Infection and Drug Resistance

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

The Role of Yinqiao Powder in Modulating *Pseudomonas aeruginosa* Biofilm and Virulence Factors

Honglin Li[1], Shebin Zhang[2], Qiwei Li[2], Yurong Shu[3], Song Li[2], Bo Wu[4], Zhenjie Xu[2]

[1]Department of Clinical Laboratory, Mianyang Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Mianyang, 621000, People's Republic of China; [2]Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, 510120, People's Republic of China; [3]Department of Clinical Laboratory, South China Agricultural University Hospital, Guangzhou, 510000, People's Republic of China; [4]Department of Transfusion, The Fourth Affiliated Hospital, Guangzhou Medical University, Guangzhou, 511300, People's Republic of China

Correspondence: Zhenjie Xu, Department of Laboratory Medicine, The Second Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine, 111 Dade Road, Yuexiu District, Guangzhou, 510120, People's Republic of China, Tel/Fax +86-02039318325, Email 15989085911@163.com; Bo Wu, Department of Transfusion, The Fourth Affiliated Hospital, Guangzhou Medical University, Guangzhou, 511300, People's Republic of China, Tel/Fax +86-02062287542, Email meteor.bullet@163.com

Purpose: It is now understood that the primary challenges in treating *Pseudomonas aeruginosa* (*P. aeruginosa*) infections are the secretion of multiple virulence factors, the formation of biofilm, and the emergence of multi-drug resistance. Small regulatory RNAs (sRNAs) and quorum sensing (QS) play an important role in regulating bacterial biofilms and multiple virulence factors, presenting potential targets for novel anti-*P. aeruginosa* therapies. Yinqiao Powder has demonstrated inhibitory activity against various bacteria and viruses. The objective of this study was to elucidate the precise mechanism of Yinqiao Powder's impact on *P. aeruginosa* virulence and to ascertain its clinical utility.

Methods: First, the effects of Yinqiao Powder on various virulence factors of *P. aeruginosa* were assessed through virulence phenotype experiments, including biofilm formation assay, pyocyanin production assay, rhamnolipid assay, and motility assay. Then, a cytotoxicity assay was used to evaluate the effect of *P. aeruginosa* treated by Yinqiao Powder on cells. Finally, an RT-qPCR assay was used to detect the effects of Yinqiao Powder on QS system and virulence-related gene expression.

Results: This study revealed that sub-minimum inhibitory concentration (sub-MIC) levels of Yinqiao Powder significantly inhibit biofilm formation, swarming motility, pyocyanin and rhamnolipid production in a dose-dependent manner. The cytotoxicity assay also confirmed that Yinqiao Powder weakened the cytotoxicity of *P. aeruginosa*. Furthermore, Yinqiao Powder was found to modulate the *P. aeruginosa* sRNA-QS-virulence network. Specifically, it repressed the *lasI*, the *rhlI*, and sRNA P27 while upregulating sRNA PhrD. Additionally, the *phzA* and *pqsA* genes, associated with pyocyanin and rhamnolipid/biofilm regulation, respectively, were repressed by Yinqiao Powder.

Conclusion: Yinqiao Powder effectively inhibits QS system-related regulatory genes, sRNAs, biofilm formation, swarming motility, pyocyanin and rhamnolipid production at specific concentrations. These results support the potential of Yinqiao Powder as a quorum-sensing inhibitor.

Keywords: Yinqiao Powder, quorum sensing, sRNA, biofilm formation, virulence, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a Gram-negative, opportunistic pathogen that ranks among the leading causes of nosocomial infections. Human health and food processing industries like dairy, poultry, ready-to-eat food, meat, and aquaculture have been severely affected by biofilm-producing *P. aeruginosa*.[1–3] This bacterium is particularly prevalent in immunocompromised individuals with cystic fibrosis (CF), implanted medical devices, and chronic wound infection.[4] The primary challenges in treating *P. aeruginosa* infections are the secretion of multiple virulence factors, the formation of biofilm, and the development of multi-drug resistance (MDR).[5] The biofilms of *P. aeruginosa*

Received: 28 November 2024
Accepted: 4 March 2025
Published: 12 March 2025

confer bacterial drug resistance by impeding antimicrobial penetration, inducing a slow-growth phenotype through microenvironmental alterations, and stimulating adaptive stress responses and cellular differentiation.[6,7]

In addition, the bacterium's motility and secretion of extracellular virulence factors, such as lectin, elastase, alkaline protease, pyocyanin, rhamnolipids are critical to its pathogenesis. Consequently, it is widely believed that elucidating the mechanisms by which *P. aeruginosa* regulates the expression of virulence genes is essential for developing alternative therapeutic strategies to control and prevent bacterial infections.[8]

Quorum sensing (QS) is a cell-density-dependent intercellular communication system that relies on the synthesis and detection of diffusible signal molecules, including the LasI/LasR (*las*), and RhlI/RhlR (*rhl), Pseudomonas quinolone signal* (*pqs*), and *iqs* systems.[9–11] An increasing body of evidence suggests that these QS systems are critical regulators of virulence, swarming motility, biofilm formation, and antibiotic resistance in *P. aeruginosa*.[12–14] Notably, two homologous N-acylhomoserine lactone (AHLs) QS systems, the LasI/LasR and RhlI/RhlR systems, are primarily responsible for activating these associated behaviors.[15]

In the *P. aeruginosa* QS systems LasI/LasR and RhlI/RhlR, the signal molecules N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-L-homoserine lactone (C4-HSL), respectively, interact with the transcriptional regulators LasR and RhlR to activate multiple virulence factors, including lectin, elastase, pyocyanin, alkaline protease, rhamnolipids, and exotoxin A.[16] In addition, the *pqs* system, a group of compounds known as 4-hydroxy-2-alkylquinolines (HAQs), upregulates the expression of virulence-associated genes by interacting with the LasI/LasR and RhlI/RhlR systems. Furthermore, QS systems interact with small regulatory RNAs (sRNAs) to modulate virulence factor expression. Studies have shown that sRNA PhrD stimulates the translation of *rhlR*, while another sRNA, P27, directly represses the translation of *rhlI* mRNA by *P. aeruginosa*.[17,18]

In recent years, the widespread application of antibiotics in clinical practice has led to the emergence of multi-drug resistant *P. aeruginosa*, which has caused great difficulties in clinical treatment. With the onset of the "post-antibiotic era", there has been a growing inclination towards non-antibiotic therapeutic treatments. Anti-QS drugs are a novel anti-virulence and anti-biofilm approach to target pseudomonal infections.[10] Inhibition of the QS could be achieved by-quorum quenching enzymes (such as AHL-lactonases, AHL-acylases, and AHL-oxidoreductase) and quorum sensing inhibitor which could be synthesized or derived from microorganisms, animals, or bioactive phytochemicals (such as curcumin, geraniol, and ajoene).[10,11,19] Yinqiao Powder, a classical formulation of Traditional Chinese Medicine (TCM) renowned for its heat-clearing and detoxification properties, exhibits low toxicity and a reduced propensity to induce antibiotic resistance.[20] Contemporary research has unveiled its anti-inflammatory and anti-allergic attributes.[21] Previous studies suggest Yinqiao Powder may inhibit *P. aeruginosa* biofilm formation, potentially influencing the pathogen's virulence.[22,23] Nevertheless, comprehensive clinical and fundamental investigations are imperative to elucidate the precise mechanism of Yinqiao Powder's impact on *P. aeruginosa* virulence and to ascertain its clinical utility.

Herein, we demonstrated that Yinqiao Powder effectively suppressed *P. aeruginosa* biofilm formation and virulence, as evidenced by reduced pyocyanin and rhamnolipid production and impaired swarming motility. Notably, sRNAs PhrD and P27 may contribute to Yinqiao Powder's suppression of *P. aeruginosa* virulence.

Materials and Methods

Bacterial Strains and Growth Conditions

Strain utilized in this study is *P. aeruginosa* wild-type strain PAO1 as described in our previous work.[12] Unless otherwise specified, *P. aeruginosa* clones isolated from blood plates were cultured overnight at 37°C in Luria-Bertani broth (LB, Oxoid) with 200 rpm agitation, subsequently washed twice with 0.9% NaCl, added 100 μL of cultures to 3 mL LB, and incubated to mid-log phase (0.5 McFarland, 1.5×10^8 CFU/mL) prior to experimental procedures. When required, Yinqiao Powder (Fengchun, Zhongjiang, Sichuan, China) was added at concentrations of 256, 128, 64, or 32 mg/mL.

MIC of Yinqiao Powder

The MIC of Yinqiao Powder was determined using the agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (M100). Yinqiao Powder was incorporated into Mueller–Hinton agar (MHA,

Oxoid) at concentrations ranging from 16 to 512 mg/mL. About 2 μL of a *P. a*eruginosa suspension standardized to a 0.5 McFarland turbidity standard was inoculated onto each agar plate. After incubation at 37°C for 20 hours, the minimum drug concentration that exhibited no visible bacterial growth was defined as the MIC.

Biofilm Formation Assay

The detection of biofilm was performed as described previously by Jieying Pu.[24] Briefly, 100 μL of bacteria suspension at 0.5 McFarland were inoculated into LB supplemented with varying concentrations of Yinqiao Powder in 96-well plates and grown at 37°C for 24 hours. Non-adherent cells were removed by washing with 0.9% NaCl, and adherent biofilms were fixed with methanol, stained with crystal violet, and quantified by measuring the absorbance at OD600 of ethanol-extracted crystal violet.

Pyocyanin Production Assay

Pyocyanin was extracted as described previously.[25] *P. aeruginosa* cultures (0.5 McFarland) were added to the LB medium supplemented with Yinqiao Powder (60 mL culture, 6 mL LB) and incubated at 37°C with shaking (200 rpm) for 20 hours. Subsequently, 3 mL of chloroform was added to 5 mL of the culture supernatant containing pyocyanin, followed by vigorous shaking and centrifugation at 4000 rpm for 5 minutes. The chloroform layer was transferred to a test tube containing 0.5 mL of 0.2 N HCL and shaken vigorously until a pink color developed before centrifugation. The absorbance of the culture and the extracted upper layer solution was measured at 600 and 520 nm, respectively. Pyocyanin production, expressed as micrograms per milliliter of culture supernatant, was calculated using the formula: (A520/A600) × 17.072.[26]

Rhamnolipid Assay

A rhamnolipid assay was conducted following our previous experimental method.[12] Briefly, the indicated bacteria suspension at 0.5 McFarland were inoculated into M9 medium supplemented with varying concentrations of Yinqiao Powder and incubated at 37°C, with 200 rpm shaking for 16 hours. Subsequently, the culture supernatant was acidified to pH 2.5 ± 0.2 using 40 μL of 1 N HCl, followed by extraction of rhamnolipids with 4 mL of chloroform through vigorous shaking for 4 minutes. After the phase separation, 100 μL of 1 g/l methylene blue and 2 mL of distilled water were added to 3 mL of the chloroform extract. The absorbance of the chloroform layer at 638 nm was measured to determine the rhamnolipid concentration.

Motility Assay

Bacterial strains were cultured to mid-log phase in LB before a 5 μL aliquot was inoculated onto swarming medium, composed of LB supplemented with varying concentrations of Yinqiao Powder, 5 g/l glucose, and 0.5% agar.[27] After a 10-hour incubation at 37°C, swarming motility was quantified by measuring the maximum diameter of the circular bacterial migration pattern.

Cytotoxicity Assay

A549 cells (ATCC: CCL-185) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2 for 14 hours. *P. aeruginosa* cultures (0.5 McFarland) were added to LB medium supplemented with Yinqiao Powder (0, 32, 64 or 128 mg/mL) and incubated at 37°C with shaking (200 rpm) for 2 hours to reach the exponential growth phase, subsequently washed and resuspended with PBS. The resuspended PAO1 strains were inoculated into A549 cells (2×10^5 per well) in 24-well plates at a multiplicity of infection (MOI) of 100. The cultures were incubated in DMEM medium supplemented with 1% FBS for 10 hours at 37°C with 5% CO2. Supernatants were collected from 24-well plates, and cytotoxicity was determined with the LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China).

RT-qPCR Assay

Primer sequences are provided in Table S1. To assess gene expression, we added 30 μL *P. aeruginosa* cultures at 0.5 McFarland to 3 mL of LB supplemented with Yinqiao Powder (0, 32, 64 or 128 mg/mL) and re-incubated at 37°C, with

200 rpm shaking for 4, 12, or 20 hours. Total RNA was extracted using RNAiso Plus reagent (Takara, Dalian, Liaoning, China), quantified using a NanoDrop 2000 BioSpectrometer (Eppendorf), and converted to cDNA using PrimeScript RT Reagent Kit (Takara, Dalian, Liaoning, China). RT-qPCR was performed with SYBR Green qPCR Master Mix (Takara, Dalian, Liaoning, China) on a ViiA^{TM} 7 Dx system (Applied Biosystems, Foster, CA, USA), using *rpoD* as a reference gene for normalization of target gene expression via the $2^{-\Delta\Delta Ct}$ method.[28]

Statistical Analysis

Data were analyzed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) and are presented as mean ± standard deviation (SD). Statistical significance was determined by Student's *t*-test. ns, not significant; *$0.01 < P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Results

The Minimum Inhibitory Concentration (MIC) of Yinqiao Powder

To determine the MIC of Yinqiao Powder against *P. aeruginosa* strain PAO1, a microdilution assay was performed. Serial dilutions of Yinqiao Powder were prepared and inoculated with a standardized suspension of PAO1. Following 20 hours of incubation, bacterial growth was assessed visually. Colony formation was observed at concentrations ranging from 0 to 256 mg/mL, with a corresponding decrease in colony diameter as drug concentration increased. No visible growth was detected at 512 mg/mL (Figure 1). Based on these results, the MIC of Yinqiao Powder against PAO1 was established as 512 mg/mL.

Yinqiao Powder Inhibits the Biofilm Formation and the Virulence of P. aeruginosa

Yinqiao Powder Inhibits the Biofilm Formation and the Virulence Factors of P. aeruginosa

To delve into the effects of sub-MIC levels of Yinqiao Powder on *P. aeruginosa* virulence, *P. aeruginosa* strain PAO1 was exposed to concentrations of 128 mg/mL, 64 mg/mL, and 32 mg/mL, corresponding to 1/4th, 1/8th, and 1/16th of the minimum inhibitory concentration, respectively. A dose-dependent decrease in biofilm formation was observed (Figure 2A). This finding suggests that Yinqiao Powder can inhibit biofilm formation by *P. aeruginosa*. To further

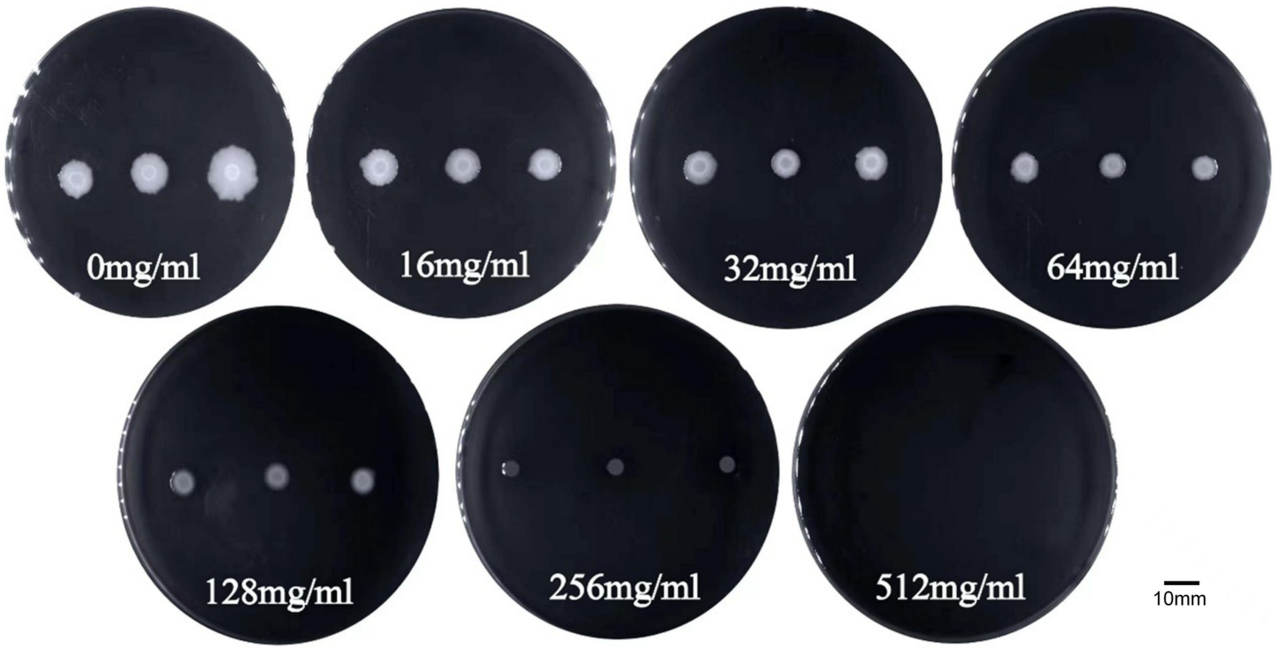

Figure 1 The MIC of Yinqiao Powder. PAO1 was inoculated quantitatively onto plates containing various drug concentrations and grown to the mid-log phase. Growth of *P. aeruginosa* was assessed after 20 hours of incubation at 37°C.

clarify the impact of Yinqiao Powder on virulence, the synthesis of rhamnolipid and pyocyanin, as well as swarming motility, were assessed in PAO1. When exposed to various sub-MIC concentrations of Yinqiao Powder, PAO1 exhibited a dose-dependent decrease in pyocyanin and rhamnolipid synthesis compared to the control group (0 mg/mL) (Figure 2B and C). Moreover, the swarming motility assay revealed a concentration-dependent reduction in colony diameter compared to the control group (Figure 3). Collectively, these findings indicate that Yinqiao Powder exerts a negative regulatory influence on the synthesis of rhamnolipid and pyocyanin, as well as swarming motility in *P. aeruginosa*.

Yinqiao Powder Protects Cell From Inactivation by P. aeruginosa

To evaluate the cytotoxic effects of *P. aeruginosa* treated with varying concentrations (0, 32, 64, and 128 mg/mL) of Yinqiao Powder on A549 cells, a lactate dehydrogenase (LDH) release assay was performed. The results demonstrated that the cytotoxicity of A549 cells induced by *P. aeruginosa* exposed to sub-MIC concentrations of Yinqiao Powder exhibited a dose-dependent reduction (Figure 4). Specifically, compared with the control group (*P. aeruginosa* without Yinqiao Powder), the cytotoxicity of A549 cells induced by *P. aeruginosa* treated with 64 mg/mL and 128 mg/mL of

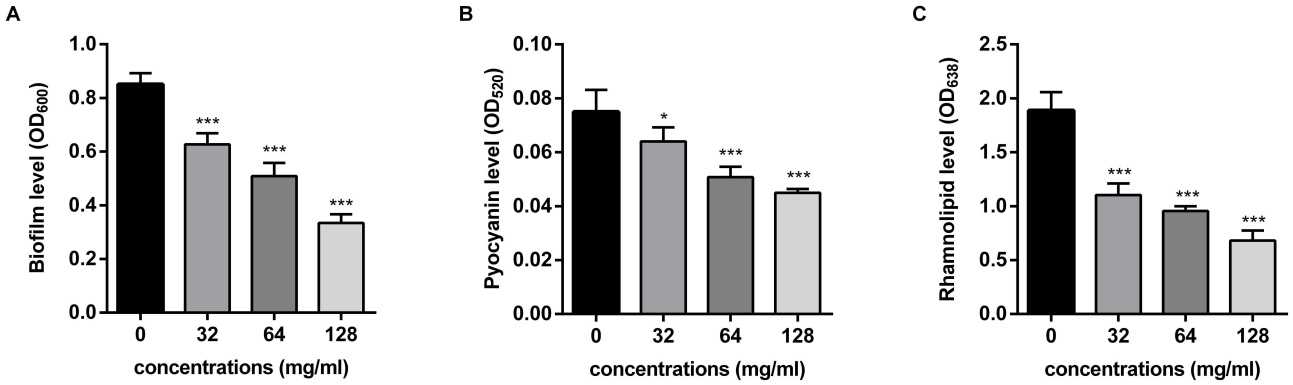

Figure 2 The sub-MIC concentrations of Yinqiao Powder influence the biofilm formation, pyocyanin and rhamnolipid synthesis of *P. aeruginosa*. (**A**) The sub-MIC concentrations of Yinqiao Powder inhibited biofilm formation. The indicated PAO1 strains were cultured in LB in the 96-well plates at 37°C for 24 hours, and biofilm in the supernatant was determined at OD600. (**B**) The sub-MIC concentrations of Yinqiao Powder inhibited pyocyanin production. The indicated PAO1 strains were cultured in LB at 37°C for 20 hours, and pyocyanin in the supernatant was determined at OD520. (**C**) The sub-MIC concentrations of Yinqiao Powder inhibited rhamnolipid synthesis. The indicated PAO1 strains were cultured in M9 medium at 37 °C for 16 hours, and rhamnolipid in the supernatant was measured. Values are the mean ± SD of three independent experiments. **P*< 0.05; ****P*< 0.001.

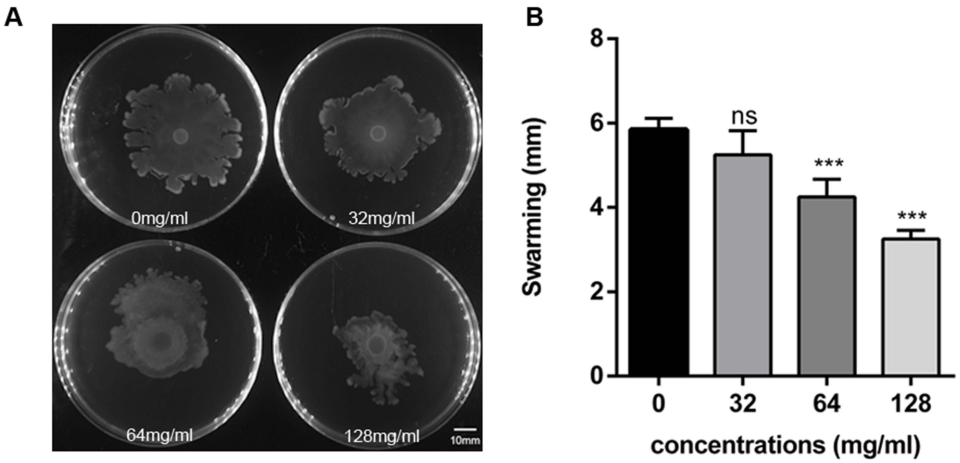

Figure 3 The sub-MIC concentrations of Yinqiao Powder influence swarming motility of *P. aeruginosa*. 5 μL cultures of the indicated strains were spotted onto the swarming medium and incubated at 37°C for 10 hours, and swarming motility was quantified by measuring the maximum diameter. (**A**) Image of swarming medium. (**B**) The swarming diameter statistics. Values are the mean ± SD of three independent experiments. ****P* < 0.001.
Abbreviation: ns, not significant.

https://doi.org/10.2147/IDR.S507257

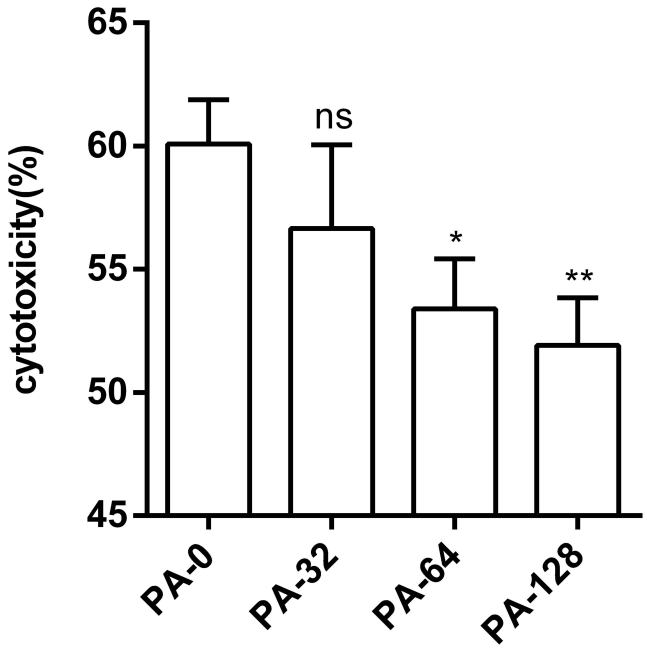

Figure 4 The cytotoxic effects of *P. aeruginosa* treated by Yinqiao Powder on cells. After the *P. aeruginosa* strains treated by Yinqiao Powder (0, 32, 64 and 128 mg/mL) were co-cultured with the A549 cells for 10 hours, the supernatants from the 24-well plates were collected, and cytotoxicity was detected by LDH Cytotoxicity Assay Kit. Cytotoxicity was quantified by measuring LDH release. Values are mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$.
Abbreviation: ns, not significant.

Yinqiao Powder was significantly decreased to 53.40% and 51.92%, respectively. These findings suggest that Yinqiao Powder can effectively attenuate the virulence of *P. aeruginosa* in a dose-dependent manner.

Yinqiao Powder Suppresses the Expression of lasI, rhlI, pqsA, and phzA Genes in P. aeruginosa

In *P. aeruginosa*, the synthesis of virulence factors and the formation of biofilm are primarily regulated by the QS system.[15,29] Based on the outcomes of the virulence tests, we hypothesized that Yinqiao Powder might exert a direct regulatory effect by modulating QS-associated virulence genes. To investigate the underlying molecular mechanism of Yinqiao Powder's influence on virulence traits, we quantified the gene expression relative levels of *lasI, rhlI, pqsA*, and *phzA* in PAO1 exposed to sub-MIC concentrations of Yinqiao Powder at 4, 12, and 20 hours (Figure 5A–C). As illustrated in Figure 5, the expression of virulence-associated genes such as *lasI, rhlI, pqsA*, and *phzA* were down-regulated following sub-MIC concentrations of Yinqiao Powder treatment. The Yinqiao Powder-treated group had the strongest inhibitory effect on QS-related gene expression at 20 h.

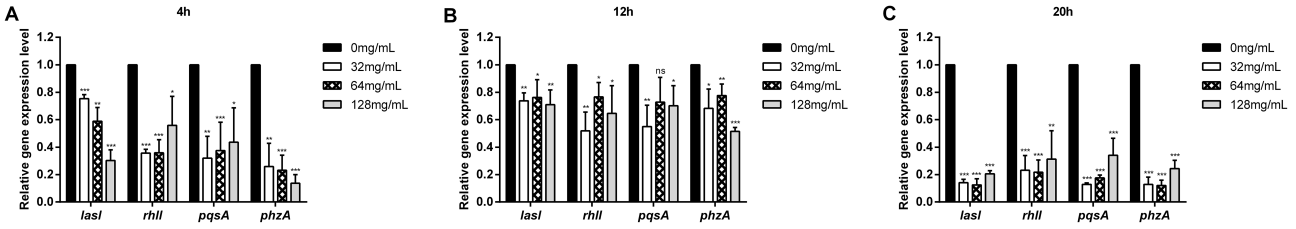

Figure 5 The influence of Yinqiao Powder on the expression of *lasI, rhlI, pqsA*, and *phzA*. (**A**–**C**) *P. aeruginosa* strain PAO1 in the exponential growth phase was treated with various concentrations (0, 32, 64, 128 mg/mL) of Yinqiao Powder for 4 hours (**A**), 12 hours (**B**) and 20 hours (**C**). Gene expression levels of *lasI, rhlI, pqsA*, and *phzA* were assessed using RT-qPCR. The *rpoD* gene was used as an internal control. Values are mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Abbreviation: ns, not significant.

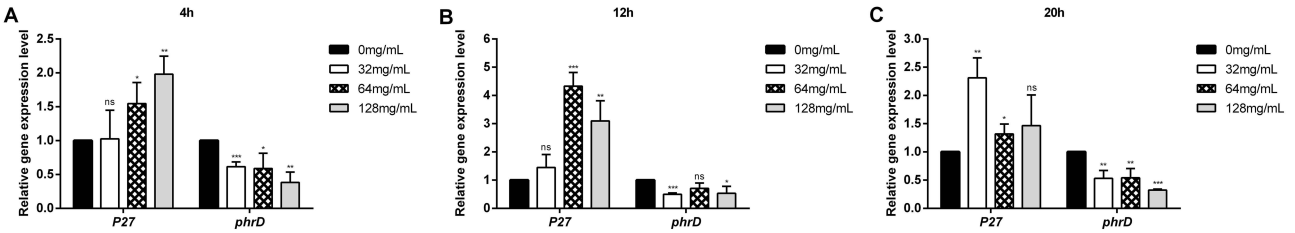

Figure 6 The influence of Yinqiao Powder on the expression of sRNA P27 and PhrD. (**A**–**C**) *P. aeruginosa* strain PAO1 in the exponential growth phase was treated with various concentrations (0, 32, 64, 128 mg/mL) of Yinqiao Powder for 4 hours (**A**), 12 hours (**B**) and 20 hours (**C**). Gene expression levels of *P27* and *phrD* were assessed using RT-qPCR. The *rpoD* gene was used as an internal control. Values are mean ± SD of three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.
Abbreviation: ns, not significant.

Yinqiao Powder Regulates sRNA P27 and PhrD Expression

Quantitative real-time PCR (RT-qPCR) and transcriptome sequencing analyses suggested a regulatory interplay between multiple small regulatory RNAs (sRNAs) and the QS system.[12,24] Based on these findings, we hypothesized that Yinqiao Powder exerts its pharmacological effects by modulating the sRNA-QS-virulence factor signaling pathway. To validate this hypothesis, we measured the expression of sRNAs P27 and PhrD under sub-MIC concentrations of Yinqiao Powder (Figure 6A–C). Our results demonstrated that sub-MIC Yinqiao Powder upregulated P27 expression while downregulating PhrD expression, suggesting a regulatory influence of Yinqiao Powder on sRNA expression.

Discussion

In this study, phenotypic experiments demonstrated that Yinqiao Powder inhibited *P. aeruginosa* biofilm formation and the synthesis of multiple virulence factors, including pyocyanin and rhamnolipid, as well as swarming motility. These processes are known to be linked to QS and sRNAs. Subsequent RT-qPCR analysis confirmed the regulatory effects of Yinqiao Powder on QS-associated genes, their downstream virulence genes, and the sRNAs P27 and PhrD. Our findings elucidate the regulatory impact of Yinqiao Powder on *P. aeruginosa* virulence and suggest that its pharmacological effects may be mediated through the sRNA-QS-virulence factors signaling pathway.

At the top of the QS systems network hierarchy in *P. aeruginosa* is the *las* system, which positively regulates the expression of the other three systems. The *rhl* system operates hierarchically under the control of the *las* system.[9] The *pqs* system can be positively regulated by *las* system and negatively by *rhl* system.[30] Similarly, the *iqs* system has a stimulatory effect on *pqs* and *rhl* systems.[14] Together, these QS systems form a complex regulatory network that allows *P. aeruginosa* to adapt to various environmental conditions. QS systems play a crucial role in regulating biofilm formation and the synthesis of virulence factors, including rhamnolipid, alkaline protease, elastase, pyocyanin, and swarming motility in *P. aeruginosa*.[31] QS represents a promising therapeutic target for suppressing biofilm and virulence. Currently, numerous studies have demonstrated that phytochemicals possess significant quorum quenching properties, which in turn inhibit biofilm formation and the production of virulence factors in *P. aeruginosa*. Carotenoid zeaxanthin has been shown to inhibit the formation of biofilm by targeting the LasI/LasR and RhlI/RhlR systems of *P. aeruginosa*. Similarly, plant flavonoid quercetin demonstrates medicinal properties by suppressing pyocyanin production and biofilm formation in *P. aeruginosa*.[32,33] Additionally, study has found that geraniol can suppress the biofilm and virulence factors of *P. aeruginosa* by inhibiting the three QS systems of *las, rhl*, and *pqs*.[19] These findings collectively indicate that the promising role of phytochemicals in the development of novel anti-virulence therapies.

Yinqiao Powder, a traditional Chinese medicine, possesses heat-clearing, detoxifying, anti-inflammatory, and detumescent properties. Although its bacteriostatic efficacy may be less potent than antibiotics, it offers several advantages, including holistic conditioning, abundant natural sources, a safe usage profile, and a reduced risk of inducing bacterial drug resistance. This study demonstrated that sub-MIC concentrations of Yinqiao Powder (64mg/mL, 128mg/mL, 256mg/mL) significantly suppressed virulence phenotypes, including biofilm formation, swarming motility, pyocyanin and rhamnolipid production, in a dose-dependent manner. The cytotoxicity assay also demonstrated that the Yinqiao Powder treatment reduced the cytotoxic effects of *P. aeruginosa* on cells. Forsythia suspense, one of the components of

Yinqiao powder, has been found to inhibit the production of QS-regulated virulence factors (such as pyocyanin, elastase and swimming motility) and biofilm formation in *P. aeruginosa*.[34] Their results were similar to ours, but we concomitantly verified the expression of virulence-associated genes, such as *lasI, rhlI, pqsA*, and *phzA*, were downregulated upon the Yinqiao Powder treatment. Those results are corroborating a previous study.[35] Nevertheless, the variance resides in that Liang's results demonstrated that the MIC of Yinqiao Powder was 500 mg/mL, while ours was 512 mg/mL. The reason for this difference was that the concentration gradients set by the two were not consistent. Our study was conducted in accordance with the requirements of CLSI, so our MIC should be more accurate. In addition, the downregulation of *rhlI* and *pqsA* did not demonstrate a dose-dependent pattern; instead, the inhibitory effect decreased with higher concentrations of Yinqiao Powder. One possible reason is that Yinqiao Powder modulates the expression of *rhlI* and *pqsA* by influencing multiple QS systems, or it may regulate the expression of *rhlI* and *pqsA* at the post-transcriptional level. In *P. aeruginosa*, the medicinal herb extracts completely inhibited the *pqs* system, moderately inhibited the *rhl* system, and slightly inhibited the *las* system, suggesting that medicinal herb extracts may selectively target the QS system to inhibit bacterial virulence and ensuring that gene expression remains stable despite external interventions.[36] Other studies have demonstrated that the used different doses of carvacrol affected the transcription levels of *lasR* gene, while, *lasI* gene was not affected. This result indicated a specific post-translational effect of carvacrol against LasI.[37] Our findings substantiate that Yinqiao Powder can effectively inhibit the formation of biofilm and the synthesis of multiple virulence factors by modulating QS system, thereby attenuating bacterial pathogenicity.

In *P. aeruginosa*, sRNAs are key regulators of virulence phenotype, interacting with the QS system to facilitate adaptation to diverse environments.[38,39] Previous studies have demonstrated that PrrH, regulated by RhlR, controls pyocyanin synthesis through its interaction with *phzC/D*,[12] while sRNAs P27 and PhrD, in complex with Hfq, respectively, downregulate and upregulate the QS system.[17,18] Our findings indicate that Yinqiao powder alters the expression of P27 and PhrD, suggesting a potential mechanism of action involving the sRNA-QS-virulence factor axis. To elucidate this further, future studies will delve deeper into the molecular underpinnings of Yinqiao powder's effects on *P. aeruginosa* virulence and biofilm formation.

Conclusion

In summary, the experimental findings substantiate the hypothesis that Yinqiao Powder possesses antibacterial properties mediated by inhibiting QS and sRNA in *P. aeruginosa*. This inhibitory effect is manifested in the suppression of biofilm formation, pyocyanin and rhamnolipid synthesis, and swarming motility. Consequently, these results validate the potential of Yinqiao Powder as a quorum-sensing inhibitor and elucidate a mechanistic pathway involving sRNA-QS-virulence factor signaling. This study provides a foundational framework for exploring the therapeutic efficacy of Yinqiao Powder in the management of chronic *P. aeruginosa* infections.

Abbreviations

QS, quorum sensing; sRNAs, small regulatory RNAs; P. aeruginosa, Pseudomonas aeruginosa; RT-qPCR, quantitative real-time PCR; pqs, Pseudomonas quinolone signal; MIC, minimum inhibitory concentration; LDH, lactate dehydrogenase; LB, Luria-Bertani broth; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum.

Acknowledgments

We are very grateful to the Clinical Laboratory of the Second Affiliated Hospital of Guangzhou University of Chinese Medicine for providing us with an experimental platform.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the Research Projects of Guangdong Provincial Administration of Traditional Chinese Medicine (Grant Number.20231111).

Disclosure

No potential conflict of interest was reported by the authors.

References

1. Rather MA, Gupta K, Bardhan P, et al. Microbial biofilm: a matter of grave concern for human health and food industry. *J Basic Microb*. 2021;61:380–395. doi:10.1002/jobm.202000678
2. Zhou J, Liang Z, Yin K, et al. Quorum sensing inhibitor: an effective strategy to attenuate the virulence and drug resistance of *Pseudomonas aeruginosa*. *Food Med Homol*. 2025. doi:10.26599/FMH.2025.9420066
3. Cai W, Arias CR. Biofilm formation on aquaculture substrates by selected bacterial fish pathogens. *J Aquat Anim Health*. 2017;29:95–104. doi:10.1080/08997659.2017.1290711
4. Pang Z, Raudonis R, Glick BR, Lin T, Cheng Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol Adv*. 2019;37:177–192. doi:10.1016/j.biotechadv.2018.11.013
5. Moradali MF, Ghods S, Rehm BHA. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Front Cell Infect Microbiol*. 2017;7:39. doi:10.3389/fcimb.2017.00039
6. Cendra MDM, Torrents E. *Pseudomonas aeruginosa* biofilms and their partners in crime. *Biotechnol Adv*. 2021;49:107734. doi:10.1016/j.biotechadv.2021.107734
7. Elborn JS, Vataire A, Fukushima A, et al. Comparison of inhaled antibiotics for the treatment of chronic *Pseudomonas aeruginosa* lung infection in patients with cystic fibrosis: systematic literature review and network meta-analysis. *Clin Ther*. 2016;38:2204–2226. doi:10.1016/j.clinthera.2016.08.014
8. Lee J, Zhang L. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell*. 2015;6:26–41. doi:10.1007/s13238-014-0100-x
9. Alfiniyah C, Bees MA, Wood AJ, et al. Quorum machinery: effect of the *las* system in *rhl* regulation of *P. aeruginosa*. *AIP Conf Proc*. 2019;2192. doi:10.1063/1.5139147
10. Chadha J, Harjai K, Chhibber S. Repurposing phytochemicals as anti-virulent agents to attenuate quorum sensing-regulated virulence factors and biofilm formation in *Pseudomonas aeruginosa*. *Microb Biotechnol*. 2022;15:1695–1718. doi:10.1111/1751-7915.13981
11. Rather MA, Saha D, Bhuyan S, Jha AN, Mandal M. Quorum Quenching: a drug discovery approach against *Pseudomonas aeruginosa*. *Microbiol Res*. 2022;264:127173. doi:10.1016/j.micres.2022.127173
12. Lu Y, Li H, Pu J, et al. Identification of a novel RhlI/R-PrrH-LasI/PhzC/PhzD signalling cascade and its implication in *P. aeruginosa* virulence. *Emerg Microbes Infect*. 2019;8:1658–1667. doi:10.1080/22221751.2019.1687262
13. Grandclément C, Tannières M, Moréra S, Dessaux Y, Faure D. Quorum quenching: role in nature and applied developments. *FEMS Microbiol Rev*. 2016;40:86–116. doi:10.1093/femsre/fuv038
14. Jurado-Martín I, Sainz-Mejías M, Mcclean S. *Pseudomonas aeruginosa*: an audacious pathogen with an adaptable arsenal of virulence factors. *Int J mol Sci*. 2021;22:3128. doi:10.3390/ijms22063128
15. Kostylev M, Kim DY, Smalley NE, Salukhe I, Greenberg EP, Dandekar AA. Evolution of the *Pseudomonas aeruginosa* quorum-sensing hierarchy. *Proc Natl Acad Sci USA*. 2019;116:7027–7032. doi:10.1073/pnas.1819796116
16. Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev*. 2012;76:46–65. doi:10.1128/MMBR.05007-11
17. Chen R, Wei X, Li Z, et al. Identification of a small RNA that directly controls the translation of the quorum sensing signal synthase gene *rhlI* in *Pseudomonas aeruginosa*. *Environ Microbiol*. 2019;21:2933–2947. doi:10.1111/1462-2920.14686
18. Malgaonkar A, Nair M. Quorum sensing in *Pseudomonas aeruginosa* mediated by RhlR is regulated by a small RNA PhrD. *Sci Rep*. 2019;9:432. doi:10.1038/s41598-018-36488-9
19. Li WR, Zeng TH, Zhang ZQ, Shi QS, Xie XB. Geraniol attenuates virulence factors by inhibiting quorum sensing of *Pseudomonas aeruginosa*. *Front Microbiol*. 2023;14:1190619. doi:10.3389/fmicb.2023.1190619
20. Lin H, Wang X, Liu M, et al. Exploring the treatment of COVID-19 with Yinqiao Powder based on network pharmacology. *Phytother Res*. 2021;35:2651–2664. doi:10.1002/ptr.7012
21. Zhang H, Xu L, Song J, et al. Establishment of quality evaluation method for Yinqiao Powder: a herbal formula against COVID-19 in China. *J Anal Methods Chem*. 2022;2022:1–19. doi:10.1155/2022/1748324
22. Lin D, Wu B, Wang L, Zhang W, Chen C. Intervention effect of modified Yinqiao Powder on outer membrane protein OprD2 of *Pseudomonas aeruginosa*. *China Trop Med*. 2017;17:1181–1183,1228. doi:10.13604/j.cnki.46-1064/r.2017.12.04
23. Zhang W, Han J, Qu P, Li S, Deng G, Chen C. Effects of Yinqiao powder against *Pseudomonas aeruginosa* biofilms and its synergism with azithromycin. *China Trop Med*. 2015;15:655–657.
24. Pu J, Zhang S, He X, et al. The small RNA AmiL Regulates Quorum sensing-mediated virulence in *Pseudomonas aeruginosa* PAO1. *Microbiol Spectr*. 2022;10:e221121. doi:10.1128/spectrum.02211-21
25. Guo Q, Wu Q, Bai D, et al. Potential use of dimethyl sulfoxide in treatment of infections caused by *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2016;60:7159–7169. doi:10.1128/AAC.01357-16
26. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol*. 1990;172:884–900. doi:10.1128/jb.172.2.884-900.1990

27. Rashid MH, Kornberg A. Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci*. 2000;97:4885–4890. doi:10.1073/pnas.060030097
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *Methods*. 2001;25:402–408. doi:10.1006/meth.2001.1262
29. Nam S, Ham S, Kwon H, et al. Discovery and characterization of Pure RhlR antagonists against *Pseudomonas aeruginosa* infections. *J Med Chem*. 2020;63:8388–8407. doi:10.1021/acs.jmedchem.0c00630
30. Sabir S, Subramoni S, Das T, Black DS, Rice SA, Kumar N. Design, synthesis and biological evaluation of novel Anthraniloyl-AMP Mimics as PQS biosynthesis inhibitors against *Pseudomonas aeruginosa* resistance. *Molecules*. 2020;25:3103. doi:10.3390/molecules25133103
31. Martínez E, Cosnahan RK, Wu M, et al. Oxylipins mediate cell-to-cell communication in *Pseudomonas aeruginosa*. *Commun Biol*. 2019;2:66. doi:10.1038/s42003-019-0310-0
32. Ouyang J, Sun F, Feng W, et al. Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. *J Appl Microbiol*. 2016;120:966–974. doi:10.1111/jam.13073
33. Thi MTT, Wibowo D, Rehm BHA. *Pseudomonas aeruginosa* Biofilms. *Int J mol Sci*. 2020;21:8671. doi:10.3390/ijms21228671
34. Zhang A, Chu WH. Anti-quorum sensing activity of Forsythia suspense on Chromobacterium violaceum and *Pseudomonas aeruginosa*. *Pharmacogn Mag*. 2017;13:321–325. doi:10.4103/0973-1296.204547
35. Liang S, Li D, Chen J, Deng W, Liu M. Studies on the Yinqiao powder against *Pseudomonas aeruginosa* biofilm targeting to quorum sensing molecule. *Infectious Dis Inf*. 2023;36:410–416,422. doi:10.3969/j.issn.1007-8134.2023.05.005
36. Wei Q, Bhasme P, Wang Z, et al. Chinese medicinal herb extract inhibits PQS-mediated quorum sensing system in *Pseudomonas aeruginosa*. *J Ethnopharmacol*. 2020;248:112272. doi:10.1016/j.jep.2019.112272
37. Tapia-Rodriguez MR, Bernal-Mercado AT, Gutierrez-Pacheco MM, et al. Virulence of *Pseudomonas aeruginosa* exposed to carvacrol: alterations of the Quorum sensing at enzymatic and gene levels. *J Cell Commun Signal*. 2019;13:531–537. doi:10.1007/s12079-019-00516-8
38. Murina VN, Nikulin AD. Bacterial Small Regulatory RNAs and Hfq Protein. *Biochemistry*. 2015;80:1647–1654. doi:10.1134/S0006297915130027
39. Fröhlich KS, Vogel J. Activation of gene expression by small RNA. *Curr Opin Microbiol*. 2009;12:674–682. doi:10.1016/j.mib.2009.09.009