for innovative treatments [8].

To combat MDR, anti-virulence strategies have been developed to disarm pathogens by disrupting their ability to colonize, communicate, or damage host tissues [9]. For instance, QS inhibitors can block the production of virulence factors controlled by the *las* and *rhl* QS systems, without affecting bacterial survival [10]. Natural products, especially phytochemicals, are a rich source of anti-virulence agents due to their structural diversity and evolutionary optimization for biological interactions [11]. *Ammi visnaga* (Apiaceae), a Mediterranean medicinal plant, has been used since antiquity for treating renal colic, asthma, and angina, primarily due to its vasodilatory furanochromones, such as khellin (KH) and visnagin [12]. Recent phytochemical studies have identified furobenzopyrans, structurally related to furanochromones as key bioactive constituents with antimicrobial, anti-inflammatory, and calcium channel-modulating properties [13–15]. Emerging evidence suggests that plant-derived molecules can disrupt bacterial virulence by interfering with QS signaling [16,17]. Building on these findings, we hypothesize that KH, a furobenzopyrans from *A. visnaga,* attenuate *P. aeruginosa* pathogenicity by targeting virulence factor secretion and this study addresses a critical gap in understanding the anti-virulence potential of *A. visnaga* phytochemical.

2. MATERIALS AND METHODS

2.1. Chemicals, reagents, and strain details

The chemicals used were of analytical grade from Sigma–Aldrich Chemicals Private Limited, India. The solvents used of spectroscopic grade from Sigma–Aldrich Chemicals Private Limited, India. Luria–Bertani (LB) media, Hexa G-minus 28, agar, and Mueller Hinton (MH) media, were sourced from Hi-Media Laboratories Private Limited, India. Elastin congo red (ECR) was procured from Sigma–Aldrich Chemicals Private Limited, India. Ciprofloxacin and KH were obtained from Tokyo Chemical Industry, India. The experiments were conducted using the *P.aeruginosa* PAO1 strain (MTCC 2453) from the microbial type culture collection, Institute of Microbial Technology, India.

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2.2. Antibiotic Sensitivity Analysis

The susceptibility to antibiotics was assessed in triplicates using Hexa G-28 sensitivity disc as per the reported procedure [15,18]. A bacterial suspension was prepared in sterile saline to achieve a turbidity equivalent to the 0.5 McFarland standard, which corresponds to approximately 1 × 10^8 colony forming unit (CFU)/mL. Using a sterile swab, excess liquid was removed by pressing alongside the walls of the tubes. The swab was then used to spread the bacterial suspension on the surface of MH agar plates, ensuring a uniform bacterial lawn. The plates were rotated 60° and swabbed two more times for even coverage. After allowing the plates to dry for 5–10 minutes at room temperature, Hexa G-28 sensitivity discs were placed on the agar surface using sterile forceps, ensuring even spacing and firm contact. The plates were subsequently incubated at 37°C for 18 hours. Post-incubation, the plates were inspected for zones of inhibition around the discs, indicating bacterial susceptibility to the antibiotics.

2.3. Effect of KH on *P. aeruginosa* Growth

The broth microdilution method as per standard guidelines was used to determine the MIC of KH against *P.aeruginosa* PAO1 [18,19]. The MH broth was used to prepare the working dilutions of KH between 0 µg/ml and 900 µg/ml. These concentrations were selected based on a broad range of preliminary screenings to identify the optimal dosage that could inhibit bacterial growth. The MH broth containing various concentrations of KH and control (no treatment) was inoculated with the overnight-grown culture of *P.aeruginosa* in triplicates. Then, the mixture was incubated at 37°C overnight and the optical density (OD) was measured at 600 nm (SpectraMax® Plus 384 Absorbance Plate Reader, Molecular Devices, US).

Table 1. Primers for quantitative real-time polymerase chain reaction.

Gene	Primer direction	Sequence (5'-3')	Amplicon size (bp)
lasR	Forward	ACGCTCAAGTGGAAAATTGG	111
	Reverse	TCGTAGTCCTGGCTGTCCTT	

2.4. Effect on Pyocyanin Content

Pyocyanin production was measured following the established methods [20]. The *P.aeruginosa* culture was incubated at 37°C overnight, with 20 µl of the overnight culture then added to 2 ml of fresh medium (2% peptone, 0.14% magnesium chloride, 1% potassium sulphate, and 1% glycerine, pH 7.4) treated with the KH (9 µg/ml) at 37°C along with shaking at 150 rpm in triplicates. The cells were then centrifuged at 12,000 rpm for 15 minutes at 4°C. The cell-free supernatants were then analyzed for pyocyanin production at 695 nm using a spectrophotometer (SpectraMax® Plus 384 Absorbance Plate Reader, Molecular Devices, US).

2.5. Effect on Elastase Activity

To analyze the elastinolytic activity, a reaction mixture was prepared by adding ECR buffer (900 µL) to the KH(9 µg/mL) treated culture supernatant (100 µL) in triplicates [21]. The ECR buffer comprised 100 mM Tris, 1 mM $CaCl_2$, and 20 mg ECR at a pH of 7.5. Following the incubation period (37°C for 3 hours), centrifugation was carried out at 10,000 rpm for 10 minutes to remove the insoluble ECR. The elastinolytic activity was then estimated by measuring the OD at 495 nm.

2.6. Impact on Secretion of 3-oxo-C12-HSL

The potential anti-QS activity of KH was evaluated by measuring the levels of N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) secreted by *P. aeruginosa* PAO1 [21]. Briefly, 0.1% overnight cultures of *P. aeruginosa* PAO1 were inoculated into 50 ml of LB medium with or without KH(9 µg/mL) in triplicates and incubated at 37°C for 48 hours. After incubation, cells were removed by centrifugation at 4°C for 15 minutes. The supernatant underwent three successive extractions using ethyl acetate. The ethyl acetate was evaporated under reduced pressure, and the residues were dissolved in methanol. Quantification of 3-oxo-C12-HSL was performed using high-performance liquid chromatography coupled with an ultraviolet detector [21]. Peaks corresponding to 3-oxo-C12-HSL were identified based on retention times of 3-oxo-C12-HSL standard (Sigma-Aldrich, India). Peak areas were calculated using the extracted chromatograms and the results were quantified.

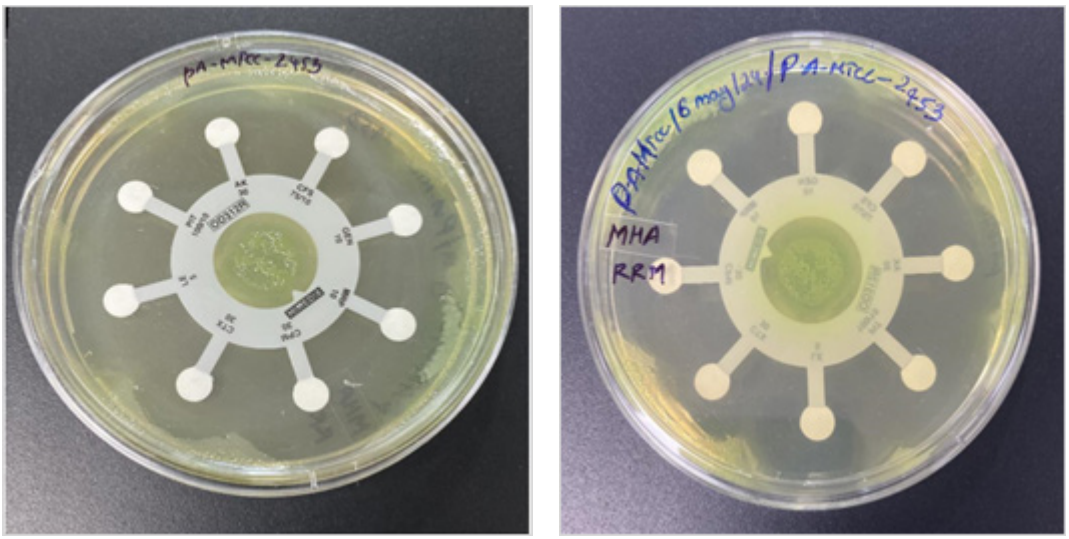

Figure 1. Effect of antibiotics (gentamicin, meropenem, cefepime, cefotaxime, levofloxacin, piperacillin/tazobactam, amikacin, and cefoperazone) against *Pseudomonas aeruginosa* showing top(left image) and back (right image) side of the plates.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as per the method described by Zhou *et al.* [22]. The PAO1 strain was cultured in LB medium, with or without the addition of KH(9.0 µg/ml) in triplicates, at 37°C and 180 rpm for 24 hours. After incubation, the cells were washed three times with sterile PBS (pH 7.2) and collected by centrifugation at 4°C for 10 minutes. Total RNA was extracted, genomic DNA was removed and cDNA was synthesized as per the protocol briefed by Zhou *et al.* [22]. The primers listed in Table 1 were sourced from the previous studies and a SYBR Green Master Mix kit (Vazyme Biotech, Nanjing, China) was used to run qRT-PCR. The fold changes of target genes were calculated using the $2^{-\triangle\triangle Ct}$ method, as previously described [23].

2.8. Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with the STATGRAPHICS® Centurion XVI software (Version 16.2.04, Statgraphics Technologies, Inc., The Plains, VA) to assess statistical significance. Post-hoc comparisons were carried out using Fisher's least significant difference test to identify specific differences between groups after the ANOVA. All experiments were performed in triplicate, and at a 95% confidence interval, a statistically significant was considered if the *p*-value was less than 0.05.

3. RESULTS

3.1. Antibiotic Sensitivity

The antibiotic sensitivity profile of *P. aeruginosa* was studied, revealing susceptibility to a range of antibiotics (Fig. 1). Specifically, *P. aeruginosa* demonstrated good sensitivity to the tested antibiotics such as gentamicin, meropenem, cefepime, cefotaxime, levofloxacin, piperacillin/tazobactam, amikacin, and cefoperazone. This broad spectrum of sensitivity indicates that the strains used for the studies were not antibiotic-resistant strains.

3.2. Effect of KH on Growth

The antibacterial activity of KH was inferred based on the growth visually, measuring OD at 600 nm, and colony count (CFU/ml) at various concentrations, and the results are summarized in Table 2. In the control group (0 µg/ml), there was significant bacterial growth, with an OD of 0.73 and a colony count of 351*10^6 CFU/ml, indicating normal bacterial proliferation without any antimicrobial intervention. The positive control, ciprofloxacin at 0.1 µg/ml, effectively inhibited bacterial growth, as evidenced by an OD of 0.09 and a colony count of less than 10 CFU/ml, demonstrating the antibiotic's strong antibacterial activity. When tested with KH at 0.9 µg/ml, bacterial growth was still observed, with an OD of 0.75 and a colony count of 368 × 10^6 CFU/ml, which was similar to the control group, suggesting

Table 2. Effect of khellin on *P. aeruginosa* growth. Each experimental data represents the average of triplicates.

	Concentrations (µg/ml)	Growth	OD@600 nm	Colony count (CFU/ml)
Control	0.0	Yes	0.73	351*10^6
Positive control – Ciprofloxacin	0.1	No	0.09	<10
KH	0.9	Yes	0.75	368*10^6
	9.0	Yes	0.74	361*10^6
	90.0	Yes	0.71	348*10^6
	900.0	No	0.29	136*10^3

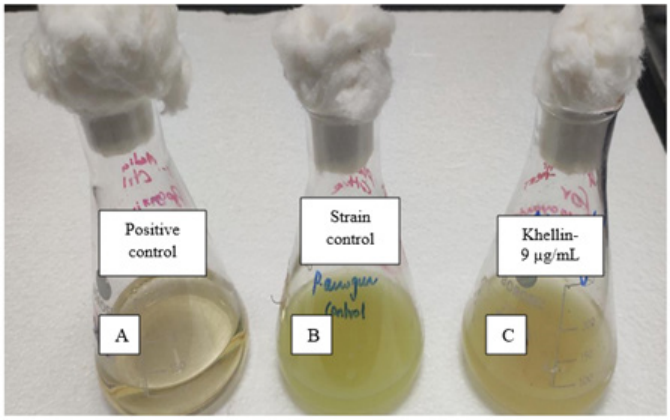

Figure 2. Culture flask showing the grown cultures of *Pseudomonas aeruginosa* . A) Positive control treated with ciprofloxacin; B) Strain control with no treatment; C) *P. aeruginosa* treated with Khellin.

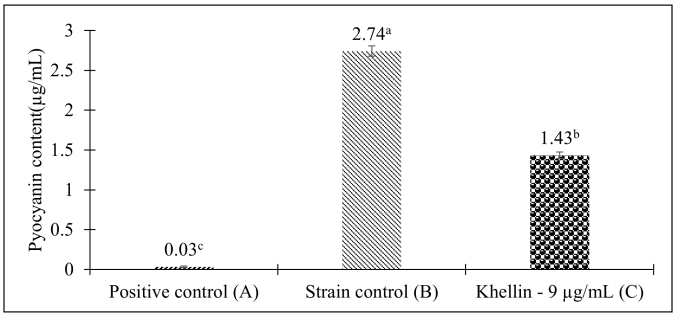

Figure 3. The effect of Khellin on pyocyanin content in *P. aeruginosa* was evaluated, with ciprofloxacin (0.1 µg/ml) serving as the positive control (A) and the control group receiving no treatment (B). Each experimental data is expressed as mean ($n = 3$) and the error bar denotes standard deviation ($n = 3$). Different lowercase alphabets in superscripts are used to indicate significant differences between groups ($p < 0.05$).

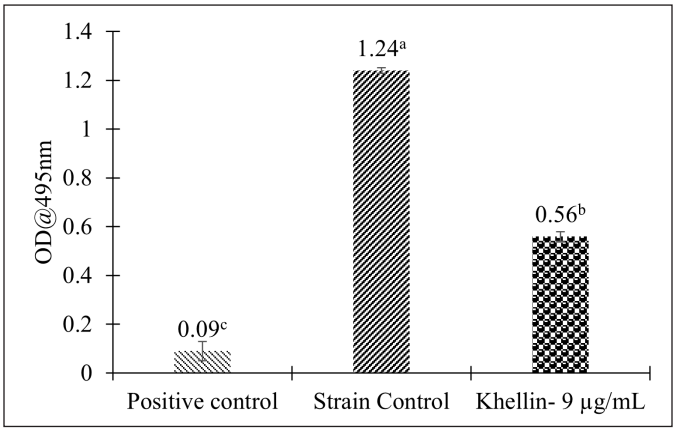

Figure 4. Khellin's effect on the elastase activity of *Pseudomonas aeruginosa.* Ciprofloxacin (0.1 µg/ml) served as the positive control and the control group received no treatment. Each experimental data are expressed as mean ($n = 3$) and the error bar denotes standard deviation ($n = 3$). Different lowercase alphabets in superscripts are used to indicate significant differences between groups ($p < 0.05$).

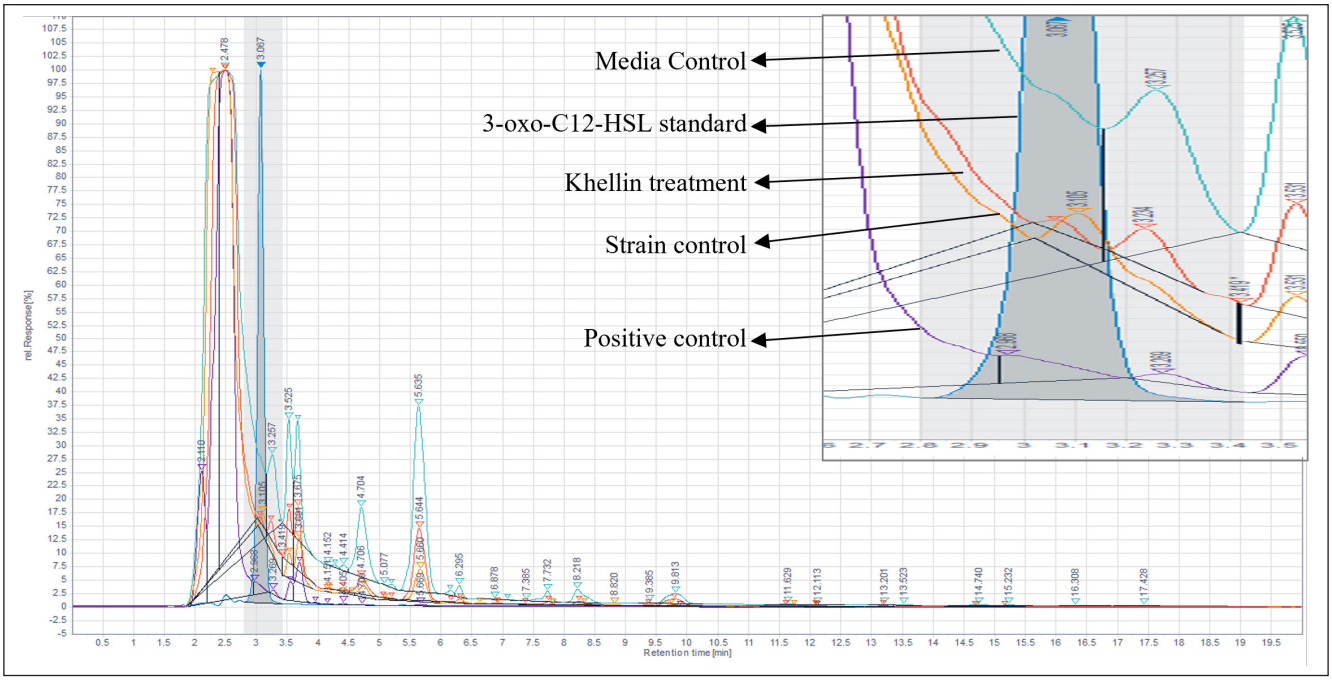

Figure 5. Chromatogram showing the impact of khellin on 3-oxo-C12 HSL signaling molecule in *P. aeruginosa*. Ciprofloxacin (0.1 µg/mL) served as the positive control and the control group received no treatment.

that this concentration of KH did not inhibit bacterial growth. At KH concentrations of 9.0 µg/ml and 90.0 µg/ml, bacterial growth remained evident, with OD values of 0.74 and 0.71, respectively, and colony counts of 361 × 10^6 and 348 × 10^6 CFU/ml. These results indicate that KH, at these concentrations, did not substantially reduce bacterial growth. However, at a higher concentration of KH (900.0 µg/ml), growth was significantly inhibited, with an OD of 0.29 and a reduced colony count of 136 × 10^3 CFU/ml. This suggests that at high concentrations, KH exhibits antibacterial activity, although it was not as potent as ciprofloxacin in fully preventing bacterial growth. Overall, the results demonstrate that while KH has some antibacterial potential at higher concentrations, its effectiveness at lower concentrations is limited, and it is less potent than the positive control.

3.3. Effect On Pyocyanin Content

The ability of KH to inhibit pyocyanin production was studied and the results are shown in Figure 2 and Figure 3. *Pseudomonas aeruginosa* produces pyocyanin which is in green color. As shown in Figure 2, the KH-treated group showed less green color compared to the strain control.

The overnight-grown cultures were subjected to pyocyanin content estimation, and the results are shown in Figure 3. We could see a marked reduction in the concentrations from 2.74 to 1.43 µg/ml in the KH-treated group($p < 0.05$).

3.4. Effect on Elastase Activity

The effect of KH on elastase activity was studied and the results are presented in Figure 4. A statistically significant reduction of elastase activity was observed for the KH-treated group (OD of 0.56) compared to the control OD of 1.24 ($p < 0.05$). These results indicate a general trend of decreasing elastase activity with increasing concentration, after an initial slight increase at the lowest concentration.

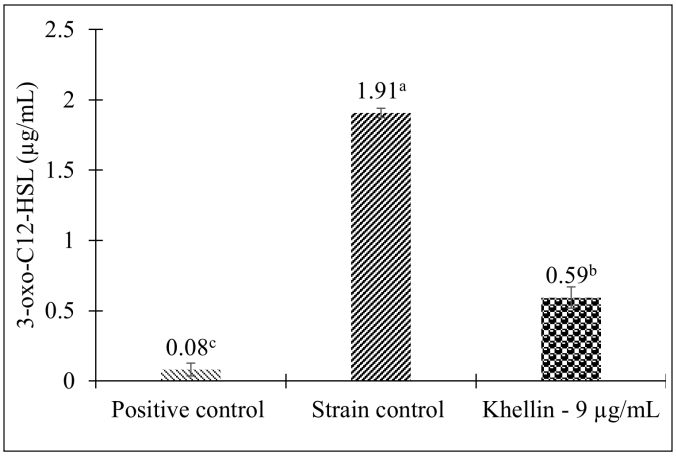

Figure 6. Effect of khellin on the secretion of 3-oxo-C12 HSL signaling molecule in *Pseudomonas aeruginosa*. Ciprofloxacin (0.1 µg/ml) served as the positive control and the control group received no treatment. Each experimental data are expressed as mean ($n = 3$) and the error bar denotes standard deviation ($n = 3$). Different lowercase alphabets in superscripts are used to indicate significant differences between groups ($p < 0.05$).

3.5. Impact on Secretion of 3-oxo-C12-HSL

The levels of 3-oxo-C12-HSL produced by *P.aeruginosa* PAO1 were quantified to assess the potential anti-QS activity of KH. Chromatography analysis revealed the presence of 3-oxo-C12-HSL, in the culture supernatants, and treatment with KH at 9 µg/mL for 24 hours led to a significant reduction of 3-oxo-C12-HSL (Fig. 5). Relative quantification analysis showed that KH reduced 3-oxo-C12-HSL levels by approximately 69% compared to the control (Fig. 6). These results indicate that KH has anti-QS activity, likely by inhibiting the production of signaling molecules.

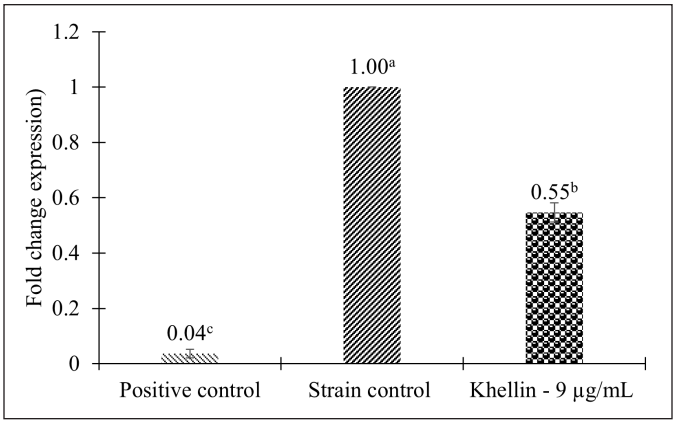

Figure 7. Effect of khellin on the *lasR* gene expression of QS-related circuits in *P. aeruginosa*. Khellin's effect on the elastase activity of *Pseudomonas aeruginosa.* Ciprofloxacin (0.1 µg/ml) served as the positive control and the control group received no treatment. Each experimental data are expressed as mean ($n = 3$) and the error bar denotes standard deviation ($n = 3$). Different lowercase alphabets in superscripts are used to indicate significant differences between groups ($p < 0.05$).

3.6. Effect on QS-Related Gene Expression

A qRT-PCR assay was conducted to assess the effect of khellin on the expression of the QS-related gene *lasR* in *P.aeruginosa* PAO1. The results revealed a significant reduction in *lasR* expression, with a 45% down-regulation observed after exposure to 9 µg/ml of khellin (Fig. 7).

4. DISCUSSION

The antibiotic susceptibility profile of *P. aeruginosa* outlined in Figure 1 demonstrated that *P. aeruginosa* used for the experiments were sensitive to gentamicin, meropenem, cefepime, cefotaxime, levofloxacin, piperacillin/tazobactam, amikacin, and cefoperazone. These antibiotics are known for their effectiveness against various bacterial infections, and their efficacy against *P. aeruginosa* aligns with the published reports [24]. In the current dose-response study with KH, the growth of *P. aeruginosa* cultures was reduced at 900 µg/ml (Table 1). In the low concentration (<900 µg/ml), no visible impact on *P. aeruginosa* growth was noticed (Table 1), indicating the sub-MIC for KH. Rafiee *et al.* [25] investigated the anti-QS activity of a ciprofloxacin–copper complex against *P. aeruginosa* PAO1 [25]. They found that 1/4 and 1/16 of MIC (sub-MIC) of the complex significantly reduced virulence factors while having minimal impact on bacterial growth. Similarly, Similarly Naga *et al*. studied the effects of methyl gallate (MG) from *Mangifera indica* on QS in *P. aeruginosa* [26]. They found that MG at sub-MIC concentrations (1/4 and 1/2 MIC) significantly inhibited QS-regulated virulence factors without affecting bacterial viability. In the current study, at 1/10, 1/100 of MIC, KH had minimal or no impact on the growth of *P. aeruginosa* supporting the published reports [27–30]. These findings underscore the potential of using sub-MIC concentrations of QS inhibitors to reduce *P. aeruginosa's virulence*.

Pyocyanin is a key virulence factor that contributes to the *P. aeruginosa* pathogenicity. Recent studies have explored the potential of quorum-sensing inhibitors to reduce pyocyanin production. For instance, Morkunas *et al.* tested an inhibitor that competitively inhibits the native autoinducer, leading to a reduction of pyocyanin production in wild-type *P. aeruginosa* cultures [31]. Fekete-Kertész *et al.* demonstrated that α-cyclodextrin (ACD) and β-cyclodextrin significantly reduced pyocyanin production, with ACD showing a higher inhibitory effect, highlighting the potential of CDs as antivirulence agents [32]. In the current study, the concentration of pyocyanin pigment was reduced in the KH-treated group. Based on all this information, the findings observed in the current study on the reduction of pyocyanin production by KH (Figs. 2 and 3) could be through suppression of virulence gene expression.

In *P. aeruginosa* pathogenesis, another major virulence factor that is involved is elastase, especially in lung-related severe infections [33,34]. Elastase breaks down elastin, a key extracellular matrix component, contributing to tissue destruction and inflammation. A recent study by Zhou *et al.* demonstrated that a phyto-compound (resveratrol), when exposed to *P. aeruginosa* cultures, significantly reduced elastase production [35]. Ren *et al.* studied the effects of quercetin on the reduction of elastase activity by 57% when *P. aeruginosa* was exposed at sub-MIC concentrations [36]. In the current study, KH showed elastase inhibition of 54.83% in comparison to control ($p < 0.05$, Fig. 4) which suggests that KH would have mediated its action similar to that of quercetin and resveratrol.

Pyocyanin and elastase play crucial roles in colonizing host tissues, and biofilm formation and are regulated by the *las* QS system mediated through *lasR* gene [22]. To confirm the mechanism of action of KH's reduced virulence factors, gene expression studies were performed and the KH-treated group showed suppression of *lasR* gene expression compared to the control (Fig. 7). These results closely aligned with KH impacting the expression of QS-related genes, confirming that KH treatment inhibits the transcriptional levels of virulence factors.

In *P. aeruginosa* QS systems, the *las* QS system modulates the synthesis of the QS signaling molecule (3-oxo-C12 HSL). Through these signaling molecules, *P. aeruginosa* regulates the secretion of virulence factors [10]. In this study, the KH-treated *P. aeruginosa* group secreted lower levels of 3-oxo-C12 HSL compared to the control (Fig. 6), likely due to the suppression of *lasR* gene expression observed in the study. In a normal QS circuit, *lasR*, upon binding to 3-oxo-C12 HSL, activates genes responsible for virulence factor production and enhances *lasI* expression, which encodes the enzyme responsible for synthesizing more 3-oxo-C12 HSL [5,10]. KH likely interferes with *lasR* expression, leading to reduced *lasR* availability and disrupting this positive feedback loop, ultimately lowering 3-oxo-C12 HSL synthesis and secretion. By targeting *lasR*, KH effectively disrupts bacterial communication, reducing virulence and potentially enhancing the susceptibility of *P. aeruginosa* to host defenses or antibiotic treatments, making it a promising anti-virulence agent.

While KH effectively reduces virulence, its bioavailability, *in-vivo* efficacy, stability, and potential cytotoxicity require further investigation. Findings suggest that KH could be combined with antibiotics to treat resistant strains causing persistent infections, warranting a more detailed study.

5. CONCLUSION

This study demonstrated that KH, at sub-MIC concentrations, effectively reduced virulence factors such as pyocyanin content and elastase activity, which are mediated by the *las* QS system. Mechanistic studies revealed that KH exerted its anti-virulence activity by suppressing *lasR* gene expression, as confirmed by the decreased production of 3-oxo-C12 HSL signaling molecules. Hence, by targeting QS pathways, it may be possible to develop

novel therapeutic strategies that weaken the pathogen's defenses and improve treatment outcomes.

6. ACKNOWLEDGMENTS

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7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. FUNDING

The authors declare that no funding was received for this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data are available with the author and will be provided upon request.

11. AUTHOR CONTRIBUTION STATEMENT

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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