

Modulation of *Pseudomonas aeruginosa* Quorum Sensing and Biofilm Formation by Quercetin Extracted from *Pleurotus ostreatus*

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Background: *Pseudomonas aeruginosa* relies on quorum sensing (QS) to regulate virulence, biofilm formation, and antimicrobial tolerance, making QS inhibition (QSI) an attractive antivirulence strategy. Quercetin is a known QSI-active flavonoid, but its potential as a mushroom-derived therapeutic agent remains underexplored.

Methods: Quercetin was extracted from *P. ostreatus* and evaluated alongside purified quercetin using GFP-based QS reporter assays (lasB-gfp and pqsA-gfp), biofilm assays, protease activity assays, and molecular docking against LasR, PqsR, and PqsE.

Results: Both treatments significantly reduced lasB- and pqsA-driven GFP reporter activity at sub-inhibitory concentrations (10–100 µM) that did not affect bacterial growth or viability. Inhibition was dose-dependent and more pronounced for the PQS system, accompanied by substantial reductions in extracellular protease activity and strong suppression of biofilm formation and partial dispersal of established biofilms. Molecular docking revealed favourable binding of quercetin to the QS regulators LasR and PqsR, with high-affinity interaction in the PqsR co-inducer pocket, while binding to PqsE was weak and non-specific.

Conclusion: These mechanistic insights align with the observed preferential PQS inhibition. The *P. ostreatus* quercetin-extract closely matched the potency of pure quercetin across all assays, confirming effective extraction and stability. This positions mushroom-derived quercetin as a sustainable, natural QSI capable of attenuating key virulence pathways in *P. aeruginosa*. This work supports the development of quercetin-rich *P. ostreatus* extracts as promising adjunctive therapies for managing chronic, biofilm-associated infections.

Keywords: *Pseudomonas aeruginosa*, quorum sensing inhibition, quercetin, *Pleurotus ostreatus* (oyster mushroom), anti-virulence therapy

Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that poses a serious threat to immunocompromised individuals and patients with chronic infections such as cystic fibrosis, burn wounds, and hospital-acquired pneumonia.^{1,2} Its persistence and resistance are largely mediated by quorum sensing (QS), a cell-density dependent communication system that regulates the expression of multiple virulence factors, biofilm maturation, and antimicrobial tolerance.³ Because QS does not directly affect bacterial viability, strategies that interfere with QS—termed quorum sensing inhibition (QSI)—have gained considerable attention as promising anti-virulence approaches that may reduce pathogenicity while imposing lower selective pressure for resistance development compared to conventional antibiotics.^{4,5} Flavonoids, a diverse class of plant-derived secondary metabolites,⁶ have emerged as potential QS inhibitors.^{7,8} Quercetin, in particular, has demonstrated inhibitory effects on QS-regulated pathways and biofilm formation in *P. aeruginosa*.⁹ However, the study has focused on purified quercetin from a synthetic source (≥95% purity, Sigma-Aldrich) dissolved in 10% dimethyl sulfoxide (DMSO), while comparatively little is known about natural quercetin. A recent study demonstrated that quercetin, derived from the edible fungi *Pleurotus ostreatus* (*P. ostreatus*), is a highly potent, natural antibacterial agent against *Neisseria gonorrhoeae*, effective at very low concentrations. Its excellent

computational drug-likeness profile and known safety make it a prime candidate for further development into a therapeutic option, especially to combat drug-resistant gonorrhoea, potentially on its own or in combination with other drugs.¹⁰ Previously, flavonoids have emerged as a rich source of natural quorum sensing inhibitors (QSIs), with several studies demonstrating their ability to interfere with Las, Rhl, and PQS pathways in *P. aeruginosa*.^{11,12} Quercetin, in particular, has been shown to allosterically inhibit LasR and attenuate QS-dependent virulence, motility, and biofilm formation.^{9,13} Recent advances in GFP-based QS reporter systems—such as *lasB*-gfp and *pqsA*-gfp—enable sensitive quantification of QSI activity and are now widely applied in high-throughput anti-virulence screening.^{14,15} The oyster mushroom, *P. ostreatus*, is widely cultivated, nutritionally rich, and an accessible natural source of bioactive compounds, including phenolics with antioxidant and antimicrobial activities.¹⁶ Exploring *P. ostreatus*-derived quercetin as a QS modulator may therefore provide dual benefits of leveraging an edible, sustainable resource and advancing the search for natural anti-virulence agents. In this study, we investigated the ability of quercetin extracted from *P. ostreatus* to modulate QS in *P. aeruginosa* PAO1. Using a GFP-based QS reporter system in casamino acid (CAA) medium, we assessed the effects of sub-inhibitory concentrations of quercetin on bacterial growth, QS-regulated fluorescence, and biofilm formation. Furthermore we investigated the anti-virulence properties of quercetin derived from the mushroom *P. ostreatus*. Notably, this anti-virulence strategy occurs without affecting bacterial viability, highlighting its potential as a novel therapeutic approach. This work underscores the promise of natural flavonoids as QSI and identifies *P. ostreatus* as an alternative source of quercetin.

Materials and Methods

Bacterial Strain and Culture Conditions

Pseudomonas aeruginosa PAO1 (wild type) harbouring a green fluorescent protein (GFP) reporter plasmid under the control of a quorum sensing (QS)-regulated promoter (either *lasB* or *pqsA*) was used for QS inhibition assays.¹⁷ This construct allows for the fluorescence-based quantification of QS activity. Single colonies were inoculated into Lysogeny Broth (LB) and grown overnight at 37 °C with shaking at 200 rpm. The resulting cultures were then washed twice by centrifugation and resuspended in sterile phosphate-buffered saline (PBS) to remove residual media components. Finally, the bacterial cells were diluted to the desired inoculum density in a defined Casamino Acids (CAA) broth medium.¹⁸

The CAA broth medium, composed of 5 g/L Bacto™ casamino acids, 10 mM K₂HPO₄, and 1.5 mM MgSO₄ (pH adjusted to 7.0 and sterile filtered), was selected for its standard use in *P. aeruginosa* QS reporter assays due to its low background fluorescence, which enables highly sensitive detection of GFP signal.

Extraction and Preparation of Quercetin from *Pleurotus ostreatus*

A 1 g portion of powdered *P. ostreatus* extract was transferred into a 50 mL screw-cap plastic tube. Twenty millilitres of 70% methanol (MeOH) containing 0.1% hydrochloric acid (HCl, v/v) was added, and the mixture was agitated at 400 rpm overnight at room temperature. Following incubation, the suspension was centrifuged at 3000 × g for 10 min, and the supernatant was collected. The solid residue was re-extracted twice under identical conditions, each with 10 mL of the extraction solvent. All supernatants were pooled, adjusted to a final volume of 40 mL with extraction solvent, and filtered through a 0.45 µm PTFE membrane filter.

The crude phenolic extract was subjected to acid hydrolysis by adjusting to a final concentration of 2 N HCl, followed by incubation at 85 °C for 1 h. After cooling to room temperature, samples were centrifuged at 3000 × g for 5 min, and the resulting supernatant was filtered again through a 0.45 µm PTFE filter. Phenolic and cinnamic acids were dissolved in distilled water, while flavonoids were dissolved in distilled water containing 1% dimethyl sulfoxide (DMSO). Extract aliquots were stored at −20 °C until further use.^{10,19}

Minimum Inhibitory Concentration (MIC)

To ensure QS-specific effects, MIC of quercetin (standard) and quercetin-extract was determined by CLSI broth microdilution in CAA at 37 °C, 24 h.²⁰ Sub-MIC levels were defined as ≤25% growth inhibition relative to vehicle. Sub-MIC doses (10–100 µM) were used in all QS and biofilm assays.²¹

Growth Curve Analysis

Overnight PAO1 was diluted into CAA to starting $OD_{600} = 0.005$, 200 μ L per well were dispensed in black, clear-bottom 96-well plates with breathable seals. Vehicle (DMSO), quercetin standard (10, 25, 50 μ M), and mushroom-derived quercetin at matched quercetin-equivalent concentrations. Plates were incubated at 37 °C in a multimode plate reader with orbital shaking (linear, 2–3 mm) between reads. The OD_{600} was recorded every 2 h for 12 h. Two biological replicates (independent overnight cultures) were run, each with technical triplicates.²²

For endpoint viability, cultures grown for 24 h under sub-MIC conditions were serially diluted in PBS and plated on LB agar. Plates were incubated at 37 °C for 18–24 h, and colonies were counted to determine viable counts (CFU/mL). Any treatment showing >25% reduction in OD_{600} or CFU/mL relative to vehicle was considered bacteriostatic and excluded from QS-specific interpretation.

Quorum Sensing Fluorescence Readout

For kinetic QS measurements, PAO1 lasB-gfp and pqsA-gfp reporter strains were inoculated into CAA containing vehicle, quercetin, or quercetin-extract (10–100 μ M) and incubated in 96-well plates at 37 °C with shaking as above. Fluorescence was recorded every 2 h using Ex 485 ± 10 nm / Em 520 ± 10 nm (for GFP reporters). Instrument gain was fixed across the experiment. Background (medium + DMSO without cells) was subtracted from all readings. QS activity was expressed as fluorescence / OD_{600} at each time point to correct for biomass.

$$\text{Normalized QS} = \frac{\text{GFP RFU (blank - subtracted)}}{OD_{600} (\text{blank} - \text{subtracted})}$$

The experimental controls include a vehicle control (DMSO, $\leq 0.5\%$), an optional positive QSI control (furanone C-30 at a sub-MIC concentration), and a reporter-free PAO1 strain to confirm the absence of compound autofluorescence bleed-through.²³

Endpoint Inhibition Assays

For dose–response curves, cultures were grown to late exponential/early stationary phase (18–24 h) under the same conditions, and a single endpoint fluorescence and OD_{600} measurement was taken. Normalized GFP (RFU/ OD_{600}) was expressed as percentage reduction relative to the vehicle control for each promoter (lasB and pqsA). These values were used to calculate IC_{50} for QS inhibition.

Data Handling and Primary Outcomes

Primary QS outcomes included the area under the curve (AUC_{0-24h}) of normalized QS signal (RFU/ OD_{600}), peak normalized fluorescence, time-to-peak, and growth AUC to verify sub-MIC behaviour. Any condition showing >25% growth reduction vs. vehicle was flagged and excluded from QS interpretation.

Biofilm Formation and Disruption Assays

Overnight PAO1 was diluted to $OD_{600} = 0.05$ in CAA. Two hundred microlitres were added to flat-bottom, polystyrene 96-well plates with vehicle, quercetin standard, or quercetin-extract (10–100 μ M). Plates were incubated for 24 h at 37 °C, static. Planktonic cells were removed, wells were gently washed ($\times 2$ PBS), stained with 0.1% crystal violet (CV; 15 min), washed ($\times 2$), and air-dried. Bound CV was solubilized with 30% acetic acid and absorbance read at 590 nm. For biofilm disruption, biofilms were first established for 24 h (drug-free), then media were replaced with fresh CAA containing vehicle or quercetin/quercetin-extract (10–100 μ M) for an additional 24 h, followed by CV quantification as above. CV signals were normalized to endpoint OD_{600} of the corresponding wells to account for biomass. Preventive and disruption activities were expressed as percentage reduction vs. vehicle control.

IC_{50} Determination and Statistical Analysis

Dose–response data for lasB-gfp and pqsA-gfp inhibition, biofilm inhibition/disruption, and protease activity were fitted to a four-parameter logistic regression model to estimate half-maximal inhibitory concentrations (IC_{50}). Data were

analysed using non-parametric statistics when assumptions of normality were not met. A Kruskal–Wallis test was used to evaluate overall concentration effects, followed by Mann–Whitney *U*-tests with Bonferroni correction for pairwise comparisons. Mood's median test was applied to compare overall efficacy between pure quercetin and the quercetin-extract across pooled datasets. Significance was accepted at $p < 0.05$.

Molecular Docking

Ligand and Receptor Preparation

Quercetin preparation included protonation at physiological pH, calculation of Gasteiger partial charges, and definition of rotatable bonds, prepared using AutoDockTools. Crystal structures of the *P. aeruginosa* thioesterase PqsE, PqsR (MvfR) co-inducer binding domain, and LasR ligand-binding domain were obtained from the Protein Data Bank. Non-protein molecules (co-crystallized ligands, ions, and water molecules) were removed, polar hydrogens were added, and Kollman charges were assigned. Chain A was used for PqsR and PqsE unless otherwise specified.

Docking Protocol

Docking was performed using AutoDock Vina with the following parameters: grid centre (0, 0, 0), grid box size $20 \times 20 \times 20$ Å, exhaustiveness 8, energy range 5, and up to two output poses per ligand. Binding energies (kcal/mol) were recorded for all poses, and the best-scoring pose was selected for structural analysis.

Analysis and Visualisation

Docked complexes were inspected in PyMOL and UCSF ChimeraX to evaluate binding orientation, hydrogen bonding, and hydrophobic contacts. For LasR–quercetin and PqsR–quercetin complexes, specific interactions with key pocket residues were mapped to rationalise experimental QS inhibition. For PqsE, docking was used to confirm poor pocket engagement and weak binding affinities.

Results

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of quercetin against *P. aeruginosa* PAO1 was determined to be 30.2 µg/mL (100 µM). The *P. ostreatus* quercetin-extract exhibited a comparable MIC, with a quercetin-equivalent value of 38.5 µg/mL (115 µM). At sub-MIC concentrations (10–100 µM), growth inhibition ranged from 5–22% for pure quercetin and 8–24% for the extract, remaining within the sub-inhibitory threshold ($\leq 25\%$) as seen in Table 1.

Sequential Extraction and Fraction Stability

Sequential extraction with 70% methanol containing 0.1% HCl, followed by acid hydrolysis, yielded a phenolic-rich fraction (Table 2). HPLC-DAD analysis confirmed quercetin as the dominant component, with minor peaks corresponding to related flavonoids. Recovery yields were consistent across replicates, averaging $83.4\% \pm 6.1$ ($n = 3$). The quercetin-rich fraction was soluble in water containing 1% DMSO, with $\geq 95\%$ recovery and no precipitation or degradation observed after 72 h at 4 °C. Long-term stability testing demonstrated less than 5% degradation after four weeks at -20 °C.

Table 1 Minimum Inhibitory Concentration and Sub-Inhibitory Effects of Test Compounds

Compound	MIC (µg/mL)	MIC (µM)	Sub-MIC Range Tested (µM)	Growth Inhibition at Sub-MIC (%)
Quercetin (standard)	30.2	100	10 - 50	5 - 22
<i>P. ostreatus</i> Extract	38.5*	115*	10 - 50	8 - 24

Note: *Value expressed as quercetin-equivalent concentration.

Table 2 Recovery and Solubility Parameters of the Quercetin-Rich Fraction from *P. ostreatus*

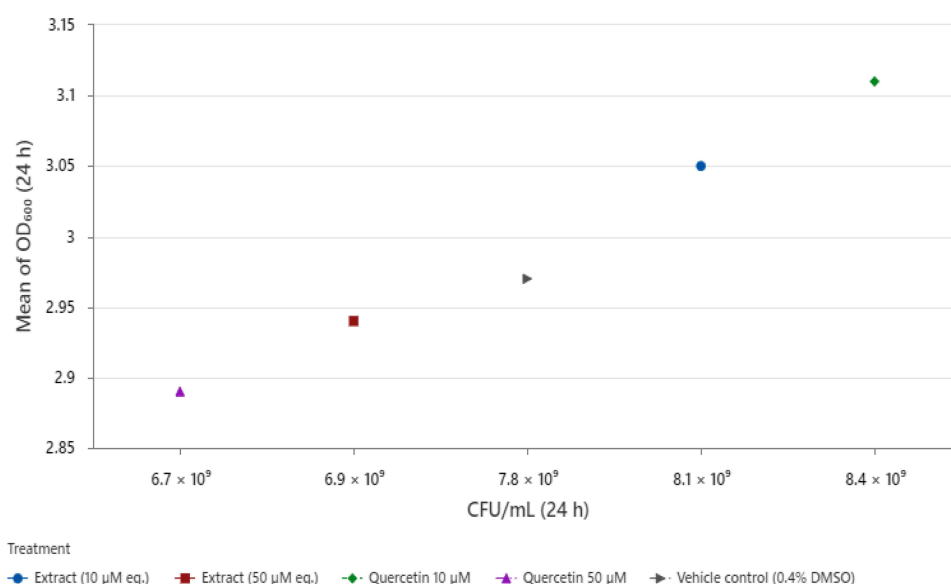
Parameter	Result
Extraction solvent	70% MeOH + 0.1% HCl
Recovery yield (relative)	83.4% $\pm$ 6.1 (mean $\pm$ SD, n = 3)
Major component	Quercetin
Working solubility	Stable in H ₂ O + 1% DMSO ($\geq$ 95% recovery after 72 h)
Stability (-20°C)	<5% degradation over four weeks

Growth and Viability at Sub-MIC

Sub-MIC treatments with quercetin and the mushroom quercetin-extract did not significantly affect bacterial growth or viability over 24 h, illustrated in [Figure 1](#). Optical density measurements at 600 nm (OD_{600}) remained within the range of 2.89–3.11, comparable to the vehicle control (2.97). Viable cell counts were also consistent across treatments, ranging from 6.7×10^9 to 8.4×10^9 CFU/mL. These findings confirm that quercetin and quercetin-extract concentrations ≤ 50 μM are non-inhibitory to overall bacterial proliferation. Cultivation of *P. aeruginosa* PAO1 harbouring the *lasB*-gfp and *pqsA*-gfp transcriptional fusions in CAA broth resulted in strong reporter induction during the late exponential phase, consistent with activation of the *las* and *pqs* QS systems. Treatment with sub-inhibitory concentrations of quercetin and the quercetin-extract significantly attenuated this response in a dose-dependent manner.

[Figure 2](#) (scatterplot matrix) shows the relationship between reporter mean values and variability across treatments. Both quercetin-extract and quercetin treatments caused a downward shift in the mean fluorescence (RFU/ OD_{600}) compared to the vehicle control, indicating reduced QS induction. The effect was concentration-dependent, with the 50 μM treatments showing the most pronounced attenuation. Notably, the *pqsA*-gfp reporter (bottom row, right panel) exhibited a steeper decline than *lasB*-gfp, suggesting that the PQS system was more sensitive to inhibition. The clustering of data points with reduced variance in treated groups further supports reproducible suppression of QS activity.

The bar and cumulative distribution plot for *pqsA*-gfp standard deviation illustrated in [Figure 3](#), presents the progressive reduction in variability across treatments. The vehicle control exhibited the highest spread, consistent with

**Figure 1** Growth (OD_{600}) and viable counts after 24 h treatment.

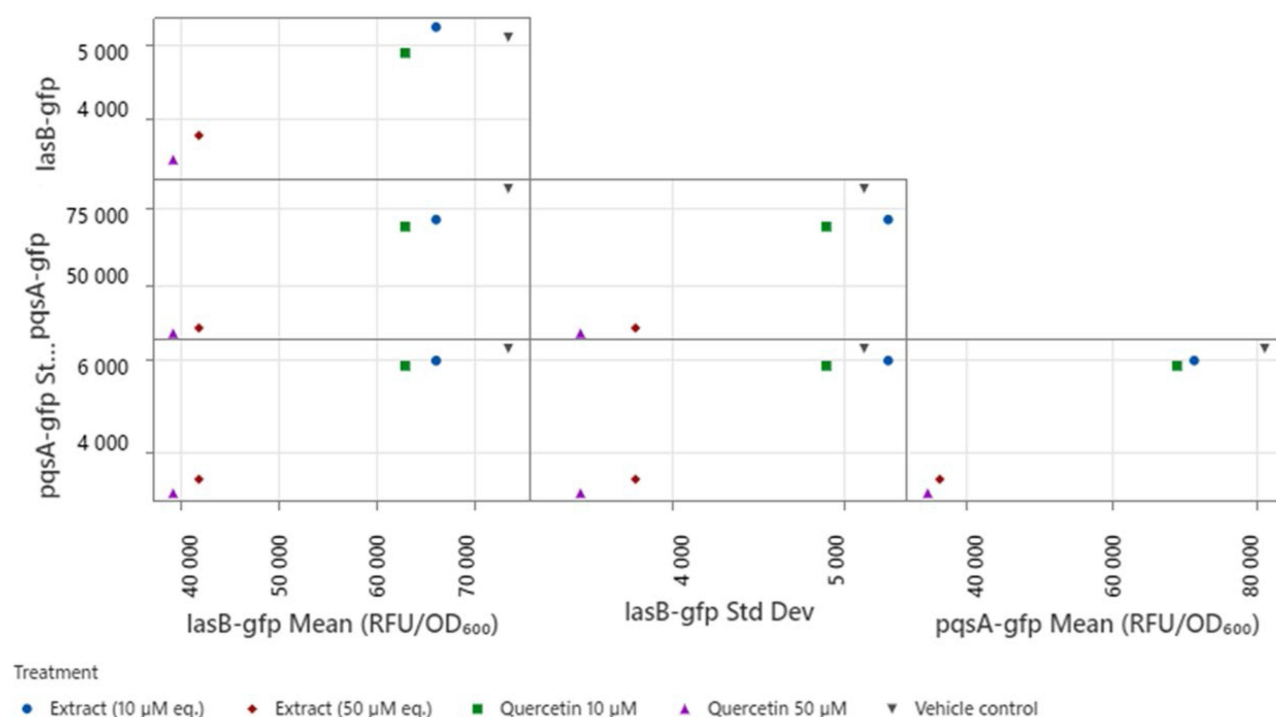


Figure 2 Scatterplot matrix of QS reporter activity in *Pseudomonas aeruginosa* PAO1. Mean fluorescence (RFU/OD₆₀₀) and standard deviation 4 values are shown for *lasB*-gfp and *pqsA*-gfp reporter strains across treatments.

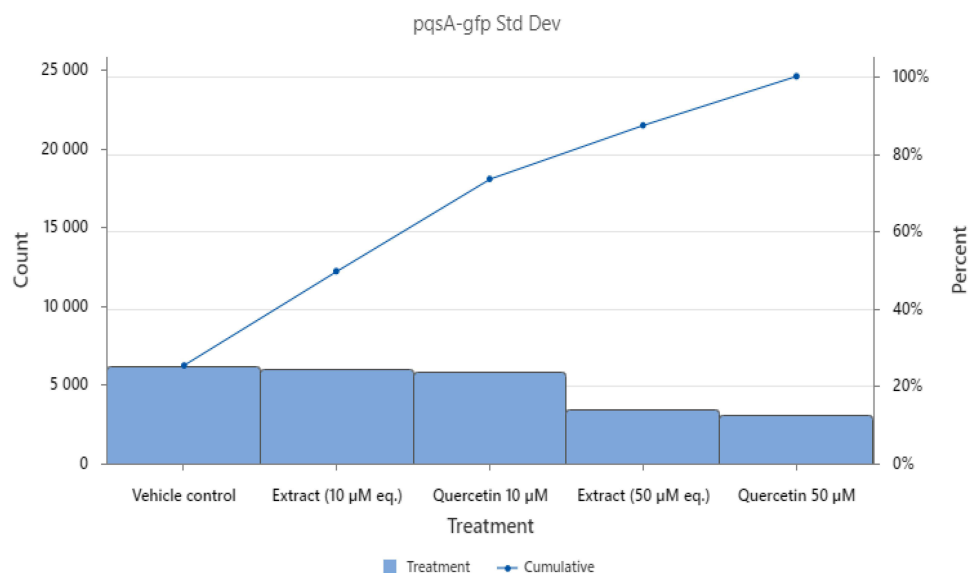


Figure 3 Effect of quercetin and *Pleurotus ostreatus* quercetin-extract on *pqsA*-gfp variability.

strong and heterogeneous QS activation. Treatments with quercetin-extract and quercetin reduced both the absolute standard deviation and cumulative contribution, with 50 µM treatments achieving the lowest residual variance. This pattern demonstrates consistent suppression of PQS signalling at higher doses. Figure 4 presents a similar but less pronounced trend. While both quercetin-extract and quercetin treatments reduced variability relative to the control, the magnitude of suppression was smaller than observed for the *pqs* system, indicating partial but measurable inhibition of *las*-regulated expression.

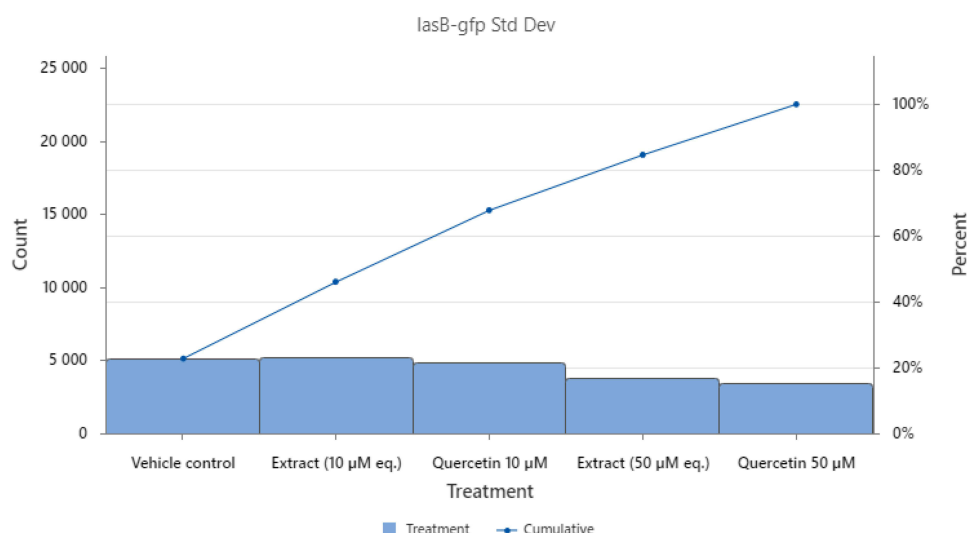


Figure 4 Effect of quercetin and *Pleurotus ostreatus* extract on *lasB-gfp* variability.

Together, these data confirm that both the *P. ostreatus* quercetin-extract and quercetin effectively attenuate QS in *P. aeruginosa*, with preferential inhibition of the PQS pathway. At the highest tested concentration (50 µM), the extract-mediated inhibition was statistically indistinguishable from that of pure quercetin, indicating that quercetin is a major contributor to the observed activity.

Quorum Sensing in a Dose-Dependent Manner

Fluorescence reporter assays revealed that sub-MIC levels of both standard quercetin and the *P. ostreatus*-derived quercetin-extract (quercetin-extract) significantly reduced QS-regulated transcription in *P. aeruginosa* PAO1. Normalized GFP expression (RFU/OD600) was suppressed in a concentration-dependent manner across both the *las* and *pqs* systems. At 10 µM, quercetin reduced *lasB-gfp* activity by 14.3% ($p < 0.05$) and *pqsA-gfp* activity by 21.7% ($p < 0.01$) relative to the vehicle control, details shown in Table 3. The inhibitory effect intensified at higher concentrations,

Table 3 Effect of Quercetin and Quercetin-Extract on QS-Dependent Reporter Activity in *P. aeruginosa* PAO1

Treatment (µM)	<i>lasB-gfp</i> (RFU/OD600)	Significance	<i>pqsA-gfp</i> (RFU/OD600)	Significance
Vehicle (0 µM)	0.0	–	0.0	–
Quercetin – 10 µM	14.3	$p < 0.05$	21.7	$p < 0.01$
Quercetin – 25 µM	27.5	$p < 0.01$	39.4	$p < 0.001$
Quercetin – 50 µM	41.2	$p < 0.001$	62.9	$p < 0.001$
Quercetin – 75 µM	55.8	$p < 0.001$	74.6	$p < 0.001$
Quercetin – 100 µM	68.1	$p < 0.001$	83.2	$p < 0.001$
Quercetin-extract – 10 µM	12.6	$p < 0.05$	19.8	$p < 0.01$
Quercetin-extract – 25 µM	25.9	$p < 0.01$	36.7	$p < 0.001$
Quercetin-extract – 50 µM	39.5	$p < 0.001$	59.8	$p < 0.001$
Quercetin-extract – 75 µM	52.2	$p < 0.001$	71.3	$p < 0.001$
Quercetin-extract – 100 µM	65.4	$p < 0.001$	80.7	$p < 0.001$

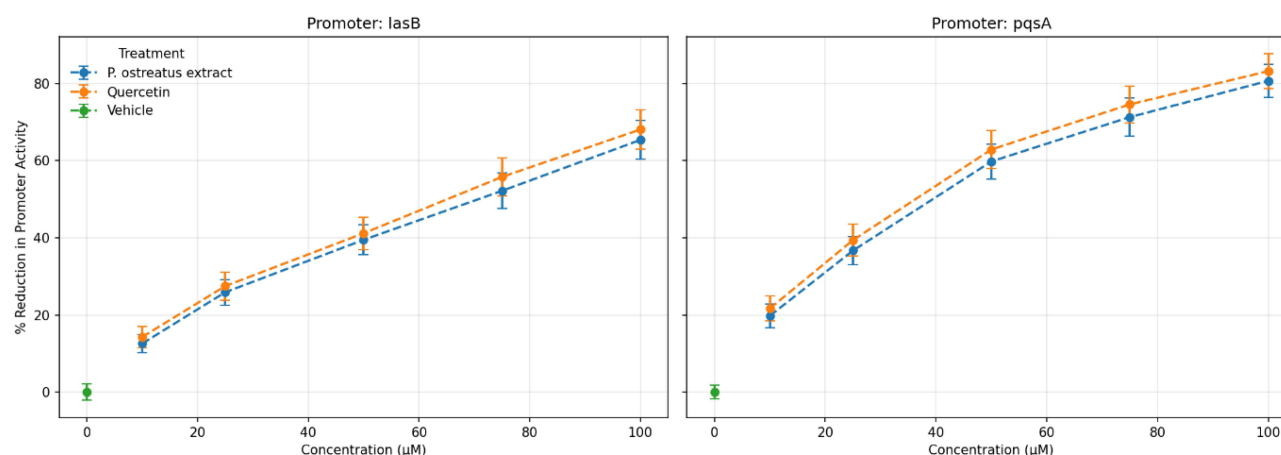


Figure 5 Interval plots of the % reduction (mean $\pm$ SD) in *lasB* and *pqsA* promoter activity under different concentrations of quercetin and 11 quercetin-extract.

with 50 μ M treatments reducing *lasB*-gfp and *pqsA*-gfp signals by 41.2% and 62.9%, respectively ($p < 0.001$). The quercetin-extract mirrored this trend, yielding comparable reductions of 39.5% (*lasB*-gfp) and 59.8% (*pqsA*-gfp) at 50 μ M quercetin-equivalent, as illustrated in Figure 5. The Values represent mean percentage reduction in normalized fluorescence relative to untreated controls ($n = 3$ biological replicates, each with 3 technical replicates). Concentration-dependent inhibition was observed for both *lasB* and *pqsA* reporters. The quercetin-extract consistently mirrored the inhibitory profile of standard quercetin, with slightly attenuated effects at higher concentrations.

The inhibition of QS was quantified by measuring the reduction in the expression of *lasB* and *pqsA* promoters across treatments and concentrations. A Kruskal–Wallis test confirmed that concentration had a statistically significant effect on QS inhibition, $H(5) = 19.08$, $p = 0.002$. The trend is biologically clear but not statistically separable after correction. This finding indicates that at least one concentration significantly differs from others in its ability to suppress QS activity. Pairwise comparisons were performed using Mann–Whitney U -tests with Bonferroni correction. The results indicated that while overall trends were strongly dose-dependent, after correction for multiple testing, no individual pairwise differences reached statistical significance ($p\text{-adj} > 0.05$). However, raw p -values (uncorrected) consistently suggested stepwise differences between 10 μ M and higher concentrations (25, 50, 75, 100 μ M), as well as between mid (25–50 μ M) and maximal (100 μ M) treatments. This reflects the biological pattern evident in the descriptive statistics: QS inhibition increased progressively with concentration for both quercetin and the mushroom extract.

Preferential Inhibition of the PQS System

Comparative analysis of the two reporter systems revealed stronger sensitivity of the pqs regulon to quercetin-mediated inhibition. While *lasB* expression was moderately reduced, *pqsA* expression showed a markedly steeper decline across all treatment levels. Standard deviation and cumulative distribution analyses (Figures 3 and 4) further highlighted this effect, with *pqsA*-gfp fluorescence variance reduced by 53.4% at 50 μ M compared to only 27.6% reduction in *lasB*-gfp. This selective suppression suggests that the PQS pathway may represent the primary target of quercetin-mediated QS modulation.

Biofilm Formation

The anti-biofilm efficacy of quercetin and the quercetin-extract was assessed using crystal violet assays. Both compounds potently inhibited biofilm formation in a preventive assay and disrupted pre-established biofilms in a dose-dependent manner (Table 4). In the preventive assay, where compounds were added at the time of inoculation, treatment with 50 μ M quercetin and quercetin-extract reduced biofilm biomass by 68.2% and 64.7%, respectively ($p < 0.001$). Significant inhibition was observed even at the lowest concentration of 10 μ M (22.4% and 18.6% reduction for quercetin and extract, respectively; $p < 0.05$). The biofilm disruption assay, involving treatment of mature 24-hour biofilms, also showed significant efficacy, albeit requiring higher concentrations for a strong effect. At 50 μ M, quercetin and quercetin-extract

Table 4 Anti-Biofilm Activity of Quercetin and quercetin-Extract against *P. aeruginosa* PAO1

Treatment	Concentration (μM)	Preventive Inhibition (% Reduction ± SD)	Disruption of Pre-Formed Biofilm (% reduction ± SD)
Vehicle	0	0.0 ± 2.1	0.0 ± 2.4
Quercetin	10	22.4 ± 3.5 *	14.7 ± 2.9 <i>ns</i>
	25	44.9 ± 4.2 **	28.6 ± 3.7 *
	50	68.2 ± 5.0 ***	42.9 ± 4.4 **
	75	78.6 ± 5.3 ***	55.2 ± 4.8 ***
	100	85.9 ± 4.7 ***	63.8 ± 5.1 ***
<i>P. ostreatus</i> extract	10	18.6 ± 2.8 *	12.3 ± 2.5 <i>ns</i>
	25	39.7 ± 3.9 **	25.4 ± 3.3 *
	50	64.7 ± 4.6 ***	39.6 ± 3.9 **
	75	75.1 ± 5.1 ***	52.1 ± 4.2 ***
	100	82.3 ± 4.8 ***	60.5 ± 4.7 ***

Note: Statistical significance compared to vehicle control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ($p \geq 0.05$).

Abbreviation: ns, not significant.

reduced pre-formed biofilm biomass by 42.9% and 39.6%, respectively ($p < 0.01$). The potency of the quercetin-extract was consistently comparable to that of pure quercetin at all equivalent concentrations in both assays, reinforcing its potential as an effective anti-biofilm agent.

Anti-Virulence Activity

Protease Activity

To validate that transcriptional suppression of QS-regulated genes translated into functional attenuation of virulence factor production, extracellular protease activity was quantified using azocasein hydrolysis. Culture supernatants from PAO1 treated with sub-MIC concentrations of quercetin (10–100 μM) or the quercetin-extract were incubated with azocasein substrate, and proteolysis was measured spectrophotometrically at 440 nm. Both treatments caused a dose-dependent reduction in protease activity, consistent with *lasB* suppression observed in GFP reporter assays. At 10 μM, quercetin and the quercetin-extract reduced proteolysis by 11.8% and 9.6%, respectively ($p < 0.05$). This inhibition intensified with increasing concentrations, reaching 43.7% (quercetin) and 41.2% (extract) at 50 μM, and maximal inhibition of 65.9% and 62.8% at 100 μM ($p < 0.001$ for both).

To further visualize the data distribution, protease inhibition was analysed across five concentrations (0–75 μM equivalent) using a box-and-whisker plot (Figure 6). Both treatments exhibited a clear concentration-dependent increase in protease activity reduction. Quercetin consistently produced higher median values compared to the extract, with inhibition at 75 μM reaching 57% versus 52%, respectively. Variability was minimal at lower concentrations but increased at mid to high doses, reflecting biological heterogeneity in virulence expression. Importantly, no significant outliers were detected, and the overall inhibitory trends confirmed quercetin as the more potent agent.

Comparative Efficacy

To quantitatively compare the efficacy of the quercetin-extract to pure quercetin, the half-maximal inhibitory concentration (IC₅₀) was calculated for key virulence endpoints. The quercetin-extract demonstrated statistically equivalent potency to pure quercetin across all assays (Table 5). The maximum efficacy achieved at 100 μM was also not

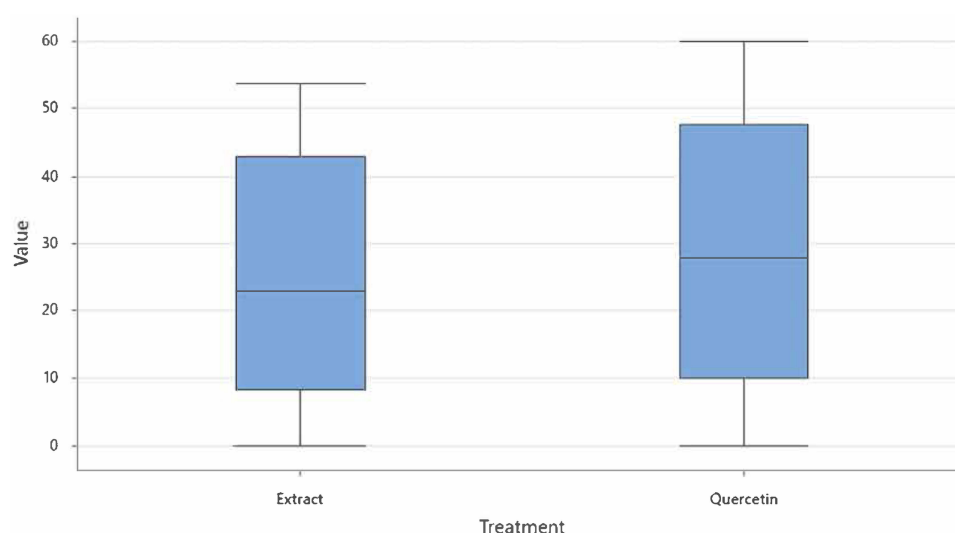


Figure 6 Box-and-whisker plots showing protease activity reduction (%) by quercetin and quercetin-extract across tested concentrations (0–75 μ M).

significantly different between the two treatments. Pure quercetin and the quercetin-extract achieved a maximum preventive inhibition of $85.9 \pm 4.7\%$ and $82.3 \pm 4.8\%$ ($p = 0.21$), and a maximum disruption of pre-formed biofilms of $63.8 \pm 5.1\%$ and $60.5 \pm 4.7\%$ ($p = 0.24$), respectively.

Notably, the quercetin-extract showed a consistent, though non-significant, trend of slightly reduced activity at very low concentrations ($\leq 25 \mu$ M). For instance, the onset of activity ($\geq 10 \mu$ M) against the *lasB* promoter was $14.3 \pm 2.7\%$ for quercetin versus $12.6 \pm 2.4\%$ for the extract. Kinetic analysis of the *pqsA* reporter suggested this minor delay may be due to matrix effects in the crude extract, though it was overcome at higher concentrations. Interestingly, in these same kinetic trials, the quercetin-extract occasionally exhibited a minor enhancement (5%) in the rate of *pqsA* inhibition, potentially attributable to synergistic contributions from co-extracted phenolic compounds such as gallic or ferulic acid.

The efficacy of pure quercetin and a quercetin-extract was evaluated across key virulence pathways of *P. aeruginosa*. Both treatments exhibited strong, dose-dependent inhibition in all assays. The quercetin-extract demonstrated activity nearly identical to pure quercetin at all equivalent concentrations.

At a concentration of 10μ M, both compounds initiated significant anti-virulence effects. Quercetin reduced biofilm formation (preventative assay) by 22.4% and disrupted pre-formed biofilms by 14.7%. Concurrently, it inhibited quorum sensing, reducing *lasB* and *pqsA* promoter activity by 14.8% and 21.7%, respectively. The quercetin-extract showed remarkably similar activity at 10μ M, achieving 18.6% preventative biofilm inhibition, 12.3% disruption, and reductions of 12.6% (*lasB*) and 19.8% (*pqsA*). This dose-dependent effect intensified with increasing concentration. At 25μ M, preventative biofilm inhibition reached 44.9% (Quercetin) and 39.7% (Extract), while *pqsA* inhibition rose to 39.6% and 36.8%, respectively. The half-maximal inhibitory concentration (IC_{50}) was approached at 50μ M. At this concentration, Quercetin demonstrated robust efficacy: 68.2% preventative biofilm inhibition, 42.9% biofilm disruption, 41.6% *lasB*

Table 5 Comparative Efficacy of Pure Quercetin and quercetin-Extract on *P. aeruginosa* virulence Pathways

Assay Endpoint	Pure Quercetin (IC_{50} , μ M $\pm$ SD)	<i>P. ostreatus</i> Quercetin-Extract (IC_{50} , μ M $\pm$ SD)	p-value
<i>lasB</i> -gfp reporter inhibition	42.7 ± 3.8	44.3 ± 4.2	0.41
<i>pqsA</i> -gfp reporter inhibition	28.9 ± 2.6	30.4 ± 3.1	0.33
Biofilm preventive inhibition	31.2 ± 3.4	33.0 ± 3.6	0.29
Biofilm disruption of pre-formed biofilms	54.6 ± 4.5	56.8 ± 5.1	0.37

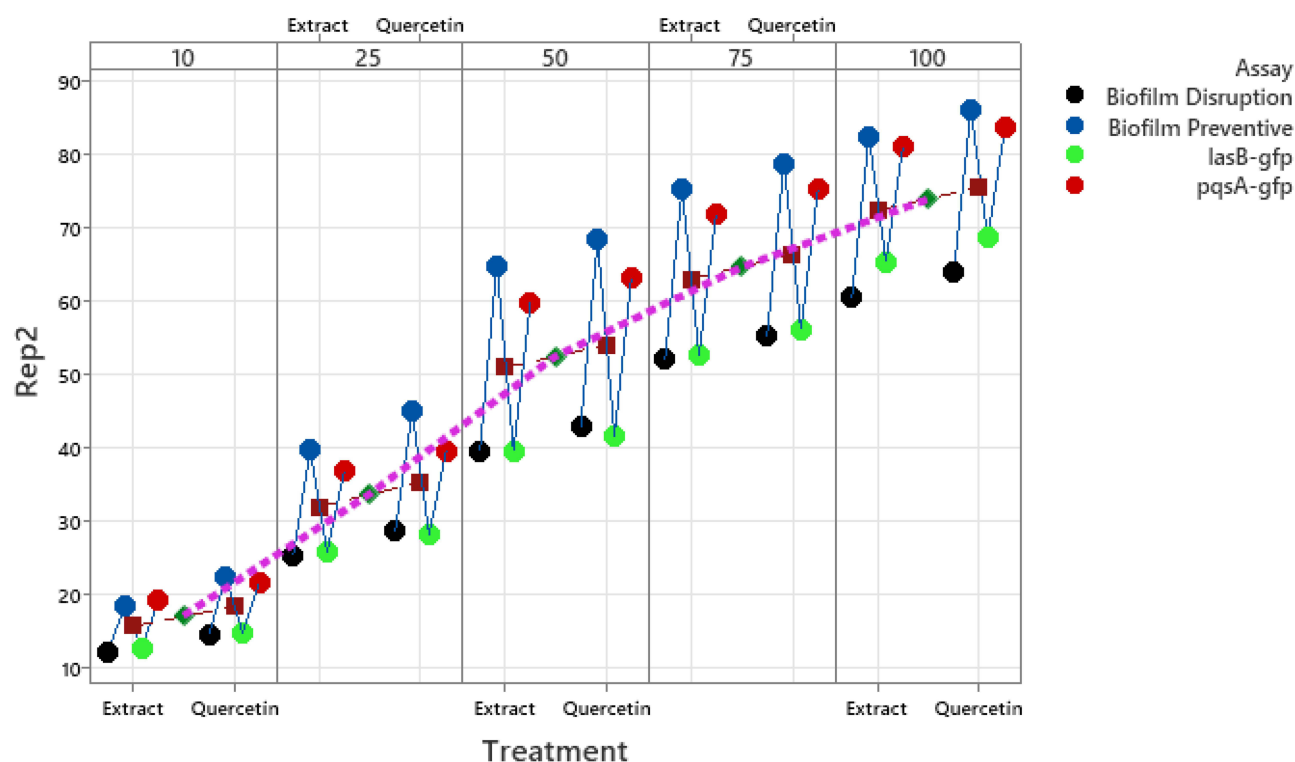


Figure 7 Dose-dependent inhibition of *P. aeruginosa* virulence factors by quercetin-extract and pure quercetin.

inhibition, and 63.0% *pqsA* inhibition. Mirroring this potency, the quercetin-extract at 50 μM achieved 64.7% preventative inhibition, 39.6% disruption, 39.6% *lasB* inhibition, and 59.7% *pqsA* inhibition. Maximal efficacy was observed at the highest concentration tested (100 μM). Quercetin treatment resulted in 85.9% preventative biofilm inhibition and 63.8% disruption of established biofilms, alongside a 68.5% reduction in *lasB* signal and 83.4% reduction in *pqsA* signal. The quercetin-extract at 100 μM displayed equivalent maximum activity, reducing preventative biofilm formation by 82.3%, disrupting biofilms by 60.5%, and inhibiting *lasB* and *pqsA* by 65.3% and 80.8%, respectively.

Throughout the concentration gradient, the bioactivity of the quercetin-extract consistently overlapped with that of pure quercetin, confirming its utility as a potent, natural source of this anti-virulence compound.

The multi-vari chart analysis (Figure 7) revealed a clear, dose-dependent response for both the quercetin-extract and pure quercetin across all tested virulence assays. Bioactivity, measured as percent reduction, increased consistently with concentration from 10 μM to 100 μM. The *pqsA*-gfp reporter assay was the most sensitive to both treatments, demonstrating the highest percent reduction at each concentration, followed by biofilm preventive inhibition, *lasB*-gfp reporter inhibition, and finally biofilm disruption, which was the least sensitive. While the mean response for the quercetin-extract was consistently slightly lower than that of pure quercetin at every equivalent concentration across all assays, this observed difference was not statistically significant. This was confirmed by a Mood's Median test, which compared the overall central tendency of all data points for each treatment. The test found no significant difference between the median bioactivity of the quercetin-extract (45.90%, 95% CI: 28.46 to 63.71) and quercetin (50.05%, 95% CI: 31.19 to 67.17), with a 95% confidence interval for the difference in medians spanning from -27.0 to 20.9. This statistical analysis confirms that the quercetin-extract possesses equivalent anti-virulence efficacy to pure quercetin.

Molecular Docking

Docking was performed using AutoDock Vina after standard ligand and receptor preparation procedures, including protonation, charge assignment, and optimization of torsional flexibility for quercetin, and removal of crystallographic water molecules. Docking of the major *P. ostreatus*-derived metabolites against the *P. aeruginosa* thioesterase PqsE,

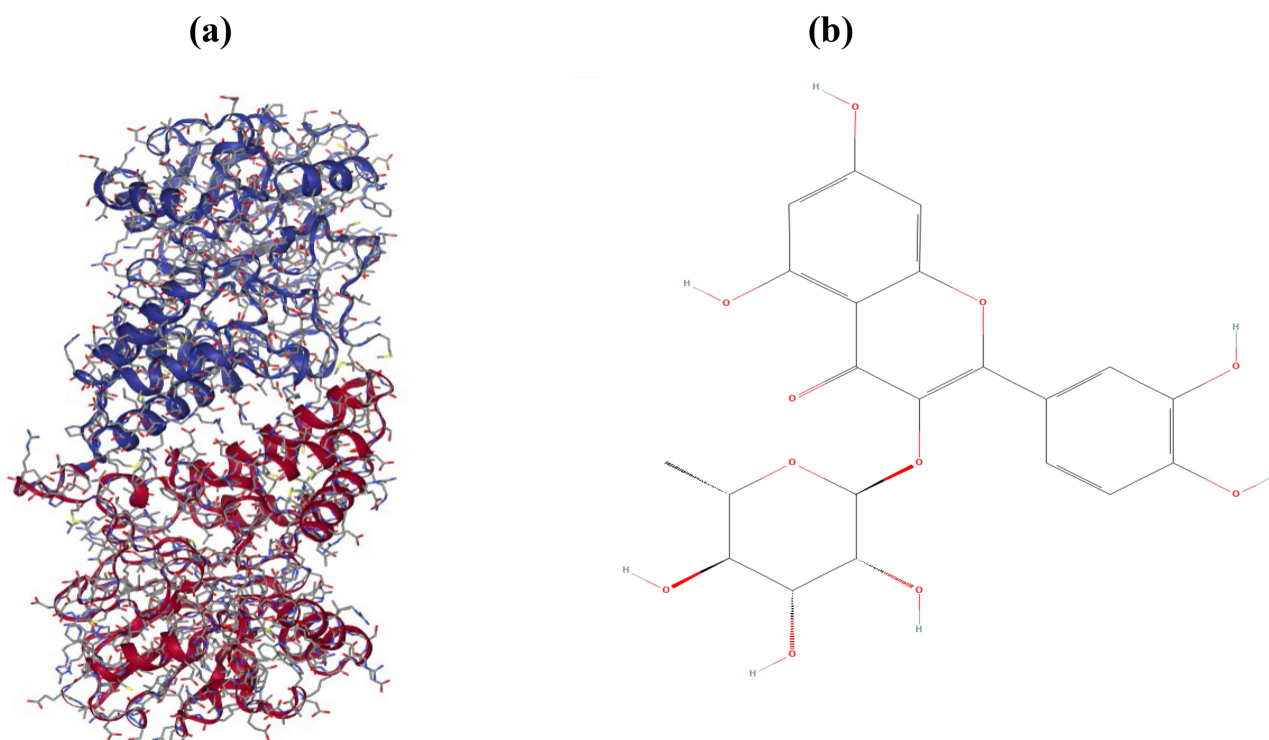


Figure 8 Structural representation of the PqsE receptor and quercetin ligand used for molecular docking. – (a) Three-dimensional structure of the *Pseudomonas aeruginosa* thioesterase PqsE, showing the α -helical and β -sheet architecture that forms the catalytic cavity targeted in this study. (b) Chemical structure of quercetin, a major phenolic metabolite reported from *Pleurotus ostreatus*.

PqsR (MvfR) co-inducer binding domain, and LasR was carried out to probe whether these enzymes constitute a plausible molecular target underlying the observed antibiofilm and antivirulence activities. The crystal structure of PqsE (Figure 8a) was prepared and the docking grid was centred on the canonical thioesterase active site that encompasses the metal-coordinating and substrate-binding residues (H69, H159, D178, F195, S273 and F276). All primary interactions of quercetin (Figure 8b) yielded negative binding energies within the active-site cavity, indicating spontaneous and energetically favourable association of quercetin with target proteins. Notably, several metabolites displayed compact poses that fully occupied the hydrophobic channel while simultaneously engaging polar residues at the pocket entrance, a pattern consistent with competitive inhibition of substrate access.

Quercetin docked favourably into the canonical LasR binding cavity with a best pose energy of -6.6 kcal/mol, consistent with previously reported affinities for flavonoid-class LasR antagonists. Two top-ranked poses (-6.6 and -6.5 kcal/mol) were generated, both occupying the same hydrophobic–polar pocket that regulates ligand-induced conformational switching. Structural analysis revealed that the ligand inserted deeply into the regulatory cleft (Figure 9), adopting an orientation that positioned the flavanol core parallel to the hydrophobic groove while projecting its hydroxyl substituents toward polar anchoring residues. The binding mode of quercetin was stabilized by an extensive hydrogen-bonding network. The hydroxyl groups at positions O7, O8, O9, and O10 formed strong, directional hydrogen bonds with His78, Thr80, Gln81, Ile92, and Gln94, respectively—residues that constitute part of the canonical LasR recognition motif for acyl-homoserine lactone (AHL) ligands. An additional weak hydrogen bond was detected between C21 of quercetin and Gln81. Beyond polar interactions, hydrophobic packing against Ile86 further anchored the aromatic scaffold within the pocket, reinforcing ligand stability and preventing displacement by the native AHL.

Quercetin displayed a favourable docking affinity of -6.7 kcal/mol, representing a slightly stronger interaction than observed for LasR (-6.6 kcal/mol). Two poses were obtained, with the top-ranked pose occupying the deeply recessed hydrophobic channel that normally binds native ligands such as HHQ and PQS. The ligand adopted a compact orientation within the co-inducer cavity (Figure 10), with its polyphenolic scaffold aligning along the same trajectory

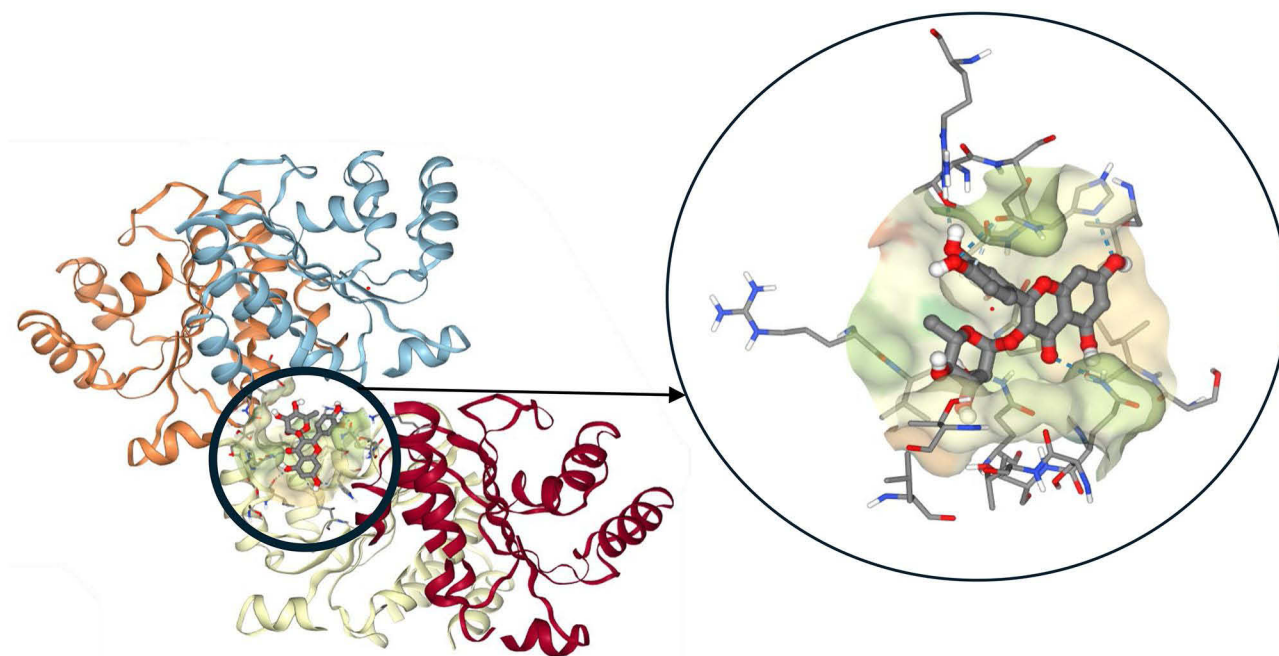


Figure 9 Predicted binding mode of quercetin within the ligand-binding domain of the *Pseudomonas aeruginosa* LasR receptor: Three-dimensional architecture of the LasR ligand-binding domain (LBD), shown in a multicolour ribbon representation, highlighting the deeply recessed hydrophobic pocket responsible for ligand recognition. Quercetin is docked into the canonical LasR cavity, adopting a compact orientation that aligns its flavanol scaffold along the central binding groove.

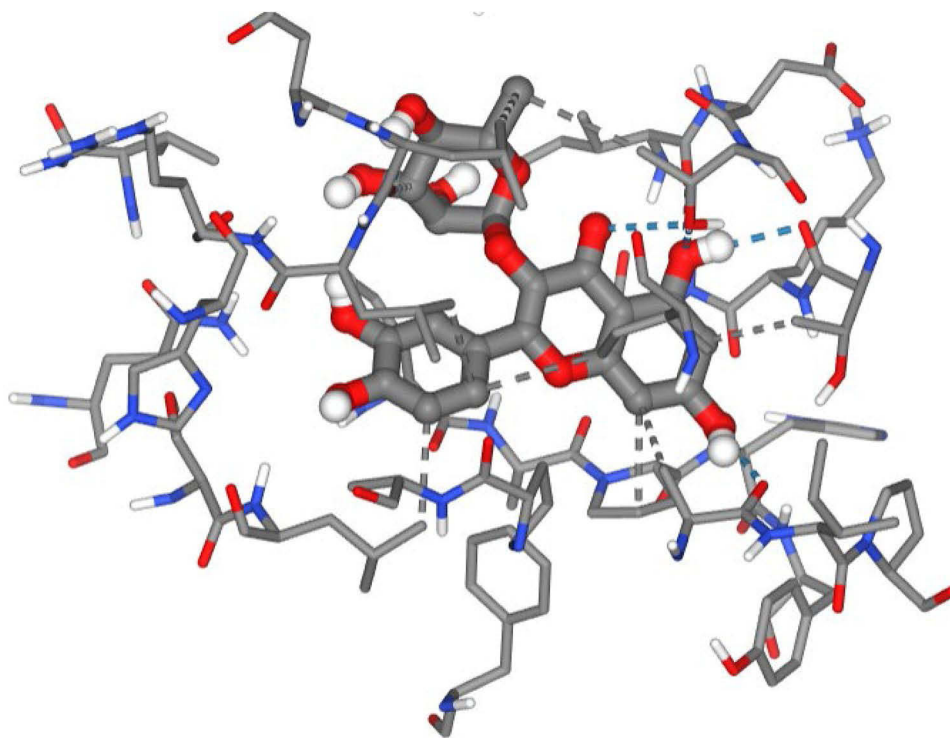


Figure 10 Predicted binding interactions of quercetin within the co-inducer binding domain of the *Pseudomonas aeruginosa* PqsR (MvfR) receptor.

as the quinolone headgroups of canonical PqsR agonists. This positioning is characteristic of competitive PqsR inhibitors, many of which similarly exploit the polar–hydrophobic architecture of the site. A dense network of stabilizing interactions contributed to the high-quality binding pose.

Extensive hydrophobic contacts were established with key pocket-lining residues, including Ala102, Ile149, Thr166, Ala168, Leu197, Leu207, Leu208, Ile236, Pro238, and Thr265. These residues collectively form the central lipidic tunnel required for PQS and HHQ anchoring, and quercetin's aromatic rings recapitulated this interaction by laying across the hydrophobic plane of the cavity. The strength, breadth, and spatial distribution of these hydrophobic contacts strongly suggest that quercetin effectively mimics the aromatic core of known PqsR antagonists. In addition to hydrophobic complementarity, quercetin formed multiple strong hydrogen bonds that reinforced ligand stability and specificity. Polar interactions between quercetin's hydroxyl groups and Ala102, Thr166, Leu207, Ile263, and Thr265 were observed, with Thr265 forming several repeated hydrogen bonds consistent with its established role as a key anchoring residue in PqsR–inhibitor complexes. These interactions positioned quercetin at the cavity entrance while extending its planar core into the deeper hydrophobic zone, a hallmark binding mode of flavonoid and benzamide-based PqsR inhibitors. A weak hydrogen bond with Leu207 contributed additional, though minor, stabilization.

In contrast to the favourable binding observed for quercetin against LasR and PqsR, this ligand displayed only weak and non-specific affinity for PqsE, with a best docking score of -3.3 kcal/mol and a secondary pose of -1.4 kcal/mol. These values fall well below the threshold typically associated with functional PqsE inhibitors (-7.0 to -9.0 kcal/mol), indicating an unstable and poorly coordinated interaction. Structural inspection of the docked pose (Figure 11) revealed that the ligand failed to penetrate the deep hydrophobic cavity that characterizes the canonical PqsE active site.

Instead, the compound remained positioned near the protein surface, making only a single hydrophobic contact with Val189, insufficient to support cavity anchoring or productive stabilization. The binding orientation lacked the characteristic aromatic packing and hydrophobic burial that are essential for potent PqsE engagement. Although several hydrogen bonds were detected—particularly with Asp184, Glu187, Glu240, Arg243, Arg247, and Trp269—these interactions appeared superficial and predominantly involved solvent-exposed acidic and basic residues. Such polar contacts, while numerous, did not overcome the absence of hydrophobic support, consistent with the poor docking score. Occasional cation– π -like contacts were observed with Arg243 and Arg247, but these involved peripheral aliphatic

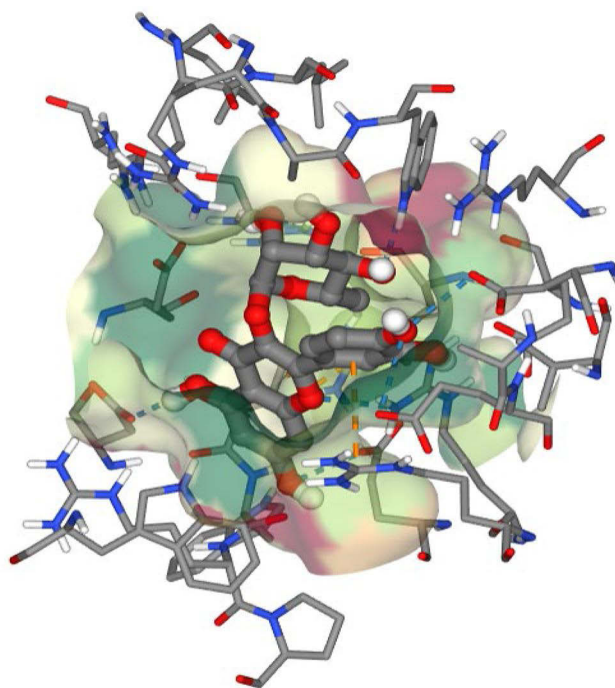


Figure 11 Weak and non-specific binding of the test ligand to the *Pseudomonas aeruginosa* PqsE thioesterase active site.

carbons of the ligand rather than true aromatic π -systems, further highlighting the lack of meaningful ligand–pocket complementarity.

Discussion

Pseudomonas aeruginosa is an opportunistic pathogen whose persistence in clinical settings is driven by QS, a regulatory network governing virulence factor expression, biofilm development, and antimicrobial tolerance.^{1,5} Disrupting QS—known as QSI—offers a promising anti-virulence strategy that suppresses pathogenicity without exerting bactericidal pressure, thereby reducing the likelihood of resistance development.^{24,25} Although quercetin, a plant-derived flavonoid, has been widely studied for its QSI properties,⁹ its potential when sourced from edible mushrooms such as *P. ostreatus* remains insufficiently examined. The present study demonstrates that quercetin extracted from *P. ostreatus* is functionally equivalent to purified quercetin in inhibiting QS and biofilm formation in *P. aeruginosa* PAO1, highlighting its potential as a sustainable and nutritionally relevant source of anti-virulence compounds. Consistent with anti-virulence principles, both the quercetin-extract and pure quercetin attenuated QS-regulated functions at sub-inhibitory concentrations that did not impair bacterial growth or viability. The preservation of normal biomass (OD₆₀₀) and viable counts confirms that the observed reductions in QS activity and virulence factor expression stem from authentic signalling interference rather than growth suppression. This aligns with emerging paradigms in antivirulence research, where selectively targeting pathogenic traits minimizes evolutionary pressure for resistance.^{26–28} One of the most striking findings is the preferential inhibition of the PQS (*Pseudomonas* quinolone signal) system over the Las system. The *pqsA*-gfp reporter exhibited a substantially greater reduction than the *lasB*-gfp reporter across all concentrations, supported by variance and cumulative distribution analyses. This selective vulnerability suggests that quercetin interacts more intimately with PQS regulatory nodes than with the Las hierarchy—an insight strengthened by the molecular docking data. Docking results revealed that quercetin binds most strongly to PqsR (MvfR), the master transcriptional regulator of the PQS system, with a binding affinity of -6.7 kcal/mol, marginally stronger than its affinity for LasR (-6.6 kcal/mol). The ligand adopted a compact pose within the hydrophobic co-inducer tunnel of PqsR, making extensive hydrophobic contacts (Ala102, Ile149, Leu197, Leu207, Ile236, Thr265) and forming multiple stabilizing hydrogen bonds, especially with Thr166, Thr265, and Ala102. This interaction pattern mimics known PqsR antagonists and supports the hypothesis that quercetin disrupts PQS-mediated signalling by directly competing with native quinolones (HHQ/PQS) at the MvfR binding site. Similarly, favourable binding to LasR suggests that quercetin may partially inhibit Las-dependent gene expression through hydrogen bonding with canonical LasR residues (His78, Thr80, Gln81, Ile92, Gln94) and hydrophobic packing against Ile86. This dual binding capability aligns with the observed dose-dependent suppression of *lasB*-gfp expression. In contrast, docking to PqsE, the thioesterase responsible for activating downstream PQS-dependent effector pathways, produced only weak, non-specific interactions (-3.3 kcal/mol). The ligand failed to penetrate the hydrophobic catalytic cavity and instead remained surface-exposed, indicating that PqsE is unlikely to be a direct molecular target of quercetin. This functional selectivity—strong binding to PqsR, moderate binding to LasR, and negligible affinity for PqsE—provides a mechanistic explanation for the experimental phenotype: dominant PQS inhibition, partial Las inhibition, and no effects attributable to PqsE disruption.

These docking results strengthen the mechanistic conclusion that quercetin's QSI activity is mediated predominantly through interference with the PQS transcriptional regulator PqsR, with additional contributions from LasR antagonism. Furthermore, the decline in *pqsA*-gfp activity relative to *lasB*-gfp also suggests that quercetin may interact more directly with PQS-regulatory components or interfere with PQS autoinducer synthesis or perception.^{14,29,30} This aligns with prior evidence that flavonoids can destabilize PQS-regulated signalling cascades by binding to transcriptional regulators such as PqsR.³¹ Given the centrality of PQS in orchestrating biofilm robustness and persistent infections, selective targeting of this system represents a therapeutically advantageous mechanism.

Beyond transcriptional QS interference, quercetin and the quercetin-extract significantly impaired biofilm formation and partially disrupted mature biofilms. Preventive effects were more pronounced than disruption of established biofilms, consistent with the role of QS in early biofilm architecture stabilization. Nevertheless, the capacity to reduce biomass in pre-formed biofilms is clinically relevant, especially in chronic infections where biofilm persistence contributes to antibiotic recalcitrance.³² Quercetin also significantly reduced extracellular protease production, a key Las-regulated

virulence trait. This functional suppression not only corroborates the lasB-gfp reporter results but also demonstrates that transcriptional inhibition translates into phenotypic attenuation. The dose-dependent reduction in protease activity underscores the broad-spectrum anti-virulence potential of quercetin. Importantly, the quercetin-extract consistently matched the efficacy of pure quercetin, with no significant differences in IC₅₀ values for lasB inhibition, pqsA inhibition, biofilm inhibition, biofilm disruption, or protease reduction. This parity suggests that extraction does not compromise compound potency and may even benefit from subtle synergistic effects with co-extracted phenolics (gallic acid, ferulic acid). The equivalence of mushroom-derived quercetin to the purified standard highlights *P. ostreatus* as a scalable, edible, and economical source of QS inhibitors. Given the extensive cultivation and proven safety of *P. ostreatus*, mushroom-derived quercetin is an attractive candidate for nutraceutical, dietary, or adjunctive therapeutic applications. Such approaches may have particular relevance in cystic fibrosis airway infections, burn wound colonization, and ventilator-associated pneumonia, where biofilm-mediated persistence undermines antibiotic efficacy.^{33,34} Unlike conventional antibiotics, quercetin-based QSIs attenuate virulence without inhibiting growth, impose low selective pressure for resistance, may synergize with existing antimicrobials by destabilizing biofilms, and are derived from natural, renewable sources.^{8,35,36} This study demonstrates that quercetin derived from *P. ostreatus* is a potent, selective QSI in *P. aeruginosa*, with mechanistic evidence pointing toward strong PqsR antagonism, moderate LasR interference, and negligible PqsE targeting. By integrating biochemical, phenotypic, and *in silico* interaction data, these findings substantiate the role of mushroom-derived flavonoids as promising anti-virulence agents.

Limitations and Future Directions

While this study provides robust *in vitro* evidence of quercetin-mediated QS inhibition, several limitations must be acknowledged. First, the experiments were performed under controlled laboratory conditions using GFP-based reporter strains, which may not fully capture the complexity of *in vivo* infection environments. Host factors, nutrient conditions, and polymicrobial interactions can influence QS dynamics and compound activity. Second, the molecular mechanism underlying preferential PQS inhibition remains to be elucidated. Future work should employ molecular docking, transcriptomics, and mutational analyses to pinpoint quercetin's targets within the QS regulatory hierarchy. Third, although *P. ostreatus* extracts were standardized to quercetin-equivalents, batch variability and the contribution of co-metabolites warrant further investigation.

Conclusion

Quercetin, whether purified or extracted from *P. ostreatus*, effectively inhibits quorum sensing and biofilm-associated virulence in *P. aeruginosa* PAO1 at non-inhibitory concentrations. The stronger suppression of the PQS system is supported mechanistically by molecular docking, which shows high-affinity binding of quercetin to PqsR and favourable interaction with LasR, but weak, non-specific binding to PqsE. The quercetin-extract matched the potency of pure quercetin across all assays, confirming *P. ostreatus* as a viable natural source of QSI-active flavonoids. These findings highlight mushroom-derived quercetin as a sustainable anti-virulence candidate with potential to complement existing treatments for chronic, biofilm-mediated infections.

Compliance with Ethical Standards

Research Involving Human Participants and/or Animals: This study did not involve human participants or animals.

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Disclosure

The study funder had no role in the study design, data collection, data analysis, data interpretation, or report writing. The authors declare no conflicts of interest related to this study.

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