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RESEARCH

In vitro modulatory effects of lichen secondary metabolite vulpinic acid on pyoverdine production and quorum sensing-regulated traits in *Pseudomonas aeruginosa*

Aleyna Nazlı Koçöz[1] · **Barış Gökalsin**[2]

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Abstract

Pseudomonas aeruginosa is a major opportunistic pathogen, especially involved with nosocomial infections. Siderophores such as pyoverdine are crucial for iron acquisition in bacteria and play an important role in virulence regulation. Therefore, targeting siderophore pathways has been suggested as a potential antivirulence approach. Lichens produce secondary metabolites with various effects, including the ability to influence quorum sensing (QS). In this study, lichen secondary metabolite vulpinic acid was investigated for its in vitro effects on pyoverdine production and specific QS-regulated traits of *P. aeruginosa*. Pyoverdine production, fluorescence quenching, QS-related virulence traits, and biofilm formation were evaluated using wild-type and GFP-expressing biosensor strains. In addition, quantitative qRT-PCR was performed to analyse QS and pyoverdine gene expressions. Results show that vulpinic acid application decreased pyoverdine production by 53.65% ± 5.45 and quenched the produced pyoverdine by 67.55% ± 2.98. The QS regulated genes *lasB* and *rhlA* were significantly downregulated. No biofilm inhibition at any applied concentration was observed, suggesting a selective influence on the bacteria rather than a global attenuation of virulence. These findings suggest that vulpinic acid can modulate specific virulence factors of *P. aeruginosa in vitro*, and provides a basis for further investigation of lichen secondary metabolites as antivirulence agents.

Keywords Anti-virulence · *Pseudomonas aeruginosa* · Pyoverdine · Quorum sensing · Siderophore

Introduction

Pseudomonas aeruginosa is a nosocomial, Gram-negative, oxidase-positive, rod-shaped pathogen that causes serious infections in elderly people, immunocompromised patients, people with chronic illnesses, and children who have inadequate immune systems (Krell and Matilla 2024). *P. aeruginosa* can be isolated from water or soil and is the main cause of high mortality and morbidity rates in cystic fibrosis (CF) disease, which affects the respiratory and digestive systems (Jakobsen et al. 2013; Gökalsın et al. 2017). Although *P. aeruginosa* can grow in various media, its high adaptability allows it to rapidly establish chronic infections and develop resistance within the host environment (Jeong et al. 2023). In 2024, the World Health Organization (WHO) prepared a list of several pathogens against which new antimicrobial developments are needed (Sati et al. 2025). Pathogens with high virulence factors, like *P. aeruginosa* has been added to this list. Due to its features that pose a threat to hosts through antimicrobial resistance development, restricting treatment options for infections, and increasing mortality rates due to treatment unresponsiveness. (García-Fontana et al. 2019; Diggle and Whiteley 2020; Sainz-Mejías et al. 2020; Jeong et al. 2023).

P. aeruginosa's pathogenicity is regulated by a system called "quorum sensing" (QS), which is based on the production of small and diffusible molecules that can be detected by the organisms in its environment (Miller and Bassler 2001). QS is a form of intercellular communication that enables coordinated bacterial behavior

✉ Barış Gökalsin
baris.gokalsin@marmara.edu.tr

[1] Institute of Pure and Applied Sciences, Department of Biology, Marmara University, Istanbul 34730, Turkey

[2] Faculty of Science, Department of Biology, Marmara University, Istanbul 34730, Turkey

in response to cell density through the production and detection of autoinducer (AI) molecules. (Papenfort and Bassler 2016; Gökalsın et al. 2017). The virulence of *P. aeruginosa* is regulated by multiple QS pathways. The two main LuxR and LuxI-type enzyme systems that include LasIR, in which the N-(3-oxododecanoyl)-1-homoserine lactone (3-oxo-C12-HSL) autoinducer is produced by LasI, and RhlIR, in which the N-butanoyl homoserine lactone (C4-HSL) autoinducer is produced by RhlI (Heeb et al. 2011; Papenfort and Bassler 2016). The AIs in these systems are acyl-homoserine lactones (AHLs), while the *Pseudomonas* quinolone signal (PQS) system, encoded by the *pqsABCDH* genes, uses a quinolone molecule as its autoinducer. These interconnected *las*, *rhl*, and *pqs* pathways collectively regulate virulence factor expression and biofilm formation (Heeb et al. 2011).

Pyocyanin is one of the unique virulence characteristics of *P. aeruginosa*. Pyocyanin is a redox-active molecule capable of electron exchange and produced only by *P. aeruginosa.* (Dietrich et al. 2006). It is a greenish-blue molecule (Abdelaziz et al. 2023), which has the ability to induce oxidative stress due to its capability of increasing intracellular levels of reactive oxygen species (ROS) (Alatraktchi et al. 2020). Furthermore, pyocyanin forms a complex with extracellular DNA (eDNA), which is a key component of biofilms in *P. aeruginosa*, and thus has a significant impact on biofilm formation (Das and Manefield 2012). Biofilm formation is defined as organized microbial communities embedded within a self-produced extracellular polymeric matrix (EPS) and adherent to surfaces (Yin et al. 2022). Biofilm formation enables *P. aeruginosa* to resist stress conditions and can act as a permeability barrier and a restricting factor against antimicrobial agents (Pang and Yuk 2019).

Cystic fibrosis (CF) patients showing evidence of biofilm development and low levels of planktonic *P. aeruginosa* in their sputum were additionally found to be iron-deficient (Høiby et al. 2010). This diagnosis has been associated with *P. aeruginosa* chelating the iron in the host to survive and form biofilms (Høiby et al. 2010; Oglesby-Sherrouse et al. 2014). In addition, various studies indicate that sufficient iron conditions serve as a signal for biofilm formation (Lyczak et al. 2000; Singh et al. 2002; Lebeaux et al. 2014; Nairz and Weiss 2020). Iron is shown as one of the essential elements for synthesizing virulence factors and the formation of biofilm (Banin et al. 2005; Díaz-Pérez et al. 2023; Jeong et al. 2023). Results of multiple studies show that iron stress highly affects biofilm formation due to the unstabilization of the polysaccharide matrix. In iron stress conditions, the hydrophobicity of the microbial surface decreases, and thus the surface composition changes. (Chhibber et al. 2013; Ribeiro and Simões 2019).

Iron is a significant element that is employed for several metabolic activities in bacteria. These processes include biosynthesis of enzymes that dispose of harmful radicals, nucleic acid synthesis, growth, and electron transport (Krewulak and Vogel 2008; Fardeau et al. 2011). Variations in iron levels can therefore indirectly influence bacterial physiology. It is also stated that iron stress may up/down-regulate virulence factors such as exotoxin A, elastase A, elastase B, and alkaline protease (Litwin and Calderwood 1993; Khasheii et al. 2021).

Although iron functions as a cofactor in redox metabolism, ferric iron (Fe^{3+}) is poorly soluble and does not exhibit binding properties under aerobic and physiological conditions (Lamont et al. 2002; Schalk 2008; Gasser et al. 2015). To overcome this, some pathogens produce and secrete small molecules called siderophores that chelate environmental iron with high affinity (Schalk and Guillon 2013; Bonneau et al. 2020). *P. aeruginosa* produces a type of siderophore called pyoverdine. In addition to iron uptake, pyoverdine serves as a signalling molecule for the production of endoproteinase *prpL* and exotoxin A, two vital virulence factors (Wilderman et al. 2001; Visca et al. 2007). Pyoverdine biosynthesis is controlled by the extracytoplasmic function sigma factor PvdS, whose expression is modulated by iron availability and is interconnected with QS regulation via LasR (Brumlik and Storey 1992; Sánchez-Jiménez et al. 2023).

It is known that iron also plays a regulatory role in QS systems (Bollinger et al. 2001; Oglesby et al. 2008; Patriquin et al. 2008). Thus, pyoverdine synthesis inhibition has emerged as a promising antivirulence strategy (Jeong et al. 2023). The antivirulence strategies are based on the observation that virulence factors are usually induced under stress conditions. Therefore, attenuating bacterial virulence specifically without killing or inhibiting bacterial growth may also lower bacterial resistance mechanisms (Rasko and Sperandio 2010; Gil-Gil et al. 2025).

Plants, lichens, and other natural sources produce chemical structures called secondary metabolites that protect these organisms from environmental stress conditions. These natural products are commonly preferred for antimicrobial applications because of their bioactivity and compatibility (Cragg and Newman 2013; Gökalsın and Sesal 2016; Atanasov et al. 2021). Like plants, lichens also have ethnobotanical and antimicrobial properties, making them a valuable source of bioactive compounds (Boustie et al. 2011; Shrestha and St Clair 2013). Lichens are complex organisms that are composed of a symbiotic relationship between mycobionts (fungi) and photobionts (algae or cyanobacteria), and they can survive in extreme conditions in a wide variety of regions (Gandhi et al. 2022). Many characterized properties of lichen metabolites have allowed them to be used as

potential therapeutic agents (Güvenç et al. 2012; Kosanić and Ranković 2019; Pradhan et al. 2023). One such metabolite is vulpinic acid. Vulpinic acid is a secondary metabolite that can be found in *Letharia vulpina*, and is known for its antimicrobial properties (Shrestha 2015; Shresta et al. 2016). Studies show that vulpinic acid has antiangiogenic, anticarcinogenic, antiproliferative, and antimicrobial effects (Kim et al. 2017; Kılıç et al. 2018a; Kılıç et al. 2018b; Şahin et al. 2019). Since *L. vulpina* can grow in harsh conditions, vulpinic acid may serve as a sustainable raw material (Nascimbene et al. 2008; Dakskobler et al. 2011). Thus, inhibition of pyoverdine biosynthesis via secondary metabolite vulpinic acid may be a promising approach.

This study investigates the effects of vulpinic acid to suppress pyoverdine production in *P. aeruginosa* and to elucidate the subsequent effects of impaired iron uptake on the regulation of its virulence factors. Fluorescent monitor strains were used to observe the effects of vulpinic acid on QS mechanisms, and wild-type strains were employed to assess biofilm inhibition potential and other virulence-associated phenotypes. Moreover, quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were conducted to evaluate relevant gene expression levels.

Materials and methods

Bacteria strains

For assays related to iron-dependent pyoverdine production, QS reporter activity, biofilm, and qRT-PCR analysis, *P. aeruginosa* PAO1 wild-type strains were grown in M9 minimal medium supplemented with 0.5% (w/v) glucose 0.5% (w/v) Casamino Acids. M9 minimal medium was selected as a low-iron environment for accurate assessment of iron-dependent pyoverdine production. The *lasB-gfp*, *rhlA-gfp*, and *pqsA-gfp* reporter strains, carrying mini-Tn5 insertions in which the *lasB*, *rhlA*, and *pqsA* promoters, regulated by LasR, RhlR, and PqsR, respectively, drive expression of an unstable green fluorescent protein (GFP), were used to assess QS–dependent promoter activity. These strains were grown in M9 minimal medium supplemented with 2.5 mg thiamine per liter in addition to the above-mentioned nutrients. For virulence assays (pyocyanin production, elastase activity, and protease activity), *P. aeruginosa* PAO1 was cultured in Lysogeny Broth (LB) for better growth and expression of virulence factors.

For all experiments, overnight cultures were grown at 37 °C with shaking (180 rpm), diluted into fresh medium, and then exposed to vulpinic acid as described in the relevant assay sections.

All strains used in this study are shown in Table 1.

Table 1 List of bacterial strains used in this study

Species	Bacteria strain	Structure	Reference
P. aeruginosa	*lasB*	Gmr-30γ; PAO1-ATCC, P*lasB-gfp*(ASV)-P*lac-las*Rmini-Tn5 derivative: pMHLAS	Hentzer et al. 2002
	rhlA	Gmr-30γ; PAO1-ATCC, P*rhlA-gfp*(ASV)-P*lac-las*Rmini-Tn5 derivative: pMHLAS	Yang et al. 2009
	pqsA	Gmr-30γ; PAO1-ATCC, P*pqsA-gfp*(ASV)-P*lac-las*Rmini-Tn5 derivative: pAC37	Yang et al. 2007
	PAO1	Wild-type	

QS inhibition assay

QS inhibition assays were performed using a modified protocol based on Bjarnsholt et al. (2010). Briefly, 100 µL of M9 minimal medium was dispensed into black 96-well microplates (Greiner Bio-One), followed by the addition of vulpinic acid and preparation of two-fold serial dilutions. Overnight cultures of GFP QS reporter strains were then added to each well to achieve a final volume of 200 µL. Final vulpinic acid concentrations of 800, 400, and 200 µM were obtained. Equivalent concentrations of DMSO were included as vehicle controls in all experiments.

Bacterial growth and GFP fluorescence were monitored using Cytation™ 3 multimode microplate reader at 36 °C for 15 h, with measurements recorded at 15 min intervals. Bacterial growth was assessed by optical density at 450 nm to minimize potential interference from pyoverdine-associated fluorescence, while GFP expression was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Pyoverdine assays

To analyze the effects of vulpinic acid on pyoverdine expression, the fluorescence quenching experiments were conducted using a modified approach based on the methodology described by Kirienko et al. (2019). Vulpinic acid was dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration in all assays did not exceed 1% (v/v). 1% DMSO was included as a vehicle control in all experiments. $FeCl_3$ was used as a positive control for iron repletion, which suppresses siderophore production, at a final concentration of 100 µM (Cornelis et al. 2023). *P. aeruginosa* PAO1 cultures were grown overnight at 37 °C with shaking. Overnight cultures were diluted 1:100 into M9 minimal medium supplemented with vulpinic acid at final concentrations of 800, 400, or 200 µM. Following incubation for 24 h at 37 °C with shaking, cultures

were centrifuged at 10,000 × g for 30 min. Fluorescence measurements were performed at excitation and emission wavelengths of 405 nm and 460 nm, respectively, which correspond to the characteristic fluorescence properties of pyoverdine.

To assess the direct pyoverdine quenching capacity of vulpinic acid, an equal amount of untreated bacterial supernatant was added to M9 media supplemented with vulpinic acid (800 µM, 400 µM, and 200 µM). After incubation for 5 min at room temperature, fluorescence measurements were performed using a Cytation™ 3 microplate reader (Agilent, USA).

Biofilm assay

Overnight *P. aeruginosa* PAO1 culture is added to a 1:100 dilution in the M9 medium (OD 600 nm: 0.01). Serial dilutions were made and 800 µM, 400 µM, and 200 µM final concentrations of vulpinic acid were achieved on a microplate. Equivalent concentrations of DMSO were included as vehicle controls in all experiments. Plates were incubated for 24 h at 37 °C under static (non-shaking) conditions to allow biofilm formation. Following incubation, planktonic cells were removed, and wells were gently washed with phosphate-buffered saline (PBS). Attached biofilms were stained with 0.1% (w/v) crystal violet (CV) for 5 min. Excess stain was removed by washing with distilled water, and the bound crystal violet was solubilized using 33% (v/v) acetic acid. Dissolved CV absorbance was measured at 590 nm using a microplate reader.

Virulence tests

P. aeruginosa PAO1 strains were cultured in LB medium to achieve a starting density of OD 600 nm: 0.1. Vulpinic acid (800 µM) was then added to these cultures in three biological replicates. Regular untreated LB medium was used as blank control. After the bacteria were incubated at 37 °C for 20 h, cell-free supernatants were collected via syringe filter (0.2 µm) and used for subsequent virulence tests. Virulence factor production was normalised to bacterial growth by dividing the measured values by the corresponding OD 600 nm of each culture.

Pyocyanin pigment production

3 mL of chloroform was added to the filtrates, and the mixture was centrifuged. Then, 1 mL of 0.2 M HCl was added to the separated chloroform phases, and the mixture was centrifuged one more time. The absorbance values of the separated HCl phases were measured at 530 nm.

Elastase activity

Elastase activities of the filtrates were determined and optimized in accordance with the method described by Ohman et al. (1980). 500 µl of filtrate was added to 500 µl of ECR buffer (100 mM Tris-HCl, pH 7.5) containing 10 mg of elastin-congo red (ECR). The mixture was incubated for 3 h at 37 °C with shaking at 180 rpm. Insoluble ECR was centrifugated and the absorbance values of the supernatants were measured at 495 nm.

Protease activity

Total proteolytic activity of filtrates was determined and performed by optimizing it in accordance with the method described by El-Mowafy et al. (2014). 500 µl of filtrate was mixed with 1 ml of 1.25% skim milk. After 30 min incubation at 37 °C, absorbances were measured at 600 nm.

RNA isolation

Upon 24th and 6th hours, untreated and treated PAO1 cultures were centrifuged at 10,000 g for 10 min. The supernatant was discarded and RNA stabilization solution was added for long term storage. RNA was isolated and purified using High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's protocols. RNA purity and concentration were assessed spectrophotometrically, and samples with A_{260}/A_{280} ratios between 1.8 and 2.0 were used for further analysis. The 24-hour incubated strains were designated for the evaluation of pyoverdine genes expression levels, whereas the 6-hour incubated strains were designated to examine QS gene expression levels.

qRT-PCR.

The qPCR assay was performed in two steps to allow the analysis of multiple genes. Reverse transcription was conducted using *P. aeruginosa* PAO1 RNA samples and LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA). Random hexamer primer and anchored-oligo(dT)18 primer sequences used in this study.

qRT-PCR tests were performed using Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) in a Roche LightCycler 96 (Basel, Switzerland). The cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10s annealing at 60 °C for 30s, and extension at 72 °C for 30 s. All primer amplification efficiencies were approximately 2.0, corresponding to ~100% amplification efficiency. Melting curve analysis was performed at the end of each amplification run to

Table 2 Primer sequences used in this study. *rpoD* was used as a housekeeping control gene for normalization. Oligo Design Tool (ThermoFisher Scientific, USA) was utilized to design the primers

Gene	Primer	Sequence (5′–3′)	Amplicon size (bp)
lasB	Forward	AACTCCGGGCTGATCTA	97
	Reverse	TCTTGCCGCGCATATAG	
rhlA	Forward	GAGACCGTCGGCAAATAC	111
	Reverse	CACCTGGTCGATGTGAAA	
pqsA	Forward	CCCTTTGCTCGACGATTT	119
	Reverse	TGGAACCCGAGGTGTATT	
pvdA	Forward	GTACCTGGAACACATGGCCA	108
	Reverse	TCGTTGAGGTCGATGAAGGC	
pvdS	Forward	GGAACAACTGTCTACCCGCA	139
	Reverse	GAAGAACGCATCCTGGACCA	
rpoD	Forward	GCTATATCGATCCCGATGAC	98
	Reverse	CTCGTCGTCCTTCTCTTTC	

confirm primer specificity and the absence of non-specific amplification or primer–dimer formation.

All qRT-PCR analyses were performed using three independent biological replicates, each analyzed in technical triplicate. Relative quantification was applied to assess variations in steady-state mRNA levels among samples, with expression normalized to an internal control RNA. Fold-change values were subsequently calculated according to the Pfaffl method (Pfaffl 2004). The primer sequences used in this study are shown in Table 2.

Statistical analysis

All experiments were performed using at least three independent biological replicates. Elastase and pyocyanin production assays were repeated in additional independent experiments to ensure reproducibility due to observed variability.

Data involving comparisons across multiple treatment concentrations were analysed using the Kruskal–Wallis non-parametric test, followed by Dunn's multiple comparisons post hoc test where appropriate. Pairwise comparisons were evaluated using the Mann–Whitney U test. Statistical analyses were conducted using GraphPad Prism 9 (GraphPad Software, USA). Differences were considered statistically significant at $p<0.05$. Statistical significance is indicated as follows: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

Results

QSI activity of vulpinic acid

The 800 µM concentration of vulpinic acid demonstrated inhibitory effects across all three QS systems: *las*, *rhl*, and *pqs*. Results shown in Fig. 1 are presented as relative fluorescence units (RFU) for GFP expression/bacterial cell growth to take growth rate into account and to show the decreased fluorescence is due to QS inhibition and not growth inhibition.

Measurements using fluorescent reporter strains demonstrated significant inhibition of all three *P. aeruginosa* QS systems at a vulpinic acid concentration of 800 µM. In addition, results show that other concentrations have also slightly inhibition activity on QS systems.

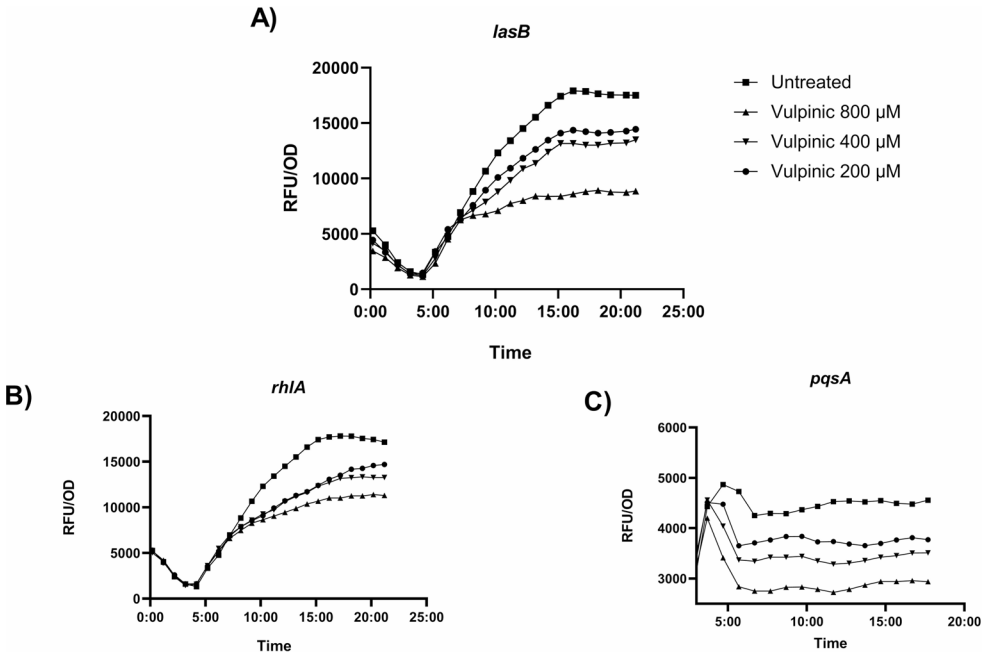

Fig. 1 QS inhibition graphics of vulpinic acid on *lasB* (**A**), *rhl* (**B**), *pqsA* (**C**) monitor strains. Data represent mean ± SD of three independent biological replicates and are shown as RFU over Optical Density (RFU/OD 450 nm)

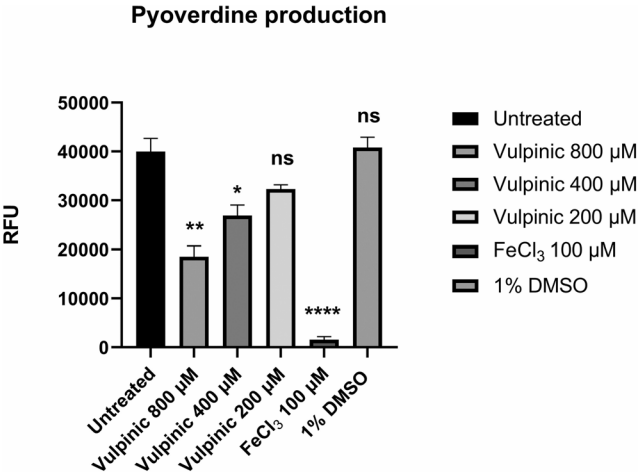

Fig. 2 Dose-dependent effects of vulpinic acid on pyoverdine production in *P. aeruginosa* PAO1. $FeCl_3$ (100 µM) was included as a positive control, and 1% (v/v) DMSO was used as the vehicle control. Data represent mean ± SD of three independent biological replicates. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Significance levels are shown as: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****), ns: not significant

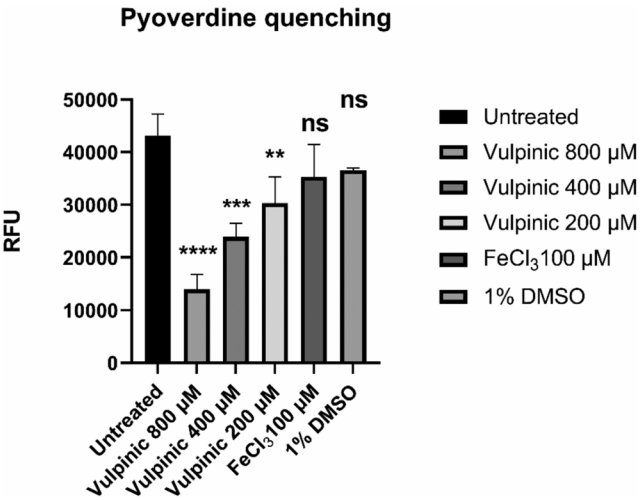

Fig. 3 Dose-dependent effects of vulpinic acid on pyoverdine quenching in *P. aeruginosa* PAO1. $FeCl_3$ (100 µM) was included as a positive control, and 1% (v/v) DMSO was used as the vehicle control. Data represent mean ± SD of three independent biological replicates. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Significance levels are shown as: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****), ns: not significant

Pyoverdine assays

Significant pyoverdine expression inhibition was observed for two concentrations of vulpinic acid. Fluorescence measurements in the graph are the means of three experiments. The results in the graph are presented as RFU. The inhibition capacity of vulpinic acid compared to the untreated control group is shown in Fig. 2.

The most pronounced inhibition effects were detected in both 800 µM and 400 µM vulpinic acid concentrations. In addition, 200 µM vulpinic acid concentration has a slight inhibitory activity on pyoverdine production, although experiment results were not statistically significant ($p>0.05$). The experimental findings indicated pyoverdine production inhibition rates of 53.65% ± 5.45 in 800 µM concentration and 32.76% ± 4.82 in 400 µM concentration of vulpinic acid.

The ability to quench pyoverdine fluorescence was observed for all concentrations of vulpinic acid. The results show the mean of three experiments. The quenching capacity of vulpinic acid compared to the untreated control group is shown in Fig. 3.

Quenching capacities for vulpinic acid were observed to be 67.55% ± 2.98 at 800 µM, 44.56% ± 3.01 at 400 µM, and 29.65% ± 5.78 at 200 µM, indicating a clear dose-response relationship.

Biofilm assay

The results show that there are no significant inhibition between the samples treated with vulpinic acid and untreated samples. Moreover, it was observed that all vulpinic acid concentrations seem to induce biofilm formation. Results are the mean of three experiments. The biofilm inhibition capacity of vulpinic acid compared to the untreated control group is shown in Fig. 4.

Virulence attenuation assays

Samples treated with vulpinic acid exhibited notable inhibition compared with the untreated negative control groups. No significant growth inhibition was observed. The activity levels of pyocyanin pigment production, protease, and elastase were quantified and are shown in Fig. 5. Data are expressed as percentages relative to the DMSO-treated control. At a concentration of 800 µM vulpinic acid, the inhibition of pyocyanin production reached 65.5% ± 8.23 (mean of five independent experiments), while elastase activity was reduced by 26.58% ± 5.66 (mean of four experiments). Furthermore, protease activity exhibited an inhibition of 85.04% ± 11.45 at the same concentration, based on the mean of three independent assays.

qRT-PCR analysis

qRT-PCR analysis revealed that treatment with vulpinic acid led to the downregulation of several genes associated

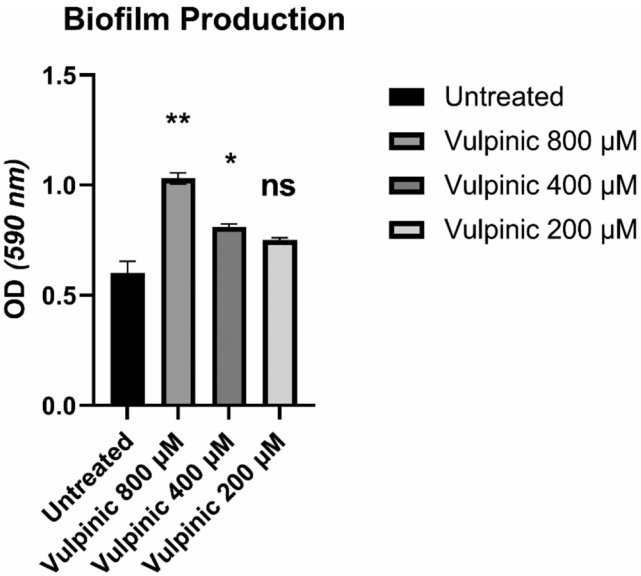

Fig. 4 Effects of vulpinic acid on biofilm formation in *P. aeruginosa* PAO1 compared to the DMSO 1% control. Data represent mean ± SD of three independent biological replicates. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Significance levels are shown as: $p < 0.05$ (*), $p < 0.01$ (**)

with QS and pyoverdine production. Gene expression levels after exposure to 800 µM vulpinic acid are presented in Fig. 6. A significant reduction in *pvdA* expression was observed compared with the 1% DMSO control ($p < 0.05$). *pvdS* expression was also decreased, but the change was not statistically significant ($p > 0.05$). The QS regulated genes *lasB* and *rhlA* were significantly downregulated ($p < 0.05$). *pqsA* expression showed a decreasing trend, although this did not reach statistical significance ($p > 0.05$).

Discussion

P. aeruginosa possesses numerous acquired resistance mechanisms including the low permeability of its outer membrane and its intrinsic, high adaptability (Sonnabend et al. 2020). Over time, it has developed antimicrobial resistance (AMR) to survive and resist various treatment methods (Jeong et al. 2023). Most of its resistance and virulence mechanisms are controlled via QS mechanisms and also by iron stress conditions (Schalk et al. 2011; Minandri et al. 2016; Gökalsın et al. 2017). In addition, pyoverdine and QS systems exhibit a functional linkage in the control of virulence (Fekete-Kertész et al. 2024). However, the QSI potential of molecules that inhibit pyoverdine production remains largely unexplored (Sepahi et al. 2015; Ramírez-Rueda and Salvador 2020; Fekete-Kertész et al. 2024).

In this research, for the first time, we tested the pyoverdine production inhibitory, pyoverdine quenching, QSI, and virulence modulation potential of vulpinic acid, a secondary metabolite of *Letharia vulpina* known for its medical uses for centuries (Hunn, 1990; Malhotra et al., 2007). Because fluorescence activity allows for the sensitive screening of gene expression inhibition, investigation using fluorescent-based biosensor strains provides an advantage. Since pyoverdine is a fluorescent pigment, monitoring the RFU rate to indicate gene expression–related inhibition level

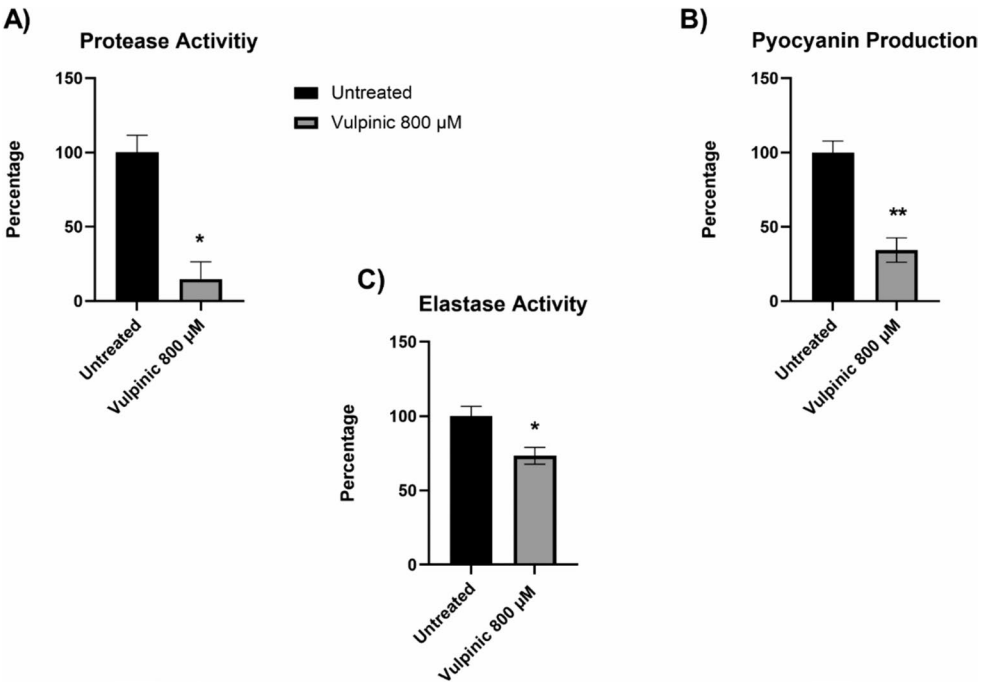

Fig. 5 Effects of vulpinic acid on selected virulence-associated phenotypes of P. aeruginosa PAO1. Pyocyanin production (**A**), elastase activity (**B**), and protease activity (**C**) were quantified following treatment with vulpinic acid. Data are presented as mean ± SD from three independent biological replicates. Comparisons were performed relative to untreated samples using an unpaired non-parametric Mann–Whitney U test. Significance levels are shown as: $p < 0.05$ (*), $p < 0.01$ (**)

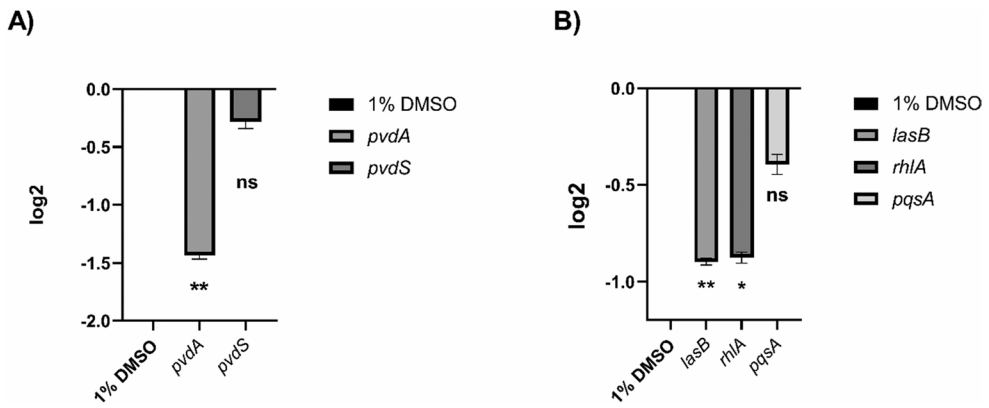

Fig. 6 Effect of vulpinic acid on the expression levels of *pvdA* and *pvdS* genes involved in pyoverdine biosynthesis (**A**), and *lasB*, *rhlA*, and *pqsA* genes associated with QS in *P. aeruginosa* PAO1 (**B**). Vulpinic acid was applied at a concentration of 800 µM, and 1% DMSO was used as control. Data are presented as mean ± SD from three independent biological replicates, each in technical triplicate. Statistical significance was evaluated using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Significance levels are shown as: $p < 0.05$ (*), $p < 0.01$ (**)

provides a reliable result (Kirienko et al. 2019). Therefore, we assessed the pyoverdine production inhibitory and pyoverdine quenching potency of vulpinic acid on *P. aeruginosa* PAO1 – Wild Type strain via monitoring RFU rates. In addition, owing to the advantage of screening RFU value, we tested QSI potency of vulpinic acid via biosensor strains (*lasB-gfp*, *rhlA-gfp*, *pqsA-gfp)*.

Our experiment results show that vulpinic acid has an inhibitory effect on in vitro pyoverdine production. Inhibitory potential on siderophore structure by using natural-based compounds has been indicated in previously conducted experiments (Adonizio et al. 2008; Xu et al. 2019b; Meena et al. 2020). In addition to inhibiting pyoverdine synthesis, vulpinic acid also demonstrated a significant pyoverdine quenching property. In this context, we conclude that vulpinic acid largely inhibits pyoverdine production and, furthermore, may also inhibit the activity of pre-existing pyoverdine. This dual function (synthesis inhibition and direct quenching) suggests vulpinic acid employs a highly effective strategy for neutralizing pyoverdine activity regardless of the pigment's concentration.

To investigate the molecular mechanism behind pyoverdine production inhibition, we used the *pvdS* and *pvdA* genes. *pvdS* is a sigma factor that regulates pyoverdine production under iron stress conditions (Cornelis et al. 2023). *pvdA* is a *pvdS*-dependent pyoverdine synthesis enzyme gene (Kirienko et al. 2019). Our results show that vulpinic acid downregulated *pvdA* gene significantly. *pvdS* gene was also downregulated slightly but the result is not statistically significant. The reason for this may be because *pdvS* is not the only iron uptake regulator for pyoverdine (Schalk and Perraud 2023). FpvI is another sigma factor for pyoverdine production (Ringel and Brüser 2018). We hypothesize that vulpinic acid may have interacted with PvdS sigma factor or downregulates FpvI sigma factor transcription; thus reducing *pvdA* expression.

Since iron is an important element for biofilm formation, we first hypothesized that pyoverdine production inhibition would also reduce biofilm formation (Das et al. 2020; Kang et al. 2021; Díaz-Pérez et al. 2023). Contrary to this expectation, vulpinic acid did not reduce biofilm formation under the conditions tested, despite its inhibitory effect on pyoverdine production. An increase in biofilm was observed at all applied concentrations. Vulpinic acid is a relatively hydrophobic molecule, with a predicted LogP value of approximately 3.0 (PubChem, XLogP3). Since *P. aeruginosa* preferentially adheres to hydrophobic surfaces, it is possible that surface interactions induced by vulpinic acid contributed to increase. This explanation remains hypothetical and requires further investigation. These results highlight that inhibition of the selected virulence factors does not necessarily result in a global reduction of biofilm formation. Therefore, it is important to evaluate multiple virulence factors when assessing the antivirulence potential of natural compounds.

We used the *gfp* strains *lasB-gfp*, *rhlA-gfp*, *pqsA-gfp* to investigate vulpinic acid's inhibitory potency on Las and Rhl systems. QS systems play a key role in managing virulence (Hamza et al. 2023). In addition, the QS system also plays a role in pyoverdine secretion, and therefore, evaluation of the QS inhibition activity of vulpinic acid was considered relevant in the context of its observed effects on siderophore biosynthesis. The reporter assays demonstrated that vulpinic acid at 800 µM significantly reduced *lasB* and *rhlA* promoter activity, while lower concentrations exhibited more modest effects. Inhibition of the Las system has previously been associated with reduced expression of QS-dependent virulence factors,

including elastase and other proteases, as well as alterations in pyoverdine production (Liu et al. 2020). Consistent with these observations, qRT-PCR analysis in the present study revealed downregulation of *lasB* and *rhlA* expression.

This study provides in vitro data that the lichen-derived secondary metabolite vulpinic acid modulates selected virulence-associated traits of *P. aeruginosa*. Since targeting siderophore biosynthesis represents a relevant antivirulence approach, identification of natural compounds that interfere with iron acquisition is an important area of investigation. Our findings show that vulpinic acid affects pyoverdine synthesis and quenching in addition to QS regulatory systems under laboratory conditions. This supports the potential of lichens as a source of natural compounds with antivirulence related activity. On the other hand, no biofilm inhibition was found. In addition, these effects were observed exclusively in vitro and do not directly demonstrate attenuation of pathogenicity or therapeutic efficacy. Consequently, the clinical relevance of vulpinic acid remains hypothetical. Further studies with host infection models will be required to determine whether such in vitro effects can be translated into meaningful antivirulence strategies against *P. aeruginosa* infections.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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