RESEARCH PAPER

Effect of Illicium verum extract upon Quorum quenching mediated bacterial attenuation

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Abstract

Aim: The study aimed to evaluate the quorum-quenching (QQ) and anti-virulence potential of *Illicium verum* aqueous fruit extract against quorum-sensing (QS) regulated phenotypes of *Chromobacterium violaceum* MCC 2290 and *Pseudomonas aeruginosa* JB9, and to optimize key variables influencing QQ activity using a Box–Behnken response surface design.

Method: A hot aqueous extract of *I. verum* fruits was prepared and subjected to phytochemical screening and activity-guided fractionation. At doses ranging from 100-300 µg/mL, the phenotypic effects of QS inhibition were examined by suppressing violacein in C. violaceum MCC 2290, inhibiting pyoverdine pigment, and reducing biofilm formation in P. aeruginosa JB9. The cumulative impacts of three independent variables—bacterial density, extract concentration, and injection frequency—on quorum-quenching efficacy were evaluated using the Box–Behnken Response Surface Methodology (RSM). The active fraction was further characterized using GC–MS to identify the major bioactive compound.

Result: *I. verum* extract exhibited strong concentration-dependent quorum-quenching activity, producing marked reductions in violacein production, pyoverdine synthesis, and biofilm formation without affecting bacterial growth. Maximum inhibition (84.38%) was observed at 300 µg/mL. The RSM model showed that all three factors significantly influenced QS inhibition, with extract concentration exerting the strongest effect, followed by bacterial density and dosing frequency. The quadratic model demonstrated excellent fit ($p < 0.0001$), confirming predictive reliability. GC–MS analysis identified the major active constituent as 1,2-Benzenedicarboxylic acid, dioctyl ester.

Conclusion: *Illicium verum* aqueous extract possesses potent quorum-quenching and anti-virulence activity against key QS-regulated phenotypes of *C. violaceum* MCC 2290 and *P. aeruginosa* JB9. Optimization through Box–Behnken design highlights the importance of extract concentration and dosing strategy in maximizing QQ efficacy. The findings support the potential of *I. verum* as a promising non-bactericidal anti-virulence agent.

Keywords: Anti-virulence activity, Biofilm inhibition, Illicium verum, Pseudomonas aeruginosa, Quorum quenching.

How to cite this article: Aswathy JK, Poongothai M. Effect of Illicium verum extract upon quorum quenching mediated bacterial attenuation. Int J Drug Deliv Technol. 2026;16(7s): 543-552; DOI: 10.25258/ijddt.16.7s.57

1. Introduction

The release of virulence factors, pigment creation, motility change, and biofilm formation are some of the coordinated behaviors governed by bacteria's communication mechanism, known as quantum sensing (QS) [1,2]. In AHL bacteria (Gram-negative) and AI-peptide bacteria (Gram-positive), there are tiny diffusible signalling molecules, which comprise the population of bacteria, to detect their numbers, and when the population reaches a critical level of density, bacteria will activate gene expression. Given that these QS-regulated traits are critical for bacterial pathogenicity but not for growth, the disruption of QS—termed quorum quenching (QQ)—has arisen as a promising antivirulence strategy capable of undermining pathogenic behavior without imposing the selection pressure linked to traditional antibiotics [3,4]. Given that antimicrobial resistance (AMR) continues to pose a significant global health problem, there is growing interest in non-bactericidal treatment options that mitigate virulence and diminish illness severity without fostering the development of resistance [5,6]. International surveillance agencies consistently report rising mortality linked to multidrug-resistant infections, highlighting the urgency of exploring

QS-targeted antivirulence approaches that can complement or enhance current antimicrobial use [7,8].

Illicium verum (star anise) is a widely consumed culinary spice with a long history of medicinal use, known for its essential oil composition rich in phenylpropanoids and terpenoids, particularly trans-anethole. Recent phytochemical investigations indicate that the fruit contains diverse aromatic compounds and phenolic acids that exhibit antibacterial, antibiofilm, and quorum-quenching properties [9–11]. These ingredients have shown the capacity to disrupt bacterial signaling, alter adhesion, and inhibit biofilm development. Plant-derived QQ agents are believed to act through varied mechanisms, including enzymatic degradation or modification of signaling molecules, competitive receptor antagonism, inhibition of signal biosynthesis, or indirect suppression of QS through interference with surface attachment and extracellular matrix production [12,13]. Because these compounds function at sub-inhibitory concentrations, their anti-QS effects are often achieved without impairing bacterial viability, further supporting their potential use as antivirulence therapeutics. Despite growing interest, much of the existing research remains preliminary, relying on crude plant extracts, limited bacterial models, and phenotypic QS assessments without detailed characterization of the active constituents or optimization of therapeutic variables [14,15].

Given these gaps, systematic evaluation of *I. verum* using well-defined QS model organisms provides an opportunity to better understand its quorum-quenching potential and identify key factors that influence its efficacy. *Chromobacterium violaceum* MCC 2290 serves as a sensitive violacein-producing reporter strain whose pigment production is directly regulated by AHL-mediated QS pathways, making it a reliable indicator for detecting QS inhibition. *Pseudomonas aeruginosa* JB9 is primarily like a pathogen that possesses clinical relevance due to its development of biofilms and pigments. Furthermore, *Pseudomonas aeruginosa* JB9 can be used to assess how effective an anti-virulence treatment will be against a medically important bacterium. A crucial aspect in optimizing the interaction between the different variables surrounding bacterial density, extract concentration, and dosage frequency is really going to depend on how these different variables influence the degree of QS inhibition. The use of RSM, specifically the Box-Behnken methodology, provides a statistically sound methodology for modeling and optimally determining biological responses, all while minimizing the amount of experimental effort required.

Consequently, this study set out to discover what active components were present by GC-MS profiling, how aqueous *Illicium verum* extract quenched the quorum of *C. violaceum* MCC 2290 and P. aeruginosa JB9, and how to optimize critical operational variables using a Box-Behnken RSM approach. By integrating phytochemical analysis, phenotypic screening, and statistical modeling, this study aims to establish the anti-virulence potential of *I. verum* and its suitability as a non-bactericidal, plant-derived quorum-quenching agenty.

2. Methodology

2.1 Collection of Plant Material and Preparation of Aqueous Extract

Illicium verum fruits were obtained from a registered herbal source and verified by the Department of Botany, where a voucher specimen was archived. The fruits were rinsed, let to dry in the shade for a week, and then ground into a powder. We prepared an aqueous extraction with 100 g of fruit powder in 500 mL of distilled water, boiling for 45 minutes. The resulting liquid extraction was filtered through No. 1 Whatman Paper and muslin. To get the crude aqueous fruit extract, the filtrate was heated in a water bath to 50 °C, drained, and stored at 4 °C for further testing.

2.2 Phytochemical Screening and GC–MS Characterisation

Preliminary qualitative phytochemical tests for the identification of tannins, phenolics, flavonoids, terpenoids and saponins in an aqueous extract were carried out and results indicated that all five classes were present. Using the violacein inhibitory activity of the crude extract originally separated by column chromatography as the basis for determining which fraction from this separation was the most biologically active, the major bioactive compounds responsible for quorum being quenched from being able to quench through GC–MS analysis have been identified from this purified biologically active fraction.

2.3 Bacterial Strains and Culture Conditions

The following quorum-sensing–dependent strains were used:

- **Chromobacterium violaceum MCC 2290** (violacein-producing biosensor) from NCCS Pune

- **Pseudomonas aeruginosa strain JB9** (biofilm- and pigment-producing strain)

At 30 °C for C. violaceum and 37 °C for P. aeruginosa JB9, sub-cultures were cultivated on LB agar, respectively. To make these cultures, we grew them in LB broth overnight while shaking them at 180 rpm until they were in the middle of the log development phase.

2.4 Determination of MIC by Violacein Pigment Inhibition Disc Method

Minimum inhibitory concentration (MIC) of the Illicium verum aqueous extract was determined using the violacein inhibition disc diffusion technique against *C. violaceum* MCC 2290.Sterile 6 mm discs were impregnated with varying concentrations of the extract (0 µg/ml-300 µg/ml) and placed on LB agar plates inoculated with the reporter strain. To form plates with 30° C for their incubation period is for the duration of 24 Hours. Loss or inhibition of purple-violet-colored pigments around the disc type was determined. The MIC was calculated according to the formula as follows: minimum concentration of felt used that produced a visible inhibition of violacein.

2.5 Violacein Pigment Quorum-Quenching Assay

To determine if the Illicium verum extract has quorum-quenching activity, lawn cultures of C. violaceum MCC 2290 were established and thereafter incubated at 30 °C for 24 h after placing discs containing different concentrations of Illicium verum extract (0 µg/ml-3 µg/ml) on the agar surface. The inhibition of violacein was measured by determining the area where violacein pigment intensity was reduced around each of the discs, indicating that violacein synthesis had been inhibited by AHL group compounds.

2.6 Inhibition of Pyoverdine Production in *Pseudomonas aeruginosa* JB9

Following the modified Bonchi et al., (2015) method, pyoverdine production was evaluated. P. aeruginosa JB9 was grown in LB broth and 0-3.0 mg/mL Illicium verum extract. A 1:10 dilution of each supernatant was generated with Tris-HCl buffer at pH 7.4 after incubating the cultures overnight at 37 °C and centrifuging. At 405 nm, the absorbance was recorded. A drop in absorbance suggested that fewer QS-dependent pigments were being produced.

2.7 Biofilm Inhibition Assay (Modified Djordjevic et al., 2002)

Biofilm inhibition was measured using sterile 96-well microtiter plates.

There were different amounts of Illicium verum extract (ranging from 0 mg/ml to 3 mg/ml), 100 µL of overnight culture of P. aeruginosa JB9, and 200 µL of LB broth in each well. All plates were left to incubate at 37 °C for one full day.

Post incubation:

1. Wells were meticulously rinsed with sterile saline to eliminate non-adherent cells.
2. Adherent biofilm was stained using **0.4% crystal violet** for 10 minutes.
3. Excess stain was removed, and the wells were washed.
4. Crystal violet was solubilized using 95% ethanol.
5. Absorbance was read at **570 nm** for biofilm biomass and **600 nm** for total bacterial growth.

Biofilm inhibition (%) was calculated relative to untreated control.

2.8 Respondent Surface Methodology Using the Design of Box-Behnken

The three independent variables were optimized for quorum quenching using a Box-Behnken response surface design.

- **X_1 — Initial bacterial density**
- **X_2 — Extract concentration**
- **X_3 — Rate of administration**

Both the analysis of variance and sequential model sum-of-squares were utilized in evaluating model fit. The estimated quadratic model demonstrated the greatest F-value (450.42), with $p < 0.0001$ demonstrating that the quadratic model provided the most accurate prediction of quorum-quenching efficiency.

2.9 Statistical Analysis

Every test was carried out three times, and the results were presented as the mean plus or minus the standard deviation (SD). A combination of SPSS 25 and GraphPad Prism 9.0 was used to conduct the statistical analysis. In order to maximize the elements that affect quorum quenching activity using the Box-Behnken approach in Response Surface Modeling (RSM). Significance was determined by statistical analysis when $p < 0.05$.

2.10 Ethical and Biosafety Considerations

All microbiological procedures were carried out in compliance with institutional biosafety regulations under Biosafety Level 2 (BSL-2) conditions. The research did not include human or animal participants; hence, ethical

approval was unnecessary. All reagents and waste materials were autoclaved prior to disposal to guarantee environmental safety.

3. Result

3.1 Evaluation and quantification of quorum-shutting processes

Aqueous fruit extract of Illicium verum was screened for their quorum quenching activity and it showed positive result. The application of a quorum sensing inhibition assay plate (as illustrated in Figure 4.1) produced a turbid, non-pigmented violacein halo that was created at the site of application and proved the ability of the positive control to demonstrate antagonism against this organism.

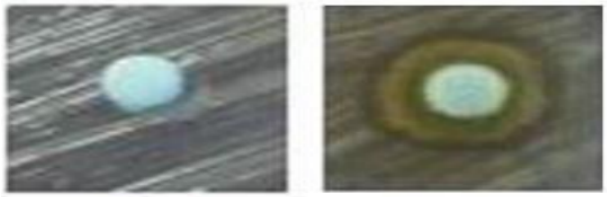


Fig 4.1 QSI plate assay of positive sample

Table 1: Levels of variables and experimental range

Factor	Code 1	Code 0	Code –1
The density of bacteria (CFU)	4.11×10^8	2.74×10^8	1.37×10^8
Concentration of drug (µg/ml)	300	200	100
Administration frequency	3	2	1

The measurement of quorum quenching activity indicated that the extract's efficacy was directly related to its concentration, as larger concentrations exhibited much greater quorum quenching activity (Figure 4.3). The IV extract sample had the most potent quorum quenching action, with 84.38% inhibition of quorum sensing at a dosage of 300 µg/ml; conversely, concentrations below 50 µg/ml showed no quorum quenching activity. The findings of the broth test corroborated those from the plate assay of superior source sample IV extract, as shown by a noticeable decrease in pigmentation, further validated by spectrophotometric analysis.

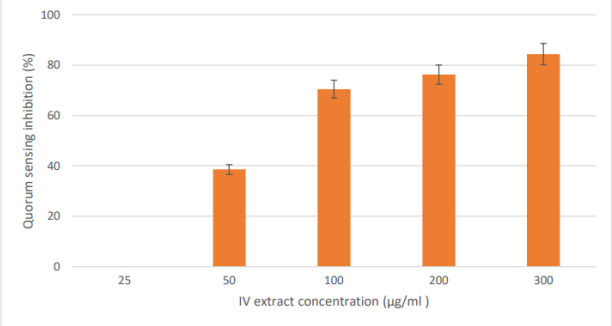


Fig.4.2: Quorum sensing broth assay with IV extract

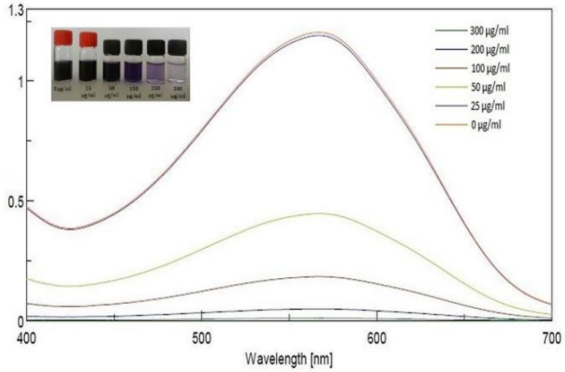


Figure 4.3 Spectrophotometric analysis of pigment reduction in different concentrations.

3.2 Characterization of bioactive extract

The purification of chromatographic fractions, determined by activity, enabled the efficient extraction of bioactive compounds from three positive samples. The positive portion was concentrated, recrystallized, and examined using gas chromatography and mass spectrometry, followed by examination against the NIST library. The primary component was identified as dioctyl phthalate. In order to elute the samples from the IV extract, solvents such as hexane, petroleum ether, ethyl acetate, methanol and water were used. The samples isolated from the IV extracts were separated into 20 possible isolates through chromatography into 5 different fractions. Dioctyl phthalate was identified as a quenching agent for all of the methanol fractions with a mean half-life of 22.891 minutes.

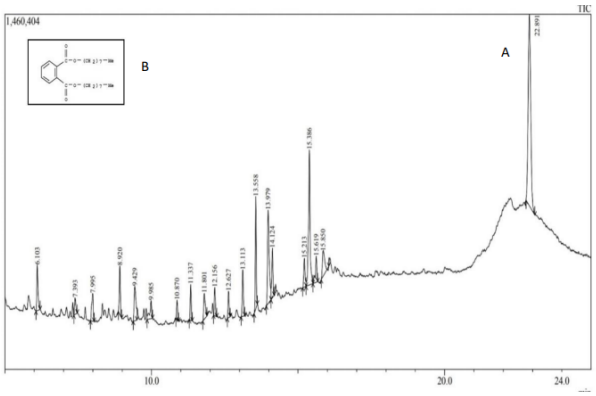


Figure 4.4: A: Chromatogram of the purified bioactive molecule derived from IV extract; B: Structure of the lead ingredient.

Table 2: The design matrix of Box-Behnken

Run	A: Density of Bacterial	B: Concentration of Drug	C: The frequency of administration
1	4.11 × 10^8	200	3
2	1.37 × 10^8	300	2
3	1.37 × 10^8	200	1
4	2.74 × 10^8	200	1
5	1.37 × 10^8	100	2
6	2.74 × 10^8	200	2
7	2.74 × 10^8	300	1
8	2.74 × 10^8	100	3
9	4.11 × 10^8	200	1
10	4.11 × 10^8	100	2
11	4.11 × 10^8	300	2
12	2.74 × 10^8	200	1
13	1.37 × 10^8	200	3
14	2.74 × 10^8	200	3
15	2.74 × 10^8	300	3
16	2.74 × 10^8	100	1
17	2.74 × 10^8	200	2

3.3 Quorum sensing hostility and attenuation of Chromobacterium violaceum

The antagonistic effect of IV extracts on quorum sensing and the attenuation of the wild strain of Chromobacterium violaceum was deemed promising. The IV extract decreased the biofilm development of Chromobacterium violaceum to 16.27% and lowered pigment production to 19.71%. Interestingly, quorum sensing inhibition was directly proportional to that of concentration of extract administrated (Figure 4.5, Figure 4.6).From the mentioned analysis it was confirmed that sample IV extract exhibited maximum quorum quenching activity in down regulating virulence factors of the bacterial strain such as biofilm formation and chromogenesis.

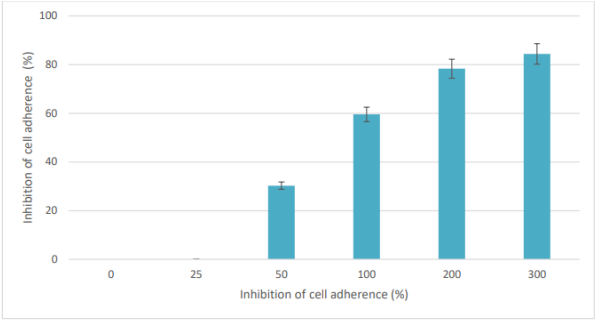


Figure 4.5: Effect of IV extracts upon cell adherence of Chromobacterium violaceum

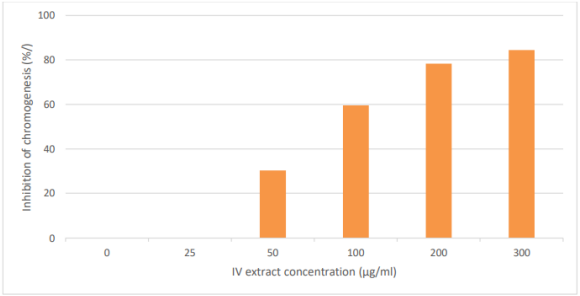


Figure 4.6: Effect of IV extracts upon chromogenesis of Chromobacterium violaceum

3.4 Impact of IV extract on bacterial growth and in vitro attenuation

The IV extract exhibited significant antagonistic effects on quorum sensing-regulated virulent traits, including

chromogenesis and biofilm development, in the Pseudomonas aeruginosa strain. The adhesion of bacterial cells decreased by 88% when exposed to a 3 mg/ml concentration of root extract (Figure 4.7).The chromogenic characteristic of the strain was clearly downregulated, as pigment synthesis decreased to 7% upon treatment with a 3 mg/ml dose of the antagonist. The extract's ability to quench quorums was concentration-dependent; nonetheless, there was no discernible difference in bacterial biomass between the treated and untreated groups, suggesting that intravenous extract had no effect on bacterial growth.

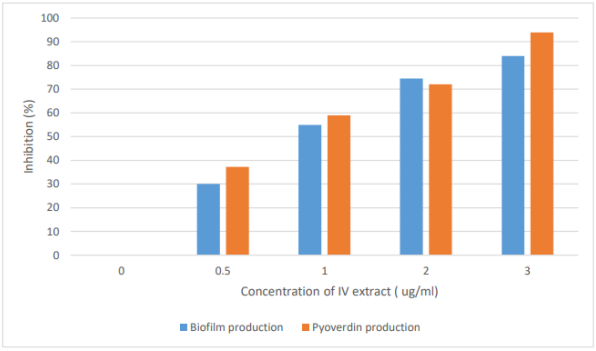


Figure 4.7: Effect of IV extract on phenotypical characters of Pseudomonas aeruginosa

3.5 Evaluation of the model's suitability for quorum quenching activities

Table 3 shows that the quadratic model was the most accurate model to fit the experimental data when examining the different scores of the model as they relate to their sequential model sums of squares.

Table 3: Sum of Squares for Models in Sequence

Source	Mean Square	Sum of Squares	df	p-value	F Value	Remarks
Residual	0.75	3.01	4	—	—	—
Linear vs Mean	2930.38	8791.15	3	< 0.0001	44.04	—
2FI vs Linear	62.19	186.58	3	0.4675	0.92	—
Quadratic vs 2FI	225.00	675.01	3	< 0.0001	450.42	Suggested
Cubic vs Quadratic	0.16	0.49	3	0.8805	0.22	Aliased
Mean vs Total	47381.66	47381.66	1	—	—	—
Total	3355.17	57037.91	17	—	—	—

The low p-value (as shown by the FI values for lack of fit, which are 0.22 and 0.88, respectively) and high F-value (450.42) suggest that the model is suitable for the study of quorum quenching.

Table 4: The Quadratic Response Surface Model using ANOVA

Source	Mean Square	Sum of Squares	df	p-value	F Value	Remarks
C – Frequency of administration	453.46	453.46	1	< 0.0001	907.74	—
B – Drug concentration	2906.27	2906.27	1	< 0.0001	5817.87	—
A – Bacterial density	5431.43	5431.43	1	< 0.0001	10872.81	—
Model	1072.53	9652.74	9	< 0.0001	2147.02	significant
AC	23.33	23.33	1	0.0002	46.70	—
BC	2.09	2.09	1	0.0802	4.18	—
AB	161.16	161.16	1	< 0.0001	322.62	—
A^2	614.73	614.73	1	< 0.0001	1230.59	—
B^2	35.52	35.52	1	< 0.0001	71.11	—
C^2	52.38	52.38	1	< 0.0001	104.85	—

When it came to quorum quenching activity, the coded variables showed that there were substantial interaction effects between the independent factors. This is seen in the formula below. When describing the amount of activity that causes quorum quenching or deterioration, the variable R1 is employed.

$$R_1 = 50.13 - 26.06 * A + 19.06 * B + 7.53 * C + 6.35 * AB + 2.42 * AC - 0.72 * BC + 12.08 * A^2 - 2.90 * B^2 - 3.53 * C^2.$$

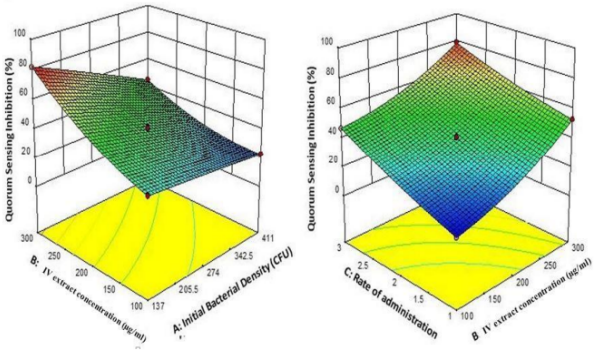
The results of the model fit calculations (leverage, studentized residuals, internally/externally available DFFITS, Cook's distance, etc.) are summarized in Table 5. Correspondingly, based on the above evidence, we can conclude that there is a satisfactory fit between the model and the data collected.

Table 5: Diagnostic case statistics

Execution Sequence	**Current Value**	**Expected Value**	**Residual**	**Utilize**	**Residual Internally Studentized**	**Residual Externally Studentized**	**Cook's Distance**	**Impact on Estimated Value**
1	42.85	42.58	0.27	0.750	0.771	0.746	0.178	1.293
2	98.38	98.08	0.30	0.750	0.845	0.826	0.214	1.431
3	79.36	79.63	-0.27	0.750	-0.771	-0.746	0.178	-1.293
4	50.17	50.13	0.036	0.200	0.057	0.053	0.000	0.026
5	72.83	72.66	0.17	0.750	0.492	0.463	0.073	0.802
6	49.55	50.13	-0.58	0.200	-0.924	-0.913	0.021	-0.456
7	55.93	55.96	-0.026	0.750	-0.074	-0.069	0.002	-0.119
8	32.92	32.89	0.026	0.750	0.074	0.069	0.002	0.119
9	22.89	22.69	0.20	0.750	0.566	0.536	0.096	0.929
10	7.55	7.85	-0.30	0.750	-0.845	-0.826	0.214	-1.431
11	58.49	58.66	-0.17	0.750	-0.492	-0.463	0.073	-0.802
12	49.01	50.13	-1.12	0.200	-1.778	-2.223	0.079	-1.111
13	89.66	89.86	-0.20	0.750	-0.566	-0.536	0.096	-0.929
14	51.02	50.13	0.89	0.200	1.402	1.530	0.049	0.765
15	69.47	69.57	-0.099	0.750	-0.279	-0.260	0.023	-0.451
16	16.49	16.39	0.099	0.750	0.279	0.260	0.023	0.451
17	50.92	50.13	0.79	0.200	1.243	1.304	0.039	0.652

3.6 Parameters influencing the suppression of quorum sensing by response surface modeling

Figure 4.8 A shows how the quorum quenching impact of a bi-daily dose of antibiotics is affected by the starting bacterial density and antibiotic concentration. Optimal activity was achieved at the lowest bacterial density, and an increase in drug concentration led to a heightened reaction, indicating that the drug response is directly proportional to concentration. Figure 4.8 B illustrates the impact of initial bacterial density and injection frequency, with the antagonist concentration maintained at a constant level of 200 µg/ml. Although the reaction was shown to be more intense with more administrations, it was also found that more frequent administrations may decrease quorum sensing, which is caused by more bacteria. Shown in Figure 4.8 C is the effect of injection frequency and medication concentration on a bacterial density of 274 x 10^6 CFU. Compared to fractionation, the graphic showed that a single large dose is more effective. The quorum quenching rate was 55.93% with a 300 µg/ml drug given once every 24 hours, however the quorum sensing suppression rate was only 32.92% with a 100 µg/ml dose given three times during the same timeframe.



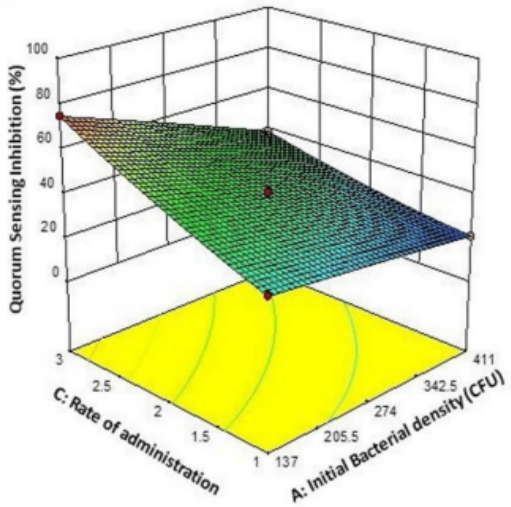


Figure 4.8 A: Three-dimensional surface graphic showing how drug concentration and bacterial population affect the suppression of quorum sensing. 4.8 B: Impact of medication concentration and dose frequency on quorum sensing inhibition shown in a 3D surface diagram. 4.8 C: A three-dimensional surface graphic is used to highlight how the suppression of quorum sensing is affected by bacterial density and dosage frequency.

4. Discussion

The current study aimed at determining the quorum-quenching and anti-virulence potential of Illicium verum aqueous extract against two important QS-regulated bacterial systems, Chromobacterium violaceum MCC 2290 and Pseudomonas aeruginosa JB9. The results herein confirm that I. verum is a strong QS inhibitor, which depressed pigment production, biofilm formation, and other virulence-associated phenotypes without affecting bacterial growth. This agrees with the recent global interest in antivirulence strategies that target bacterial communication rather than viability, thereby reducing the selective pressure that accelerates antimicrobial resistance [16,17]. Plant-derived quorum-quenching compounds have gained prominence due to the fact that such an approach modulates pathogenicity without disrupting bacterial growth, and findings of the present study further reinforce this emerging paradigm.

Reports indicate that I. verum has a large number of phytochemicals including flavonoids, phenylpropanoids, terpenoids, and phenolic acids [18]. Previous studies have documented antimicrobial, antibiofilm, and antioxidant activities. Trans-anethole is one of the major compounds of this species that have been shown to be the

active ingredient in all of these activities [18,19]. In the present study, the aqueous extract demonstrated strong violacein inhibition in C. violaceum MCC 2290, a sensitive reporter organism whose pigment production is directly controlled by AHL-mediated QS circuits. This confirms that I. verum contains components which are capable of interfering with AHL-regulated pathways. Our results corroborate previous reports where botanical extracts, such as those from Impatiens balsamina and Apium graveolens, were shown to downregulate QS-controlled phenotypes [20,21]. We observed significant attenuation of QS outputs at sub-inhibitory concentrations, substantiating the idea that plant compounds may cause disruption of signaling instead of any bactericidal stress.

The extract also caused marked inhibition of pyoverdine production and biofilm formation in P. aeruginosa JB9, a clinically relevant, highly virulent pathogen known for its biofilm-driven persistence and resistance. These observations are in parallel with earlier reports where botanical extracts impaired QS-mediated biofilm maturation and pigment production in Pseudomonas species [22,23]. As P. aeruginosa heavily relies on hierarchical QS systems for virulence, interfering with them may considerably dampen its pathogenic capabilities. Noticeably, the extract did not affect bacterial biomass, clearly showing a non-bactericidal antivirulence mechanism. Similar observations have been described for plant-derived QS inhibitors like ajoene, cinnamaldehyde, and Iberin, which suppress virulence but do not affect growth [23,24]. This is of immense clinical interest since antivirulence agents have a lesser chance of developing resistance compared to bactericidal antibiotics.

The main strength of this study is the application of RSM, through the Box–Behnken design, in understanding the combined effects of bacterial density, extract concentration, and dosing frequency on quorum-quenching activity. Its model exhibited significant quadratic fit predictive capacity, at $p < 0.0001$, and interaction of variables showing important biological trends. Extract concentration emerged as the most influential variable, which agreed with previous studies indicating that phytochemicals inhibited QS-regulated traits in a dose-dependent manner [16,19]. The higher concentrations used, up to 300 µg/mL, demonstrated the highest decrease in pigment production and biofilm formation, reflecting a typical concentration–response behavior usually exhibited by botanical antivirulence assays.

Bacterial density was another important factor. At higher cell densities (4.11×10^8 CFU), bacteria tend to produce more signaling molecules; thus, more concentrated extract or a higher frequency of dosing is required to achieve equivalent levels of inhibition. That was in agreement with the idea that QS activity increases with the number of bacterial cells, and thus high-density conditions may result in diminished success of QS interference. The RSM surface plots indicated the same trend, showing clear evidence for optimal inhibition at high extract concentration and moderate to low bacterial density.

Frequency of administration, although less influential than concentration, still contributed positively. This indicates that continued presence of effective antagonist concentrations in the medium improves overall cumulative inhibitory effect. Surprisingly, this study further showed that a single high dose of 300 µg/mL could be more inhibitive than multiple small doses within the same 24-hour period. This confirms that the initial saturation of the signaling pathways might elicit a greater level of inhibition compared to fractionated exposure. The pharmaceutical optimization research has indicated these characteristics as well. In general, the predominant active ingredient in the bioactive fraction is dioctyl phthalate as determined through activity-guided fractionation and GC-MS techniques. The principles of receptor saturation kinetics, or physiological laws of saturation for receptors, suggest that administering higher doses initially will often yield a better therapeutic outcome than administering lower doses repetitively. [25,26]. Traditionally considered a plasticizer, this molecule has, however, kept surfacing in various plant extracts and has shown antimicrobial and QS-inhibiting potential. Its detection in I. verum agrees with prior phytochemical analyses which showed similar compound profiles and suggests that this may contribute to QS-inhibiting activity. Since crude extracts are mixtures of several components, synergistic interaction among phytochemicals cannot be excluded. Further purification and mechanistic studies will be needed to pinpoint exact molecular targets. Taken together, this study reinforces the potential of I. verum aqueous extract as a natural, nontoxic, nonbactericidal antivirulence agent that has the ability to attenuate QS-controlled pathogenic traits in both environmental and clinically

significant bacteria. The ability to inhibit pigment production, biofilm formation, and adherence-without affecting bacterial viability-positions it as a strong candidate for adjunct therapeutic applications, food preservation, or infection control strategies. In addition, Box-Behnken optimization will provide a scientific background for determining the effective dosage strategy required for maximum QS inhibition in real-life applications.

5. Conclusion

All bacterial virulence mechanisms are regulated through quorum-sensing (QS) signaling; therefore, disrupting QS can effectively suppress virulence, reduce persistence, and enhance natural immunological clearance. In this study, *Illicium verum* (Star anise) aqueous extract was screened to evaluate its quorum-quenching potential, mechanism of action, and active lead compound. Initial QS inhibition was assessed using the indicator strain *Chromobacterium violaceum*, which showed marked reduction in violacein pigment production. Using activity-guided purification, gas chromatography-mass spectrometry, and NIST library analysis, it was possible to identified as the major bioactive component. The main factors influencing inhibition of QS—density of bacteria, concentration of extract, frequency of injection—were then altered through Response Surface Methodology (RSM) using Box–Behnken designs for Bacteria and Extract. Model adequacy was confirmed through ANOVA and Cook's distance diagnostics. The highest inhibitory activity was observed with 300 µg/mL administered thrice daily. Importantly, the extract exhibited no bactericidal effect, indicating that virulence reduction resulted from QS signal disruption rather than growth inhibition. In vitro assays against *Pseudomonas aeruginosa* further demonstrated significant downregulation of QS-regulated phenotypes, including pigment production and biofilm formation. Overall, *Illicium verum*, a widely used spice, exhibits strong quorum-sensing inhibitory activity with promising anti-virulence potential.