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ORIGINAL RESEARCH

Scopoletin and Scoparone from *Viola philippica* Attenuate the Virulence of *Pseudomonas aeruginosa* by Inhibiting Quorum Sensing-Related Genes

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Purpose: Quorum sensing (QS) system is crucial for biofilm formation, virulence and survival of *Pseudomonas aeruginosa* (*P. aeruginosa*). With the rise of antibiotic-resistant bacteria, QS inhibitors (QSIs) are seen as potential agents to mitigate infections. Plant-based natural products are rich sources of anti-QS compounds. *Viola philippica* Cav. (*Violaceae*) is traditionally used to treat inflammatory diseases, respiratory diseases and anti-microbials. However, its anti-QS effects against *P. aeruginosa* are poorly understood. We aimed to reveal the effects of scopoletin and scoparone from *V. philippica* Cav. extract (VPE) on QS-related virulence phenotypes and genes in *P. aeruginosa* PAO1.

Materials and Methods: Compounds in VPE were identified using ultrahigh-performance liquid chromatography coupled with Q-exactive hybrid quadrupole-Orbitrap mass spectrometry (UHPLC-Q Exactive-Orbitrap MS). Virulence factors of PAO1, including pyocyanin, rhamnolipids, elastase, hemolysin, swarming and swimming motility, and biofilm formation, were evaluated. Real-time reverse transcription-polymerase chain reaction (RT-qPCR) was used to detect virulence-related genes. Bioassays for the QS inhibitory activity of VPE and its compounds were performed using *Chromobacterium violaceum* (*C. violaceum*) CV026.

Results: Sub-minimum inhibitory concentrations (sub-MICs) of VPE inhibited QS-related virulence factors in PAO1, including pyocyanin, rhamnolipids, biofilms, and swarming motility. Scopoletin and scoparone, two coumarin derivatives identified in VPE using UHPLC-Q Exactive-Orbitrap MS, also showed anti-QS activity against PAO1. These compounds reduced pyocyanin, rhamnolipid, and hemolysin production, elastase activity, biofilm formation and swarming motility. Further RT-qPCR assays revealed that scopoletin and scoparone universally suppress QS-associated genes (*lasI, rhlI* and *pqsA*) in PAO1, primarily during the early and middle stages of growth.

Conclusion: Scopoletin and scoparone, two previously uncharacterized coumarin derivatives from VPE, were identified as novel QSIs. They are responsible for VPE's effect on weakening QS-controlled virulence of PAO1 by inhibiting QS-related genes. This study provides a laboratory basis for using scopoletin and scoparone as promising anti-virulence candidates for adjuvant therapy against *P. aeruginosa* infection.

Keywords: PAO1, natural plant, *Viola yedoensis* makino, quorum sensing inhibitor, coumarin, *Chromobacterium violaceum* CV026

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic, Gram-negative microorganism causing acute or chronic infection in immunocompromised individuals and is a major cause of morbidity and mortality in hospital-acquired

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infections.[1] Patients suffering from ventilator-associated pneumonia (VAP), bronchiectasis, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD) frequently develop chronic respiratory infections due to *P. aeruginosa*.[2] The treatments of *P. aeruginosa* infection are extremely challenging due to its rapid mutations and resistant to multiple antibiotics. Carbapenem-resistant *P. aeruginosa* has been classified as a "critical" pathogen by World Health Organization (WHO),[3] which urgently need to develop innovative effective medicines that can successfully and safely prevent *P. aeruginosa* resistance.

Quorum-sensing (QS) systems can generate signal molecule N-acyl homoserine lactones (AHLs) linked to extracellular virulence and internal communication.[4] QS modulates various biological processes, including biofilm formation, virulence factor production, and microbe-host interactions.[5] QS-inhibitory (QSI) agents, which can reduce bacterial virulence rather than kill it, may offer an effective therapeutic strategy for controlling *P. aeruginosa* infection.[4] Researchers have gradually realized that plant-derived compounds can provide abundant materials for preventing and treating various diseases.[6] Natural plants are one of the best potential sources of new drugs, as a World Health Organization (WHO) report mentioned.[7] Extracts from various natural plants, such as Baicaetin,[8] Naringenin,[9] polyphenols from *Salix tetrasperma*,[10] Falcarindiol from *Notopterygium incisum*,[11] have been regarded as potential therapeutic agents due to their ability to inhibit the QS of *P. aeruginosa.*

Viola philippica Cav. (Violaceae), the traditional Chinese medicine Zǐ Huā Dì Dīng (ZHDD, in pinyin Chinese), also synonymized as *Viola yedoensis* Makino, is a perennial flowering plant native in China, Japan and Korea. To date, approximately 162 compounds have been identified in *V. philippica*, covering a wide range of chemical categories including coumarins, flavonoids, terpenoids, phenolic acids, alkaloids, and cyclopeptides.[12,13] Recent studies have shown that extracts of *V. philippica* and its compounds exhibit a range of pharmacological effects, including anti-viral,[13] anti-inflammatory,[14,15] anti-coagulant[16] and antimicrobial effects.[14] Specifically, these extracts have shown antimicrobial efficacy against several bacterial strains, such as *Streptococcus mutans*,[15] *Staphylococcus aureu*s, *Escherichia coli, Streptococcus lactis, Streptococcus agalactiae, Streptococcus dysgalactiae* and *Salmonella*.[17] Xie et al illustrated that the petroleum ether and ethyl acetate extracts of *V. philippica* effectively inhibited *Bacillus subtilis* and *Pseudomonas syringae*, while the methanol and methanol-water extracts failed to exhibit antibacterial activity.[18] Given the rich content of bioactive compounds in *V. philippica* and its diverse antibacterial activities, its potential role in combating *P. aeruginosa* infections has attracted the attention of researchers. However, the effects of *V. philippica* on QS-regulated virulence against *P. aeruginosa* are poorly understood.

To the best of our knowledge, despite the diverse antibacterial activities of *V. philippica*, no study has yet investigated its anti-virulence effects against *P. aeruginosa* PAO1 via QS regulation. This study aims to fill this gap by investigating the effects of *V. philippica* and its compounds on QS-regulated virulence and genes in PAO1. We found that *V. philippica* granule herbal extract (VPE) acts as a QSI, inhibiting the production of QS-linked virulence traits in *P. aeruginosa* PAO1. Scopoletin and scoparone, two previously uncharacterized coumarin derivatives from VPE, were identified as novel QSIs. These compounds reduced pyocyanin, rhamnolipid, and hemolysin production, elastase activity, biofilm formation and swarming motility by inhibiting QS-associated genes (*lasI, rhlI* and *pqsA*), suggesting that scopoletin and scoparone were responsible for VPE's effect on weakening QS-controlled virulence of PAO1. This study provides a laboratory basis for using scopoletin and scoparone as promising anti-virulence candidates for adjuvant therapy against *P. aeruginosa* infection.

Materials and Methods

Bacterial Strains and Reagents

The wild-type *P. aeruginosa* PAO1 strain (Strain No.: ATCC 15692) and *Chromobacterium violaceum* (*C. violaceum*) CV026 strain, both preserved in our laboratory, which had been used in our previous studies,[19,20] were originally obtained from the American Type Culture Collection (ATCC) and the China General Microbiological Culture Collection Center (CGMCC), respectively. All strains were authenticated by 16S rDNA sequencing prior to use. The ATCC 15692 cell line was authenticated by short-tandem-repeat (STR) profiling, which confirmed 100% match with the ATCC reference profile. Luria-Bertani (LB) broth (0.5% yeast extract, 1.0% tryptone, 0.5% NaCl, pH 7.4) was used to cultivate both strains, with or without antibiotics. Unless otherwise noted, *P. aeruginosa* PAO1 and *C. violaceum* CV026 were

cultured at 37°C and 28°C, respectively, with 200 rpm, in a shaking incubator for 16 h (overnight). Overnight cultured *P. aeruginosa* was inoculated into LB broth and cultured at 37°C with 260 rpm for 2 h to obtain mid-exponential phase *P. aeruginosa* PAO1. Bacterial density was recorded at OD600 using BioTek Synergy H1 microplate reader.

V. philippica granule herbal extract (VPE, Tianjiang Pharmaceutical Co., Ltd., Jiangsu, China) was dissolved in autoclaved double-distilled water to prepare a 1000 mg/mL solution, stored at −80°C until use. The extract was diluted with the corresponding solution (eg, sterilized water or LB medium) and sterilized through 0.22 μm syringe filters (Millipore) before use. Scopoletin standards (also known as 7-Hydroxy-5-methoxycoumarin, CAS: 92–61-5) and scoparone standards (also known as 6,7-dimethoxycoumarin/6,7-dimethylesculetin, CAS: 120–08-1) were purchased from Sigma-Aldrich (St Louis, MO, USA), dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at 81.92 mg/mL and 102.4 mg/mL, respectively, and stored at −80°C until use.

Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method was used to evaluate the minimum inhibitory concentration (MIC) of VPE against *P. aeruginosa* PAO1 in Müeller-Hinton agar containing VPE (VPE-MH, in serial two-fold dilution), in accordance with the M7-A11 protocol published by the Clinical and Laboratory Standards Institute Standards (CLSI), as stated[19] with slight modifications. The final concentration of VPE ranged from 1 mg/mL to 128 mg/mL. MH agar without VPE served as control. The mid-exponential phase *P. aeruginosa* PAO1 was diluted in LB broth, and 2 μL of the diluted suspension was spotted onto the plates to achieve a final concentration of 10^4 CFU/spot. The plates were incubated statically at 37°C for 20 h. MIC was defined as the lowest concentration of VPE preventing visible growth.

Growth Curve

Growth curve of *P. aeruginosa* PAO1 with VPE treatment was constructed as previously reported.[20] Briefly, 10 μL of mid-exponential phase *P. aeruginosa* PAO1 was added to 1 mL of LB broth with or without sub-MICs of VPE, and incubated at 37°C 200 rpm. Every 3 h, cells were washed twice with sterile saline, resuspended in 1 mL of sterile saline, and 200 μL of the suspension was withdrawn into a 96-well plate, and the OD600 was measured using BioTek Synergy H1 microplate reader to generate growth curves.

Growth curves of *P. aeruginosa* PAO1 with scopoletin/scoparone treatment were performed in another way as previously described.[21] Mid-exponential phase *P. aeruginosa* PAO1 were diluted 1:200 final concentration in a 96-well plate with or without scopoletin/scoparone, incubated at 37°C with gentle shaking for 24 h. OD600 was continuously monitored using BioTek Synergy H1 microplate reader. Sub-doses lower than 1024 μg/mL scopoletin/scoparone were used in all assays in the present study.

Bioassay for QSI Activity Using *C.violaceum* CV026

C. violaceum bioassay was performed with several modifications as previously described.[22] The overnight culture of *C. violaceum* CV026 was mixed with warm LB agar supplemented with *N*-Butanoyl-L-homoserine lactone (C4-HSL) to a final concentration of 20 μmol/L. The mixture was then poured onto LB plates with a solidified layer. Wells (5 mm in diameter) were punched into the agar, and 40 μL sub-MICs of VPE or scopoletin/scoparone were added to the wells. The plates were incubated at 28°C for 20 h. The absence of purple violacein pigment in the bacterial lawn surrounding the wells indicated QS inhibition.

Pyocyanin Production Assay

The pyocyanin assay was carried out as previously described.[20] Mid-exponential phase *P. aeruginosa* PAO1 (30 μL) was added to 3 mL of LB broth with or without sub-MICs of VPE or scopoletin/scoparone, and incubated at 37°C with 200 rpm for 20 h. Pyocyanin was extracted from 2.5 mL of *P. aeruginosa* PAO1 culture supernatant using 1.5 mL of chloroform and 0.5 mL of 0.2 mol/L HCl. The absorbance of the extract was measured at OD520 nm using BioTek Synergy H1 microplate reader.

Rhamnolipid Assay

The rhamnolipid assay was performed as described with minor modifications.[23] Mid-exponential phase *P. aeruginosa* PAO1 (20 μL) was inoculated into 2 mL of M9 medium (containing 0.4% glucose, 2 mmol/L MgSO4, and 100 μmol/L $CaCl_2$) with or without sub-MICs of VPE or scopoletin/scoparone, and incubated at 37°C 200 rpm for 16 h. The centrifuged supernatant (1mL) was treated with 40 μL of 1 mol/L HCl and 4 mL of chloroform, followed by vigorous shaking. The chloroform extract (3 mL) was then reacted with 100 μL of 1 g/L methylene blue and 2.45 mL of deionized water. Finally, 200 μL of the chloroform layer was transferred to a 96-well plate to measure the OD at 638 nm using BioTek Synergy H1 microplate reader.

Elastase Assays

Elastase activity was carried out by elastin-Congo red (ECR) assays.[24] Mid-exponential phase *P. aeruginosa* PAO1 (20 μL) was inoculated into 2 mL of LB medium in the presence or absence of scopoletin/scoparone and incubated at 37°C 200 rpm for 8 h. Subsequently, 200 μL culture supernatant was inoculated with 800 μL ECR solution (Sigma-Aldrich, St. Louis, MO) containing 0.1 M Tris (pH 7.2), 1 mM $CaCl_2$ and 3 mg/mL ECR, and incubated at 37°C 200 rpm for 4 h. The reaction was terminated by adding 100 μL of 0.12 M EDTA. The insoluble ECR was removed by centrifugation, and its OD495 was measured using BioTek Synergy H1 microplate reader.

Motility Assays

Swimming and swarming motility were assessed as previously described.[20] Mid-exponential phase *P. aeruginosa* PAO1 (5 μL) was spotted onto the center of swarming medium (LB containing 0.5% agar, 5 g/L glucose) and swimming medium (tryptone broth containing 0.3% agar), both supplemented with various concentrations of VPE (16 mg/mL and 32 mg/mL), scopoletin (256 μg/mL and 128 μg/mL), or scoparone (256 μg/mL and 128 μg/mL). Media without VPE or scopoletin/scoparone served as controls. After the incubation at 37°C for 16 h, the motility zones were measured using a Vernier caliper.

Biofilm Formation Assay

Crystal violet staining was used to perform biofilm formation assay.[8] Overnight cultured *P. aeruginosa* PAO1 (100 μL, 1×10^7 CFU/mL) was cultured in a 96-well plate with or without sub-MICs of VPE and scopoletin/scoparone for 24 h at 37°C statically. Planktonic/non-adherent cells were discarded, and the wells were gently washed with sterile saline. The remaining bacterial biomass was fixed with methanol for 5 min. After discarding the methanol, the wells were air-dried and stained with 1% crystal violet. After rinsing with sterile saline three times, the crystal violet-stained biofilms were eluted with 95% ethanol, and the OD595 was measured using BioTek Synergy H1 microplate reader.

Characterization of the Extract by Ultrahigh Performance Liquid Chromatography Coupled with Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometry (UHPLC-Q Exactive-Orbitrap MS)

Qualitative identification of *V. philippica* compounds was performed using a Thermo Scientific UltiMate 3000 UHPLC system and ultra-high-performance liquid chromatography coupled with a Q Exactive Orbitrap mass (Thermo Fisher Scientific, Waltham, MA, USA). A Waters ACQUITY UPLC HSS T3 Column (2.1 mm × 100 mm, 1.8 μm) with an ACQUITY UPLC HSS T3 (1.8 μm) precolumn was employed. Gradients of solvents: Acetonitrile (A) and 0.1% formic acid aqueous solution (B) were used as the mobile phase, with a flow rate of 0.2 mL/min. The gradient elution was as follows: 0–2 min, 5–10% of A; 2–5 min, 10–30% of A; 5–15 min, 30–35% of A; 15–18 min, 35–40% of A; 18–21 min, 40–100% of A; 100% - 21–24 min, 30% of A; 24–26 min, 30–5% of A. The injection volume was 5 μL and the total analysis time was 26 min. The main parameters for Q Exactive-Orbitrap MS were set as follows: heat electrospray ionization (ESI) positive and negative ion modes; capillary temperature, 350°C; sheath gas flow rate, 40; NCE, 20, 30, 45 eV; auxiliary gas heater temperature, 350°C; auxiliary gas flow rate, 15. In the full MS-dd MS^2 experiment, the full MS scan range was set at m/z 100–2000 and the resolution was set at 70000. The resolution of the dd-MS^2 was 17500. The parameters of the excluded isotopes were operated, and the dynamic exclusion time was 10s.

Blood Hemolysis Assays

Blood hemolysis assays were performed as previously described.[20] Human whole blood was collected from healthy individuals and washed with phosphate-buffered saline (PBS, PH 7.4) to remove the leukocyte layer by centrifugation at 2,000 rpm for 5 min. The erythrocytes were then resuspended to a 4% concentration. A 300 μL aliquot of 4% washed erythrocytes was added to 300 μL of mid-exponential phase *P. aeruginosa* PAO1 resuspended in PBS (experimental group, EG), 300 μL of PBS (negative control, NC), and 300 μL of 2% Triton X-100 solution (positive control, PC), and scopoletin/scoparone was added. The mixtures were incubated at 37°C 6 h, and supernatant was measured at OD450 using BioTek Synergy H1 microplate reader. The percentage of hemolysis was determined using the following formula: $(A_{EG} - A_{NC})/(A_{PC} - A_{NC}) \times 100\%$ = hemolysis%.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Overnight cultured *P. aeruginosa* PAO1 (30 μL) was added to 3 mL of LB broth with or without scopoletin/scoparone and incubated at 37°C 200 rpm. After collecting the cultures at 8 h intervals beginning at 4 h, total RNA was extracted using RNAiso Plus reagent (Takara, Liaoning, China), and reverse-transcribed using the PrimeScript RT Reagent Kit (Takara, Liaoning, China). PCR was performed using the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Hunan, China) on a ViiATM7 Dx system (Applied Biosystems, Foster, CA, United States), with the primers listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate target gene expression levels, with the *rpoD* gene serving as an internal control.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) of three independent experiments unless otherwise indicated. One-way ANOVA was performed using SPSS Statistics (version 23.0) to compare the differences between groups. Graphs were generated using the GraphPad Prism software (version 5.0; GraphPad Software, United States). Each experiment was repeated at least thrice to yield comparable results.$P < 0.05$. P values are represented as $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

Results

VPE Inhibits the Production of QS-Related Virulence Factors in *P.aeruginosa* PAO1

First, the growth of *P. aeruginosa* PAO1 was evaluated after VPE treatment. Our results showed that the MIC of VPE against PAO1 was 128 mg/mL, using the agar dilution method. At sub-MIC concentrations (1/2, 1/4, and 1/8 MIC, corresponding to 64, 32, and 16 mg/mL, respectively), PAO1 growth kinetics remained unaffected, indicating no inhibitory effect of VPE on *P. aeruginosa* growth (Figure S1). Sub-MICs of VPE exhibited dose-dependent anti-QS activity, evidenced by the notable halo zones around the disc against purple colonies (Figure S2), indicating the extremely promising anti-QS activity of VPE. As shown in Table 2, analysis of **virulence factors** revealed that sub-MICs of VPE significantly reduced the production of pyocyanin (1/8 MIC VPE treatment: 52% decrease; 1/4 MIC VPE treatment: 80% decrease) and rhamnolipids (1/8 MIC VPE treatment: 26% decrease; 1/4 MIC VPE treatment: 28% decrease). Correspondingly, swarming motility, related to rhamnolipid production, was also inhibited by VPE treatment,

Table 1 Primers Used for Real-Time RT-PCR Analysis

Target Gene	Primer Sequence	Target Length
rpoD	F: 5′-CTGAAGATCGCCAAAGAGCC-3′ R: 5′-GTGTGGTCGGTGTTCATGTC-3′	224bp
lasI	F: 5′-CGTGCTCAAGTGTTCAAGGA-3′ R: 5′-AAAACCTGGGCTTCAGGAGT-3′	131bp
rhlI	F: 5′-CTACCGGCATCAGGTCTTCA-3′ R: 5′-GTTTCGCTGCACAGGTAGG-3′	210bp
pqsA	F: 5′-CAACACGCTCGGTTTCTGC-3′ R: 5′-CATCTTGGGAATCGAATACAGC-3′	85bp

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Table 2 Inhibitory Effect of VPE on QS-Regulated Virulence

Group	Pyocyanin (OD520)	Rhamnolipids (OD638)	Swarming Motility (mm)	Swimming Motility (mm)	Biofilm Formation (OD595)
Ctrl	0.171 (±0.021)	0.635 (±0.075)	47.9 (±12.4)	36.8 (±1.7)	1.343 (±0.150)
VPE (1/8 MIC)	0.083 (±0.024)[a]	0.469 (±0.069)[a]	27.7 (±2.5)[a]	37.9 (±2.2)[ns]	1.111 (±0.067)[a]
VPE (1/4 MIC)	0.034 (±0.019)[a,b]	0.457 (±0.068)[a]	16.4 (±2.0)[a]	38.0 (±1.4)[ns]	0.670 (±0.087)[a,b]

Notes: [a]$P < 0.05$ vs Ctrl; [b]$P < 0.05$ VPE (1/8 MIC) vs VPE (1/4 MIC); [ns], nonsignificant. The absorbance of the extracted pyocyanin, rhamnolipids, and biofilm was detected at OD520, OD638 nm and OD595 nm, respectively. For the motility assay, the diameter of the cultures was measured. Data are shown as mean ± standard deviation (SD) of at least three independent experiments.

while swimming motility was unaffected (Table 2). Biofilm formation of *P. aeruginosa* was also inhibited by VPE treatment (1/8 MIC VPE treatment: 19% decrease; 1/4 MIC VPE treatment: 51% decrease) (Table 2). RT-qPCR assays showed that QS-regulatory genes (*lasI, rhlI* and *pqsA*) were downregulated in the presence of VPE, consistent with the suppression of virulence phenotypes including pyocyanin, rhamnolipids, motility, and biofilms (Figure S3). Overall, these results indicate that sub-MICs of VPE can inhibit QS-linked virulence traits in *P. aeruginosa* PAO1.

VPE Components Assay by UHPLC-Q Exactive-Orbitrap MS

The total ion chromatograms (TIC) of *V. philippica* granule herbal extract, analyzed by UHPLC-Q Exactive-Orbitrap MS in both positive (ESI+) and negative (ESI−) ion mode, are presented in Figure 1. A total of 16 compounds were identified based on published literature[25] (Table 3), comprising six coumarins, five flavonoids, and five organic acids. The coumarins identified are esculin (2), esculetin (3), 7-hydroxy-8-methoxyecoumarin (6), scopoletin (8, known as 6-methoxy-7-hydroxycoumarin), scoparone (9, known as 6,7-dimethoxycoumarin), and prionanthoside (11). Given that coumarins are widely found in various plants and some have shown potential antibacterial and antifungal effects, we are motivated to explore the coumarin derivatives scopoletin and scoparone in VPE. However, the role of these derivatives in the QS-related virulence of *P. aeruginosa* PAO1 has not been previously studied.

Scopoletin and Scoparone Influence the Production of QS-Related Virulence Factors in *P.aeruginosa* PAO1

We further studied the effects of scopoletin and scoparone on QS-related virulence factors of *P. aeruginosa* PAO1. At 1024 μg/mL, PAO1 growth kinetics were inhibited, while no significant effects were observed at sub-doses of 512, 256, and 128 μg/mL of scopoletin/scoparone treatment (Figure 2A). *C. violaceum* bioassay revealed dose-dependent increases in halo zones under scopoletin/scoparone treatment, with more pronounced zones under scopoletin treatment, suggesting its stronger QSI activity (Figure 2B). Pyocyanin synthesis decreased dose-dependently with scopoletin/scoparone exposure (128, 256, and 512 μg/mL), though the decrease was not significant at 128 μg/mL scoparone treatment (Figure 2C). Scopoletin significantly repressed rhamnolipid production, elastase activity, and biofilm formation (Figure 2D–F), while scoparone only significantly attenuated rhamnolipid production ($P < 0.05$) and biofilm formation ($P < 0.01$) at 512 μg/mL. Elastase activity was significantly inhibited by 256 and 512 μg/mL scoparone treatment, with a slight but insignificant downregulation at 128 μg/mL (Figure 2E). Consistent with the effects of VPE on PAO1 motility, swarming motility was also dose-dependently inhibited by scopoletin/scoparone treatment (Figure 3A), whereas swimming motility remained unaffected (Figure 3B). Overall, these results indicate that scopoletin and scoparone negatively regulated pyocyanin synthesis, rhamnolipid production, elastase activity, biofilm formation, and swarming motility, with scopoletin being a more potent inhibitor than scoparone.

Scopoletin and Scoparone Inhibit Hemolytic Activity of *P.aeruginosa* PAO1

As hemolysin is another important QS-related virulence factor, hemolysin production by *P. aeruginosa* PAO1 was evaluated after scopoletin and scoparone treatment. As shown in Figure 4, scopoletin significantly downregulated the hemolysis rate of PAO1 at doses of 256 and 512 μg/mL. Low doses of scoparone had no impact on the hemolysis rate, whereas a high dose of scoparone (512 μg/mL) inhibited hemolysin production of *P. aeruginosa* PAO1. Thus, these data indicate that scopoletin and scoparone decreased hemolytic activity.

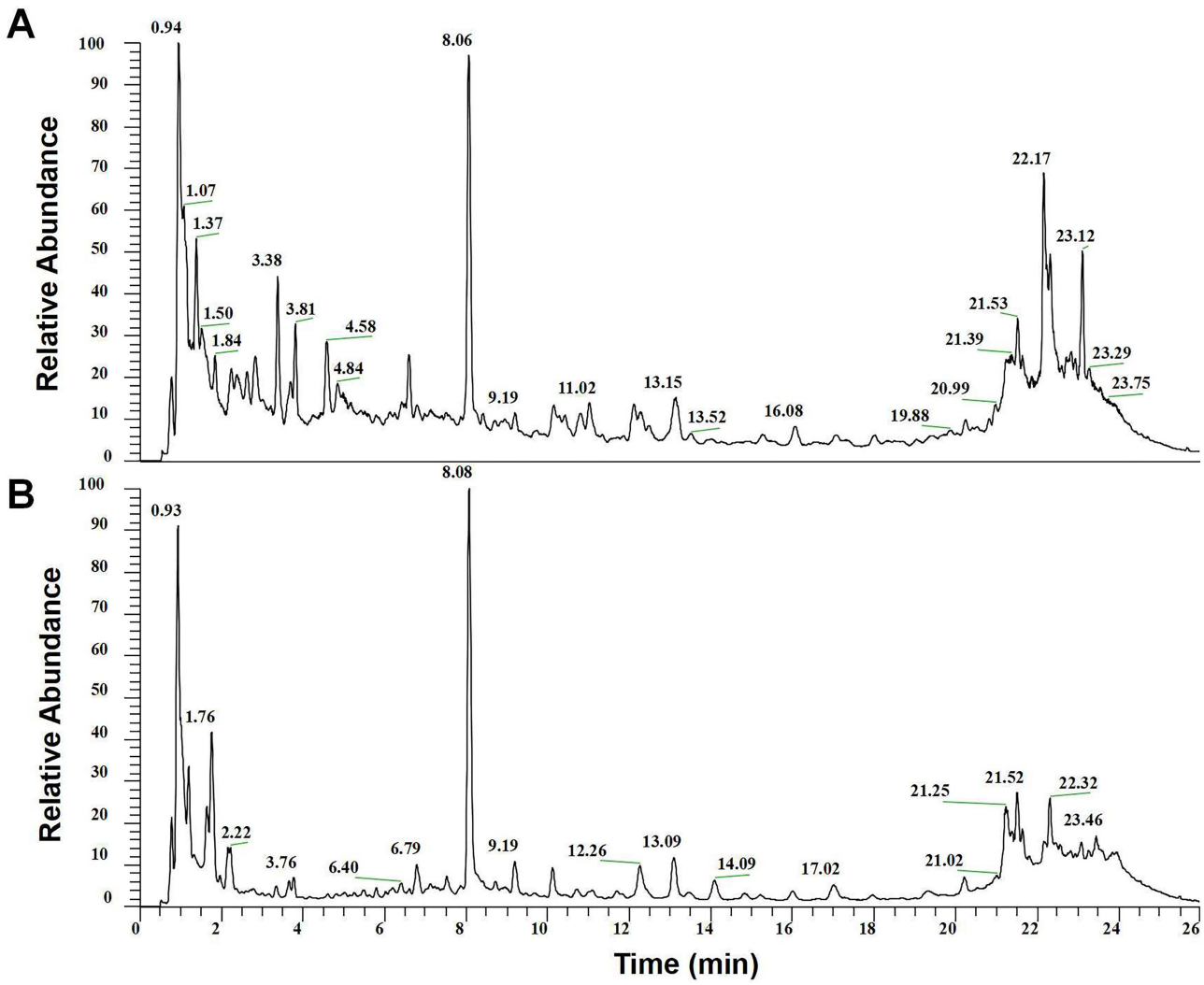

Figure 1 The total ion chromatograms (TIC) of *V. philippica* granule herbal extract detected by UHPLC-Q Exactive-Orbitrap MS in positive ion mode (**A**) and negative ion mode (**B**).

Mechanism of the Anti-QS Activity of Scopoletin and Scoparone

As QS systems in *P. aeruginosa* govern the production of virulence factors, we investigated the effects of the compounds on QS-associated genes. *P. aeruginosa* has three canonical QS systems: two LuxI/LuxR QS circuits (*LasI/LasR* and *RhlI/RhlR*), and a quinolone-based signaling (*pqs*) system. Therefore, QS regulatory genes such as *lasI, rhlI* and *pqsA* were tested. *P. aeruginosa* PAO1 was co-cultured with various concentrations of scopoletin or scoparone for 4, 12, and 20 h, corresponding to the early, middle, and late stages of bacterial growth, respectively. qRT-PCR was used to detect the expression levels of *lasI, rhlI* and *pqsA*. As shown in Figure 5, scopoletin at 256 and 512 μg/mL extensively inhibited *lasI, rhlI* and *pqsA* expression, with more pronounced effects at 4 and 12 h. Scopoletin at 128 μg/mL also downregulated *rhlI* and *pqsA* at 4 and 12 h, *lasI* was downregulated, though not significantly at all time points. Scoparone at 256 and 512 μg/mL also had inhibitory effects on *lasI, rhlI* and *pqsA*, but the inhibitory potential was weaker than that of scopoletin treatment. Scoparone at 128 μg/mL had no significant impact. Our findings suggest that scopoletin and scoparone may universally suppress QS-associated genes in *P. aeruginosa*, mainly in the early and middle growth stages, thereby downregulating virulence factors synthesis.

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Table 3 Compounds Identified from *V.philippica* Granule Herbal Extract

No.	t_R (min)	MS Mode	m/z	$^{\Delta}$ppm	MS Fragmentation	Molecular Formula	Molecular Weight	Identification
1	6.19	ESI-	311.04114	0.916	179.03392,149.00793, 135.04376	$C_{13}H_{12}O_9$	312.23	Caftaric acid
2	6.80	ESI-	339.07184	−0.93	177.01831	$C_{15}H_{16}O_9$	340.29	Esculin
3	8.08	ESI-	177.01863	−3.965	149.02309, 133.02821	$C_9H_6O_4$	178.14	Esculetin
4	8.95	ESI-	609.14551	−0.961	447.09323	$C_{27}H_{30}O_{16}$	610.52	Kaempferol-3,7-O-diglucoside
5	9.19	ESI-	353.02982	−1.33	335.01895,352.03870, 309.04050,297.03989, 177.01860	$C_{18}H_{10}O_8$	354.27	Mongolicumin A
6	10.29	ESI+	193.04945	−0.442	178.02608,161.05968, 133.02838	$C_{10}H_8O_4$	192.17	7-hydroxy-8-methoxyecoumarin
7	10.44	ESI-	473.07278	0.488	311.04153,293.03119, 179.03404, 149.00795	$C_{22}H_{18}O_{12}$	474.37	Chicoric acid
8	11.30	ESI+	193.04948	−0.286	178.02612, 133.02840	$C_{10}H_8O_4$	192.17	Scopoletin
9	12.61	ESI+	207.06491	−1.33	175.03893,161.13245, 147.04398	$C_{11}H_{10}O_4$	206.19	Scoparone
10	11.69	ESI-	353.02982	−1.33	325.03580,309.03989, 292.03726, 177.01848	$C_{18}H_{10}O_8$	354.27	Isobenzo[kl]xanthene-1,2-dicarboxylic acid,6,9,10-trihydroxy
11	11.78	ESI-	381.08307	0.919	177.01834	$C_{17}H_{18}O_{10}$	382.30	Prionanthoside
12	12.57	ESI-	447.09232	−2.157	357.06149, 327.05066	$C_{21}H_{20}O_{11}$	448.38	Isoorientin
13	13.10	ESI-	563.1413	1.192	473.10736,443.09903, 353.06689	$C_{26}H_{27}O_{14}$		Apigenin-6-C-α-L-arabinopyranosyl-8-C-β-D-glucopyranoside
14	13.44	ESI-	447.09357	0.638	429.08408,357.06110, 327.05066	$C_{21}H_{20}O_{11}$	448.38	Orientin
15	14.47	ESI-	431.09885	1.114	341.06635,323.05579, 311.05594, 283.06137	$C_{21}H_{20}O_{10}$	432.38	Isovitexin
16	19.22	ESI-	623.19757	−0.92	461.16833,179.03415, 161.02336	$C_{29}H_{36}O_{15}$	624.59	Isoacteoside

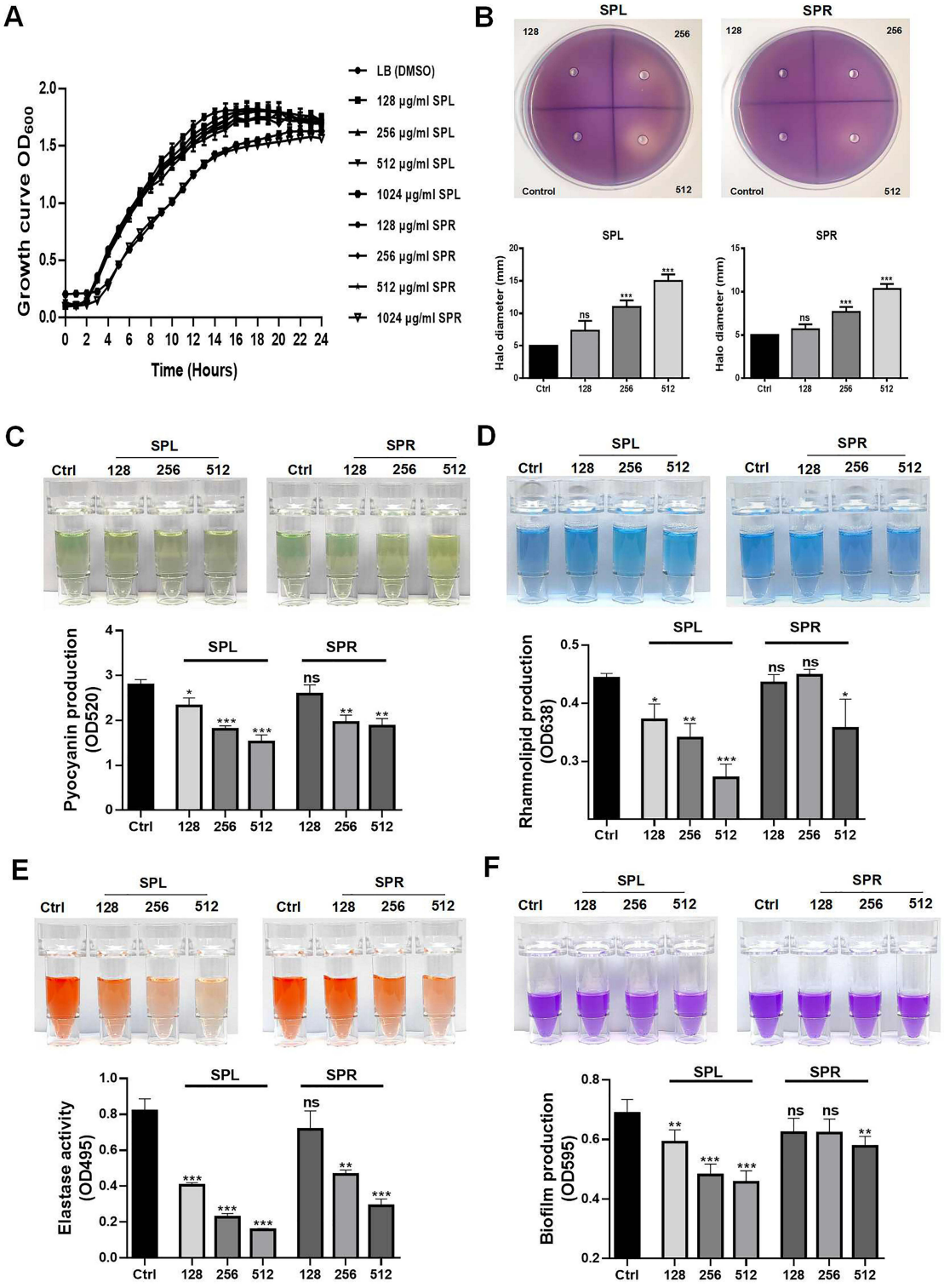

Figure 2 Scopoletin and scoparone influenced the production of QS-related virulence factors in *P. aeruginosa* PAO1. (**A**) Growth curve analysis of *P. aeruginosa* PAO1 under different concentrations of scopoletin/scoparone treatment. LB (DMSO), compound-free controls which added equal amount of DMSO for dissolving the compounds. (**B**) Different concentrations of scopoletin/scoparone were added into the well of LB agar plates containing *C. violaceum* CV026 which supplemented with C4-HSL and cultured at 28°C for 20 h. The violacein halo production was observed (top) and the diameter of the halo was measured (bottom). (**C**) Pyocyanin in the supernatant of PAO1 cultured with different concentrations of scopoletin/scoparone in LB medium for 20 h was measured. The pyocyanin was observed (top) and the extracted pyocyanin was measured at OD520 (bottom). (**D**) Rhamnolipid in the supernatant of PAO1 cultured with different concentrations of scopoletin/scoparone in M9 medium for 16 h was measured. The extracted rhamnolipid was observed (top) and measured at OD638 (bottom). (**E**) Elastase in the supernatant of PAO1 cultured with different concentrations of scopoletin/scoparone in LB medium for 8 h was measured. The elastase incubated with ECR for 4 h was observed (top) and measured at OD495 (bottom). (**F**) Biofilm formation of PAO1 cultured with different concentrations of scopoletin/scoparone in LB medium in the 96-well plate for 24 h was determined at OD595. SPL, scopoletin; SPR: scoparone; Ctrl, compound-free treatment. 128, 256 and 512: 128 μg/mL, 256 μg/mL and 512 μg/mL of scopoletin or scoparone. Data are shown as mean ± SD of at least three independent experiments. ns, nonsignificant. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

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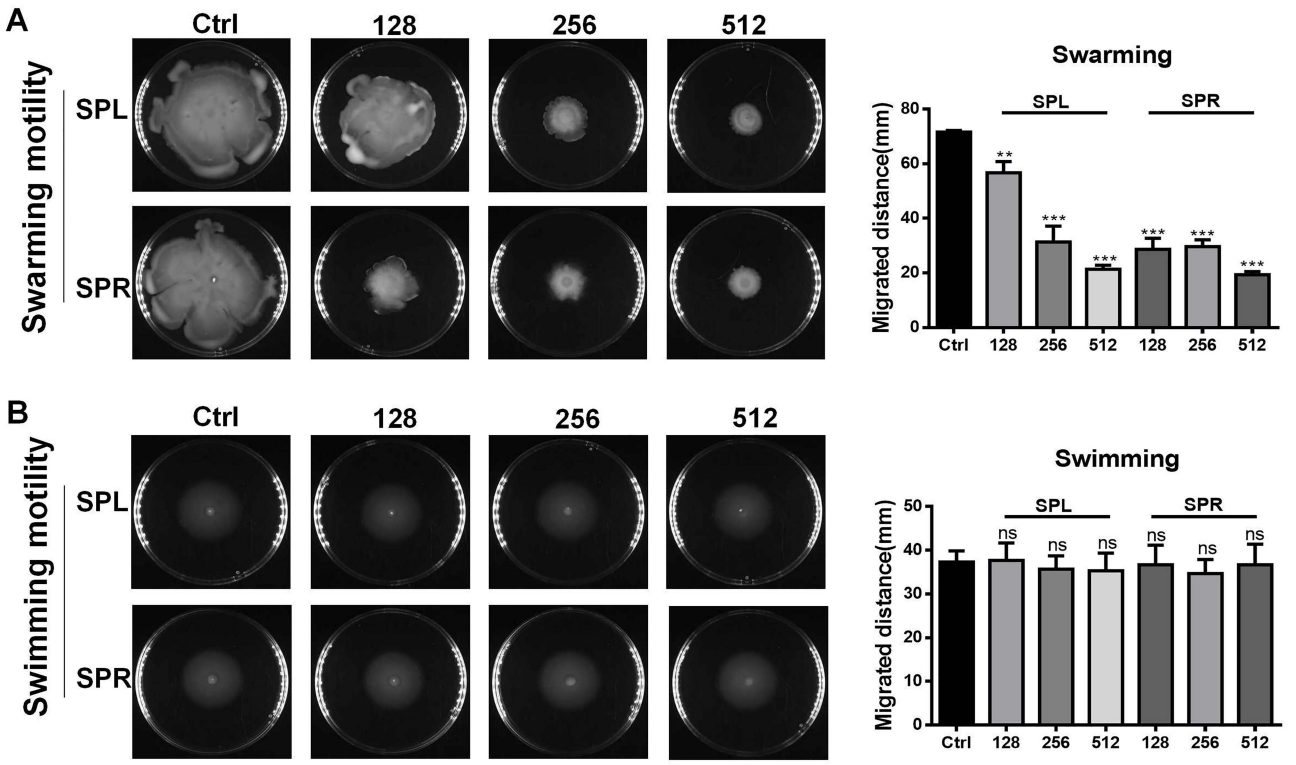

Figure 3 Scopoletin and scoparone inhibited swarming motility of PAO1, but had no impact on swimming. (**A**) PAO1 (5 μL) was spotted onto the middle of pre-prepared swarming plates with or without scopoletin or scoparone at 37°C for 16 h, and the diameters of the culture were measured. (**B**) PAO1 (5 μL) was spotted onto the middle of pre-prepared swimming plates with or without scopoletin or scoparone at 37°C for 16 h, and the diameters of the culture were measured. SPL, scopoletin; SPR: scoparone; Ctrl, compound-free plate. 128, 256 and 512: 128 μg/mL, 256 μg/mL and 512 μg/mL of scopoletin or scoparone. Data are shown as mean ± SD of at least three independent experiments. ns, nonsignificant. $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

In this study, for the first time, we focused on *P. aeruginosa* PAO1 as a model to investigate mode of action of traditional Chinese medicinal *V. philippica* and its compounds against bacterial infections. Consistent with Zhang's report on the virulence attenuation of *P. aeruginosa* by falcarindiol from an antipyretic drug *Notopterygium incisum*,[26] we report for the first time that *V. philippica*, also an antipyretic herb, as a novel QSI inhibits QS-controlled virulence in *P. aeruginosa* PAO1. Further component analysis of VPE based on UHPLC-Q Exactive-Orbitrap MS reveal that two previously uncharacterized coumarin derivatives scopoletin and scoparone in VPE are identified as novel QSIs using *C. violaceum* CV026 bioassay. Scopoletin and scoparone significantly downregulate QS-related virulence of *P. aeruginosa* PAO1, such as pyocyanin, rhamnolipid, elastase, hemolysin, biofilm formation and swarming motility by inhibiting QS-related genes. This research fills the gap in the study of anti-virulence properties of *V. philippica* and its compounds against *P. aeruginosa*. Our results position *V. philippica* and its compounds as promising candidates for adjuvant therapy against drug-resistant *P. aeruginosa* infections.

The virulence factors produced by *P. aeruginosa*, including pyocyanin, rhamnolipid, elastases and proteases, are intricately regulated by a hierarchical QS regulatory network and are directly linked to the pathogenicity of the bacteria.[27] Researchers have paid extensive attention to this elaborate regulatory strategy to develop therapeutics for infections caused by *P. aeruginosa*. Numerous bacteria use the QS cell–cell communication system to sense the density of their population. These bacteria create and recognize diffusible signaling molecules known as autoinducers, which regulate the synthesis of virulence factors, motility, and biofilm formation.[28] The discovery of the *P. aeruginosa* QS system offers an alternative treatment to traditional chemotherapy. In particular, many plant-based natural products have been shown to possess anti-QS and anti-virulence properties.[29] However, no reports have claimed *V. philippica* and its compounds to be anti-QS agents for the control of *P. aeruginosa* infection.

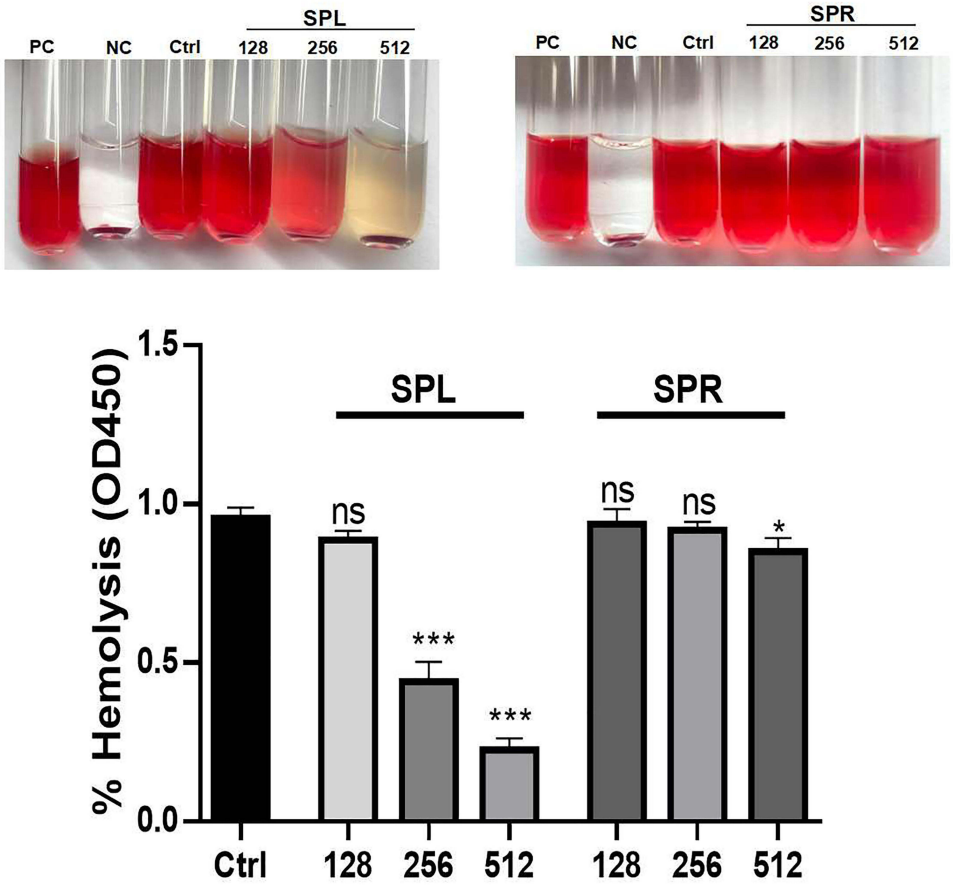

Figure 4 Scopoletin and scoparone inhibited hemolytic activity *P.aeruginosa* PAO1. 300 μL of 4% washed erythrocytes were added with 300 μL of mid-exponential phase *P. aeruginosa* PAO1 resuspended with PBS (experimental group, EG), 300 μL of PBS (negative control, NC) and 300μL of 2% Triton X-100 solution (positive control, PC) respectively, then scopoletin/scoparone was added. The cells were incubated at 37°C 6 h and supernatant was measured at OD450. SPL, scopoletin; SPR: scoparone; 128, 256 and 512: 128 μg/mL, 256 μg/mL and 512 μg/mL of scopoletin or scoparone. Data are shown as mean ± SD of at least three independent experiments. ns, nonsignificant. **P* < 0.05; ****P* < 0.001.

Currently, UHPLC-Q Exactive-Orbitrap MS system has been widely used to identify significantly different chemical compounds in various Chinese herb samples. In this study, we used UHPLC-Q Exactive-Orbitrap MS to qualitatively identify the *V. philippica* compounds. Previous research has shown that *V. philippica* contains flavonoids,[30] coumarins,[17] anthocyanins[31] and cyclotides, etc.[32] Though Wu et al reported 32 compounds within water extract of *V. philippica* using UHPLC-ESI-Q-TOF-MS/MS, including coumarin, flavonoids and organic acids,[25] we identified 16 compounds (6 coumarins, 5 flavonoids and 5 organic acids) from VPE. Coumarins are a group of natural compounds containing a variety of molecules with similar basic structures. Coumarins have been found in various plants and some have been shown to have antibacterial, antifungal, and pharmacological potential.[33] It has been identified that coumarins, aesculetin and aesculin, can interact with the signal-adhering domain of the TraR protein, thus inhibiting the biofilm formation of *P. aeruginosa*.[34] R.E. D'Almeida et al compared the QS inhibitory effects of seven structurally related coumarins (dihydrocoumarin, coumarin, 3-hydroxycoumarin, 4-hydroxycoumarin, 6-hydroxycoumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin) on *P. aeruginosa and* revealed the intensity of anti-biofilm and anti-QS activity to *P. aeruginosa was* structure-dependent.[35] Therefore, we focused on five coumarin derivatives identified in VPE. Scopoletin, also known as 6-methoxy-7-hydroxycoumarin, is a phenolic coumarin isolated from many plants and has been shown to possess antibacterial activity in inhibiting the growth of certain pathogenic bacteria, such as *S. aureus, Escherichia coli* and *Salmonella* spp.[36] Scoparone, also known as 6,7-dimethoxycoumarin/ 6,7-dimethylesculetin, is the major component of *Artemisia capillaris Thunb*. Scoparone is the main efficacious and active component of the Yin-Chen-Hao decoction (YCHD) and Yin-Zhi-Huang decoction (YZHD) for the treatment of hepatic and cholestatic disorders.[37,38] Recent studies have shown that scoparone has extensive pharmacological activities such as anti-

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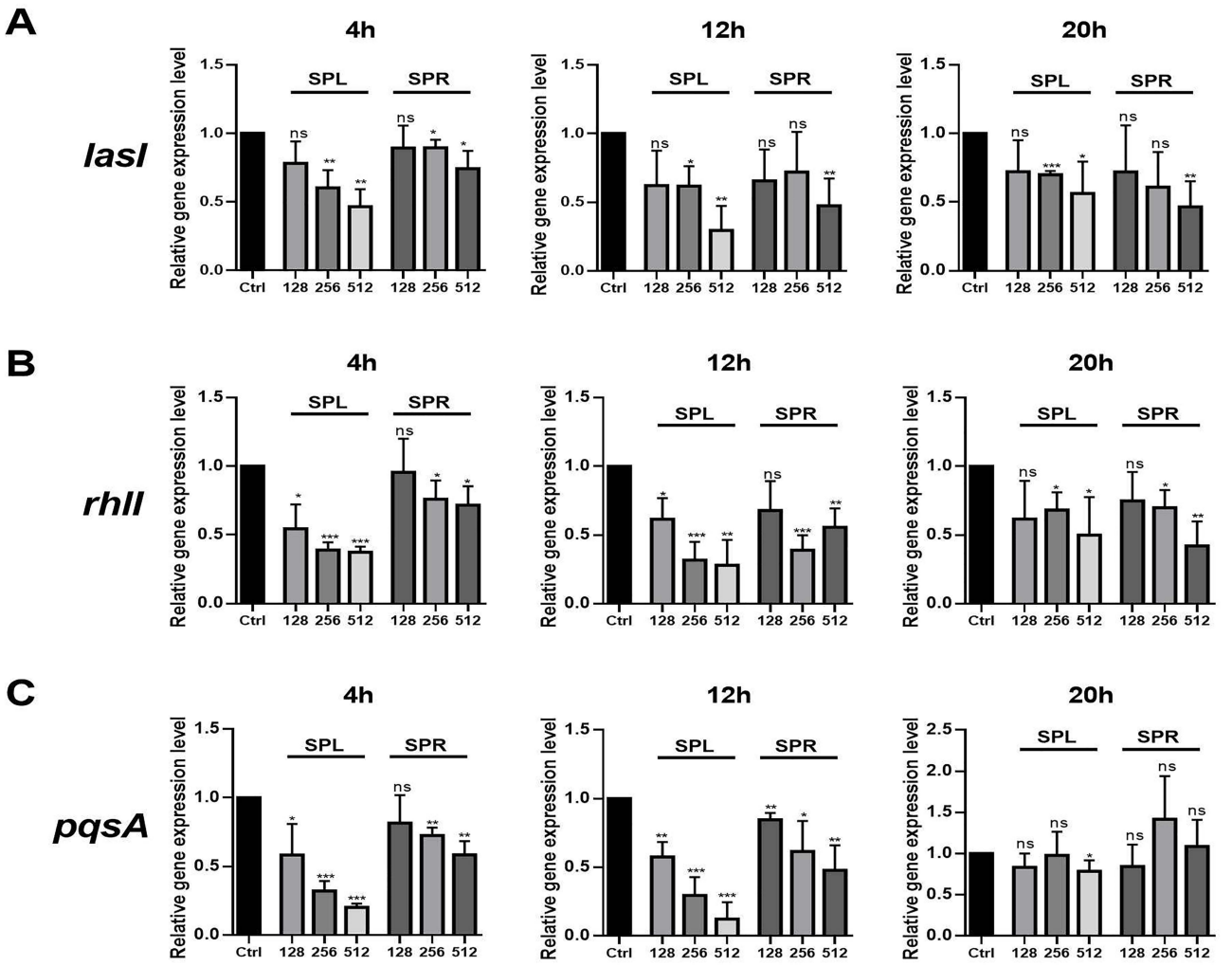

Figure 5 Effects of scopoletin/scoparone on the expression of QS regulator genes in PAO1. PAO1 was incubated with different concentrations of scopoletin/scoparone at 37°C 200 rpm for 4 h, 12 h and 20 h, then the expression levels of *lasI* (**A**), *rhlI* (**B**), and *pqsA* (**C**) were measured by qRT-PCR. PAO1 incubation without scopoletin/scoparone was used as a control. SPL: scopoletin; SPR: scoparone; 128, 256 and 512: 128 μg/mL, 256 μg/mL and 512 μg/mL of scopoletin or scoparone. Data are shown as mean ± SD of at least three independent experiments. ns, nonsignificant. **P* < 0.05; ***P* < 0.01; and ****P* < 0.001.

inflammatory, anti-fibrotic, anti-apoptotic, anti-coagulant, and anti-diabetic properties.[39–41] Scopoletin is the demethylation product of scoparone, and we hypothesized that scoparone might also exert antibacterial effects. Altogether, we investigated whether the coumarin derivatives, scopoletin and scoparone, were responsible for the anti-QS activity of VPE (Table 2).

Considering the antibacterial activity of *V. philippica*[18] and to prevent exerting excessive selective pressure on PAO1, the MIC of VPE was evaluated. Our findings revealed that the MIC of VPE against PAO1 was 128 mg/mL using the agar dilution method. Moreover, PAO1 growth kinetics were unaffected at different concentrations (1/2, 1/4, and 1/8 MIC, corresponding to 64 mg/mL, 32 mg/mL, and 16 mg/mL, respectively). Likewise, exposure of PAO1 to scopoletin/scoparone resulted in growth inhibition at a concentration of 1024 μg/mL, while no impact was observed at sub-doses of 512 μg/mL, 256 μg/mL, and 128 μg/mL. Consequently, sub-doses of VPE, scopoletin, and scoparone were employed in the subsequent phenotypic and genetic investigations, given that these doses did not exert any inhibitory effects on the growth of *P. aeruginosa*.

QS inhibitors (QSIs) are a promising alternative to antibacterial therapies, exerting less selective pressure for resistance than conventional antibiotics.[42] The biosensor *C. violaceum* CV026 is a double mini-Tn5 mutant that is limited to producing the purple pigment violacein when given an exogenous short-chain AHL under the control of the CVi/R QS system.[43] Approximately 200 studies have been published regarding its use as an indicator for QS activity.[44] Here, we observed that the range of the nonviolacein halo around *C. violaceum* CV026 progressively expanded in tandem

with the increased concentration of VPE, scopoletin, and scoparone, suggesting that VPE and its compounds impede *C. violaceum* CV026's capacity to respond to exogenously given N-hexanoylhomoserine lactone. This finding is in line with the work of Tajani AS et al,[45] who reported that a variety of coumarin derivatives exhibit anti-QS, and scopoletin and scoparone are two newly discovered coumarin derivatives with anti-QS functions.

P. aeruginosa produces many virulence factors including pyocyanin, rhamnolipids, elastases, and hemolysin. Interestingly, our findings revealed that VPE treatment decreased the production of pyocyanin and rhamnolipids without affecting cell viability (Table 2). Similarly, scopoletin and scoparone exhibited inhibitory effects against *P. aeruginosa* PAO1, resulting in reduced expression of virulence factors such as pyocyanin (Figure 2C), rhamnolipid (Figure 2D), elastase (Figure 2E), and hemolytic activity (Figure 4). Pyocyanin is synthesized from chorismate by the *phzABCDEFG* operons, along with *phzM* and *phzS*,[46] and is responsible for the blue-green color characteristic of *P. aeruginosa*. A possible mechanism of pyocyanin inhibition is that VPE and scopoletin/scoparone affect quinolone biosynthesis, resulting in reduced pyocyanin synthesis. Rhamnolipid, a so-called "secondary metabolite", is a biosurfactant regulated by *rhlA* and *rhlB* operons that cluster with *rhlI* and *rhlR*. Rhamnolipids also play multiple roles in biofilm formation by *P. aeruginosa*.[47] Scopoletin significantly inhibited the production of rhamnoolipid, whereas scoparone only showed moderate attenuation at 512 μg/mL. Hence, VPE and scopoletin/scoparone might have operated by upsetting QS systems or resulting in dysregulation of rhamnolipid synthesis. Hemolysins are *las*-regulated by various virulence factors. Our results showed that scopoletin treatment significantly inhibited the hemolysis rate of PAO1, whereas scoparone treatment only inhibited the hemolysis rate at high doses (512 μg/mL). To sum up, our research has revealed that VPE, along with its bioactive components scopoletin/scoparone, effectively inhibit QS-regulated virulence factors in *P. aeruginosa*.

Pyocyanin and rhamnolipid are virulence phenotypes in liquid media, and motility is one of the virulence phenotypes manifested by surface-associated behaviors that are crucial for colonization, biofilm formation, and production of virulence factors. Swarming motility is an uncoordinated surface-associated motility of hyperflagellated and highly motile bacterial cells.[48] Swarming motility necessitates the involvement of flagella, type IV pili, and rhamnolipid biosurfactants. In contrast to swarming, swimming motility refers to the movement of individual bacterial cells within liquid environments. Our research revealed that VPE and its compounds, scopoletin and scoparone, inhibited *P. aeruginosa* swarming motility, but not swimming motility, suggesting that VPE and its compounds may hinder flagellar functions. Considering that QS regulates bacterial motility, it is plausible that *V. philippica* inhibited the motility of *P. aeruginosa* either by either directly targeting flagella or by disrupting with the QS signaling pathway. Such interference would, in turn, hinder bacterial adhesion and colonization, biofilm formation, as well as the production of other virulence factors regulated by QS. The manifestation that anti-QS compounds only affect swarming but not swimming has likewise been reported in several other studies, such as those by Luo et al,[8] Che et al[49] and Thomas et al.[50]

Additionally, *P. aeruginosa* forms surface-attached communities known as biofilms, the so-called "bacterial cities".[51] Bacteria living in biofilms cause 80% of bacterial infections.[52] Numerous studies have found that natural products, such as baicalin, hyperoside, allicin and garlicin, exhibit an inhibitory effect on biofilms.[8,53,54] Similarly, we showed here that VPE and its compound scopoletin/scoparone can significantly inhibit the development of biofilm, suggesting their potential for treating chronic infections. The early stages of biofilm formation rely on bacterial motility and rhamnolipid production. Thus, changes in biofilm, motility, and rhamnolipid production are interrelated.

Many plant agents downregulate the *P. aeruginosa* virulence via unknown QS-inhibiting mechanisms.[29] Studies have elucidated some anti-QS molecular pathways. In *P. aeruginosa*, the *rhl* and *pqs* systems are regulated by the *las* system, and all three QS systems are required for producing virulence factors such as pyocyanin, biofilm formation, drug resistance, and full pathogenicity.[55] Our results showed that under VPE treatment, QS regulatory genes (*lasI, rhlI* and *pqsA*) were downregulated, correlating with the suppression of virulence phenotypes, including pyocyanin, rhamnolipids, motility, and biofilms. Consistent with the crude extract findings, high concentrations of compounds extensively inhibited *lasI, rhlI and pqsA*, with *pqsA* having a dramatic inhibitory effect, mainly in the early and middle stages of growth. These results suggest that scopoletin and scoparone may act as multi-target inhibitors of bacterial virulence, given the inhibition of all three hierarchical QS systems in *P. aeruginosa*. The inhibition mechanisms could be (i) top-to-bottom regulation: scopoletin/scoparone shutting down the *las* system, thereby downregulating the *pqs* and *rhl* systems; and (ii) scopoletin/scoparone inhibiting the *pqs* system, leading to the subsequent shutdown of the *rhl* system. Nevertheless, the inhibitory

mechanisms of scopoletin and scoparone on QS-regulated virulence factors may be multifaceted, including: (a) inhibiting the synthesis of signaling molecules (eg, C4-HSL and 3-O-C12-HSL; (b) enzymatic degradation of signaling molecules (eg, acylase hydrolysis of AHLs); (c) competing with signaling molecules for binding to related receptors (eg, *lasR, RhIR, PqsR*); (d) interfering with the binding of signaling molecules to promoters to repress gene expression. Further in-depth studies, such as transcriptomics and proteomics research, are needed to reveal unknown targets.

Although this study demonstrates that scopoletin and scoparone exert anti-virulence effects by broadly inhibiting QS-regulated genes, the precise molecular mechanism remains unclear. Additionally, as this study is based on in vitro results, the in vivo effects of scopoletin and scoparone are yet to be determined. Future research should focus on two main aspects: (1) Elucidation of molecular mechanisms: using molecular docking, site-directed mutagenesis, signal synthesis and degradation assays, and protein–protein interaction studies to provide a deeper understanding of the molecular mechanisms underlying the anti-QS activities of scopoletin and scoparone, and further validate their potential as effective anti-virulence agents against *P. aeruginosa* infections; (2) In vivo experiments: establishing animal models of *P. aeruginosa* infection (eg, mouse lung infection models) to investigate the in vivo mechanisms of these compounds. In summary, our findings suggest that scopoletin and scoparone from *V. philippica* can be used as potential anti-virulence agents to control harmful infections caused by *P. aeruginosa* via QS inhibition.

Conclusion

P. aeruginosa is a common nosocomial pathogen causing various infections. There is an urgent need to search for innovative effective medicines that can prevent microbial resistance owing to the rise in carbapenem-resistant *P. aeruginosa* and antibiotic abuse. The virulence of *P. aeruginosa* is regulated by QS. Plant-based natural products are repositories of QS inhibitors (QSIs), which can block the generation or transmission of QS signals. Therefore, understanding the compounds from *V. philippica* that regulate the QS-related virulence of *P. aeruginosa* is important to combat infection. In this study, two previously uncharacterized coumarin derivatives, scopoletin and scoparone from *V. philippica* were identified as novel QSIs. They have been found to inhibit QS-mediated virulence factors, including pyocyanin, elastase, rhamnolipid, hemolysin, biofilm, and swarming by inhibiting the QS-related genes *lasI, rhlI*, and *pqsA*. This study provides a laboratory basis for using scopoletin and scoparone as promising anti-virulence candidates for adjuvant therapy against *P. aeruginosa* infection. Future research will focus on elucidating the molecular mechanisms and conducting in vivo investigations.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author (email: chencha@gzucm.edu.cn) upon reasonable request.

Ethics Approval and Informed Consent

For whole blood hemolysis assays, informed consent was obtained from each volunteer prior to study commencement. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Guangdong Provincial Hospital of Chinese Medicine (BE2024-311-01).

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.

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Disclosure

The authors report no conflicts of interest in this work.

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