

Screening of quorum sensing inhibitors for *Pseudomonas aeruginosa* in the traditional Chinese medicine *Phellodendron amurense*

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Abstract

Drug-resistant bacterial infections pose a significant challenge in the field of bacterial disease treatment. Finding new antibacterial pathways and targets to combat drug-resistant bacteria is crucial. The bacterial quorum sensing (QS) system regulates the expression of bacterial virulence factors. Inhibiting bacterial QS and reducing bacterial virulence can achieve antibacterial therapeutic effects, making QS inhibition an effective strategy to control bacterial pathogenicity. This article mainly focused on the PqsA protein in the QS system of *Pseudomonas aeruginosa*. An affinity chromatography medium was developed using the SpyTag/SpyCatcher heteropeptide bond system. Berberine, which can interact with the PqsA target, was screened from *Phellodendron amurense* by affinity chromatography. We characterized its structure, verified its inhibitory activity on *Pseudomonas aeruginosa*, and preliminarily analyzed its mechanism using molecular docking technology. This method can also be widely applied to the immobilization of various protein targets and effective screening of active substances.

1. INTRODUCTION

Pseudomonas aeruginosa is an important human conditionally pathogenic bacterium that is widely found in the environment including water and soil^[1]. This bacterium is responsible for a range of infections, encompassing urinary tract infections, acute ulcerative keratitis, malignant otitis externa, ventilator-associated pneumonia in intubated patients, burn wound infections, and chronic destructive lung infections in cystic fibrosis patients^[2–4]. Antivirulence therapy represents a strategic approach that centers on suppressing the production of virulence factors by pathogens, as opposed to directly impeding bacterial proliferation. In comparison to conventional antibiotics, antivirulence therapy not only mitigates the evolutionary pressures on bacteria, thereby diminishing the emergence of antibiotic resistance, but also exhibits expeditious action on target entities^[5]. Perturbing bacterial quorum sensing (QS) is a pivotal tactic within the realm of antivirulence therapy^[6]. Quorum sensing denotes an intercellular chemical communication process that orchestrates the expression of virulence factors in bacteria. Quorum sensing inhibitors (QSIs) efficaciously disrupt the QS system, curtailing the production of virulence factors without eliciting bacterial lethality. This mechanism provides a strategic avenue for tackling drug-resistant bacterial infections.

Bacterial community sensing is an intercellular chemical communication process that depends on cell density. During bacterial growth, when the density of the bacterial body is low, bacteria synthesize and release a signaling molecule, which is called an Autoinducer (AI), which senses the change in the number of their own bacteria or other bacteria in the surrounding environmental species according to the different concentrations of AIs. When the AIs reach a certain concentration threshold, they can initiate the expression of related genes, and regulate the bacterial population behavior to adapt to changes in the environment, such as bioluminescence, production of toxins, formation of virulence factors such as biofilm, and production of antibiotics^[7–9]. The QS system of *P. aeruginosa* mainly consists of the las system and rhl system based on acyl-homoserine lacton (AHL), as well as the 2-heptyl-3-hydroxy-4-

quinolone (PQS) system. The QS systems of *P. aeruginosa* directly or indirectly control the expression of more than 10% of the genome^[10] and about 20% of the bacterial proteome^[11].

A large variety of synthetic AHL analogues and natural product libraries have been screened and a number of QS inhibitors (QSIs) have been identified^[12–14]. However, there is limited research on the screening of QSIs based on the PQS system, mainly due to the complexity of the PQS system^[15–16]. *Pseudomonas aeruginosa* produces the cell-to-cell signal molecule PQS, which is integrated within a complicated QS signaling system. PQS is synthesized via the PqsABCDE operon, which can produce the precursor of PQS, 2-heptyl-4-quinolone (HHQ). HHQ can activate the quinolone signaling receptor PqsR (also known as MvfR)^[17, 18]. In the PQS system of *P. aeruginosa*, PqsA plays an important role in the pathway to synthesize the signaling molecule PQS, and inhibition of the entry of PqsA into the downstream steps can block PQS formation, thereby reducing the production of bacterial virulence factors^[19–20]. Therefore, PqsA is an effective target for QS inhibition in the QS system of *P. aeruginosa*. PqsA serves as a protein target, and the search for substances that can interact with PqsA can effectively block the production of virulence factors by the PQS system.

Natural products are characterized by rich sources, diverse chemical compositions, and high pharmacological activities, which make them an important source of QSI screening that cannot be ignored^[21]. However, traditional screening of QSI components from natural products often requires a combination of high-throughput and biological activity screening, and the process is cumbersome and time-consuming^[22]. In addition, the loss of trace high-value components and the lack of sample quantity in activity experiments are also important factors affecting the screening results. The rapid construction of affinity chromatography separation systems using target molecules as ligands is an important approach for drug screening^[23].

Phellodendron amurense extract is a traditional Chinese herbal medicine, which contains isoquinoline alkaloids, flavonoid glycosides, phenolic compounds and other active ingredients, and has antibacterial, anti-inflammatory, antiviral and other effects^[24–25]. In this work, we utilized its simplicity and highly efficient covalent linkage properties of isopeptide bonds^[26]. First, SpyCatcher protein was immobilized on the agarose gel, and then the fused SpyTag PqsA was made to bind to SpyCatcher autonomously and specifically, so that the target PqsA was immobilized on the affinity chromatography medium. For specific data, please refer to our previous work^[23, 27]. Affinity chromatography was utilized to screen substances from herbal medicines that could interact with the PqsA target and to investigate their inhibitory activity against *P. aeruginosa* population sensing.

2. Materials and methods

2.1 Materials

Bovine serum albumin (BSA), an SDS-PAGE preparation kit, Bradford reagent and agarose were from Sangon Biotechnology (Shanghai, China). HisTrap™ HP (5 mL) columns were from GE Healthcare (United States). Other reagents were of analytical grade.

2.2 Instruments and Operating Conditions

Protein purification was performed using an ÄKTA Purifier System. A SpectraMax®M5 Multi-Mode Microplate Reader was utilized for Bradford assays at 595 nm. An Olympus CKX53 inverted microscope was used for fluorescence imaging. HPLC analyses were conducted using a Shimadzu LC 20A system equipped with a Synergi Hydro-RP 80A column (250 mm × 4.6 mm, 4 μm).

Preparation of *Phellodendron amurense* Extract

After washing, drying, and crushing *Phellodendron amurense* bark, 2 g of powder was weighed and 40 mL of 80% ethanol was added at a ratio of 1:20. The extract was centrifuged, evaporated at 50°C and then dissolved in methanol and filtered.

2.3 Protein Expression and Purification

Strains of *Escherichia coli* (*E. coli*) BL21(DE3)-pET30a(+)-SpyCatcher and *E. coli* BL21(DE3)-pET30a(+)-SpyTag-PqsA were constructed for protein expression. Induction of expression was achieved using isopropyl β-D-1-thiogalactopyranoside (IPTG). Purification was carried out using a HisTrap™ HP column (5 mL), following a protocol described in our previous experiments^[23]. Protein concentration was determined using the Bradford assay with BSA as the standard.

2.4 Preparation of Affinity Chromatography Beads

Ten mL of agarose gel (settled volume) was measured and washed repeatedly with excess distilled water to remove ethanol. Sequentially, dimethyl sulfoxide, epichlorohydrin, sodium hydroxide, and sodium borohydride were added to the processed agarose gel. The agarose gel suspension was maintained at a constant temperature of 37°C with continuous agitation at 150 rpm for 3 hours. After completion of the reaction, the agarose gel was thoroughly washed with distilled water and subsequently dried. 60 mg of purified SpyCatcher was incubated with activated agarose gel overnight at 4°C. Following this, the gel was washed with 0.02 mol/L phosphate buffer (PB) and then dried. Then, 60 mg of SpyTag-PqsA fusion protein was reacted with the gel, incubated for 3 hours, and then washed with PB buffer before drying. Ten mL of SpyTag/SpyCatcher-coupled agarose beads were packed into a glass column using an ÄKTA Purifier System at a flow rate of 2 mL/min. This process resulted in an affinity column with a height of approximately 8 cm.

2.5 Screening of Active Ingredients

Using a flow rate of 1 mL/min, the system was equilibrated with equilibration buffer (0.02 mol/L PB). Detection was conducted at UV wavelengths of 215 nm, 280 nm, and 345 nm. After achieving baseline equilibrium, 700 μL of the extract was injected into the sample loop and loaded. Once baseline

equilibrium was re-established, elution was obtained using elution buffer (1.5 mL/min, 30 min, gradient of 0 – 0.5 mol/L NaCl in 0.02 mol/L PB).

2.6 Substance Analysis

In order to identify the active components, both HPLC and mass spectrometry (MS) analyses were conducted. Following desalting treatment, a sample volume of 10 µL was injected. A mobile phase consisting of 0.1% aqueous phosphoric acid (A) and acetonitrile (B) was employed for gradient elution (30 min, 10%–100% B) at a flow rate of 1 mL/min. Detection was carried out at a wavelength of 345 nm, and the column temperature was maintained at 25°C. The positive mode was used for MS analysis.

2.7 Growth Curve Assay

Fresh LB medium was used to dilute the bacterial suspension to a McFarland turbidity of 0.5. Subsequently, the diluted bacterial suspension was added to 10 mL of LB medium at a ratio of 1:100, resulting in a final bacterial concentration of 1×10^6 CFU/mL. Three parallel groups were established for the experiment. The cultures were incubated at a constant temperature of 37°C with continuous shaking at 220 rpm. Samples were collected at specific time points, namely 4, 6, 8, 10, 12, 14, 24, and 36 hours. For each time point, a control culture medium was prepared without bacterial inoculation. The optical density of the bacterial suspension was measured at OD_{600 nm} using a spectrophotometer.

2.8 Determination of the MIC

The minimal inhibitory concentration (MIC) of the compound against *P. aeruginosa* was determined using the serial dilution method. The compound was diluted over a range of 0 – 2,560 µg/mL. After dilution, the compound was added to cultures of *P. aeruginosa*. The cultures were then incubated at 37°C with continuous shaking at 220 rpm for 16 – 18 hours. Following this incubation period, the growth of the bacteria was evaluated. The MIC was defined as the lowest concentration of the compound that effectively inhibited bacterial growth.

2.9 Toxicity Factor Assay

Biofilm inhibition assay

The culture medium was dispensed into individual wells, and different concentrations of the compound were added to the culture medium, with methanol as a control. In each well of a 96-well plate, 150 µL of bacterial suspension was added and incubated at 37°C for 18 – 24 hours.

The bacterial suspension was gently aspirated using a pipette, then the 96-well plate was gently washed with PB buffer and left to air dry. 150 µL of 1% Crystal Violet stain was then added to each well for 10 min. The Crystal Violet solution was aspirated, the wells were washed 2 – 3 times with PB buffer, allowed to air dry, and the dye was dissolved using acetic acid solution. The numerical value at OD_{495 nm} was measured, with three parallel setups for each group.

The percentage of biofilm inhibition (%) was calculated using the formula:

$$\text{Biofilm Inhibition (\%)} = \frac{\text{OD}_0 - \text{OD}_1}{\text{OD}_0} \times 100\%$$

Pyocyanin Assay

After incubating *P. aeruginosa* with varying concentrations of the compound at 37°C and 220 rpm for 16 hours, the cultures were centrifuged and the supernatant collected. 3 mL of chloroform was added for extraction, the chloroform layer was transferred to a new centrifuge tube, and mixed with 1 mL of hydrochloric acid. The solution was centrifuged to separate the upper aqueous phase and measured at OD_{520 nm}. This procedure was performed in the three parallel setups for each group.

Rhamnolipid Assay

Preparation of the Rhamnose Standard Curve involved the preparation of 0, 100, 200, 300, 400, and 500 µg/mL standard solutions of rhamnose. A total of 100 µL of each solution was placed in test tubes and 900 µL of sulfuric acid-moss black phenol reagent was added. The contents were thoroughly mixed and heated in a boiling water bath for 10 min, and then cooled at room temperature for 20 min and the OD_{421 nm} value was measured and used as the y-value. A standard curve using Rhamnose concentrations as the x-value was created.

For the copper-green *Pseudomonas* culture medium, different concentrations of the compound were added. The cultures were incubated at 37°C and 220 rpm for 16 hours. Following incubation, the bacterial suspension was collected and centrifuged at 4°C and 10,000 rpm for 10 min. The supernatant was collected and the pH was adjusted to around 2 using HCl solution. Extraction of the supernatant was performed with an equal volume of ethyl acetate by vortexing. After allowing it to settle, the organic layer was aspirated and then vacuum dried. After re-dissolving, 100 µL was obtained and 900 µL of the sulfuric acid-moss black phenol reagent was added. This was heated in a boiling water bath for 10 min, cooled at room temperature for 20 min, and the OD_{421 nm} value measured. The Rhamnose content was measured based on the Rhamnose standard curve.

Motility Assay

The melted swarming agar medium was obtained and different concentrations of the compound were added. The medium was allowed to air dry for 15 min. In the center of the agar medium, 5 µL of bacterial suspension was added. The agar plates were placed in an incubator set at 37°C and incubated upright for 24 hours. Following this incubation period, the experimental results were determined.

Molecular Docking

To retrieve the three-dimensional crystal structure of the receptor protein PqsA from the PDB database (<https://www.rcsb.org>), Pymol software was used to remove the solvent and ligand molecules from the protein structure. AutoDockTools were employed for hydrogenation and charge assignment of the

protein. Rotatable bonds in the small molecule compound were identified to enable free rotation and set it as the receptor protein.

The ligand file of the small molecule was downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>), and then AutoDockTools were used to hydrogenate the ligand and assign charges, setting it as the ligand compound. Semi-flexible docking was conducted between the processed receptor protein and ligand compound using AutoDock software. The result dialog box was used to examine the analysis outcomes between the receptor and ligand.

3. Results and Discussion

3.1 Expression and Purification

The recombinant protein was successfully expressed and purified. The molecular weights of SC (lane 1) and SP (lane 2) were approximately 18 kDa and 63 kDa, respectively. When SC and SP were combined, a higher molecular weight protein band was visible in lane 3 (Fig. 1), corresponding to SC@SP (18 + 63 kDa), thereby confirming successful formation of the complex.

3.2 Screening of Active Ingredients

The affinity chromatogram of the 700 μ L loaded extract is shown in Fig. 2. Around two min after loading, a prominent breakthrough peak appeared, indicating the initial displacement of weakly bound compounds. Subsequent gradient elution using the elution buffer resulted in the appearance of a distinct elution peak. This indicated the presence of substances in the extract that could interact with the ligand, potentially implying the presence of compounds capable of interacting with the PqsA protein.

3.3 Substance Analysis

The collected elution peak was subjected to HPLC analysis using a mobile phase of 0.1% aqueous phosphoric acid and acetonitrile, with a UV detection wavelength of 345 nm. The flow rate was set at 1 mL/min. As depicted in Fig. 3(B), a sharp and symmetric single peak appeared at the retention time of 11.465 min. This retention time coincided with that of the berberine standard compound (Fig. 3(A)). The mass spectrum of the elution compounds is presented in Fig. 3(C). The peak with the highest response value in the full scan corresponded to a mass-to-charge ratio (m/z) of 336.1230. Based on both the MS analysis results and the matching retention time, the primary component in the elution peak was identified as berberine. By utilizing MassHunter software, the molecular formula of this compound was determined to be $C_{20}H_{17}NO_4$ (Fig. 3(D)).

3.4 Growth Curve Assay

A growth curve of *P. aeruginosa* was plotted with cultivation time (hours) on the x-axis and optical density ($OD_{600\text{ nm}}$) on the y-axis. As illustrated in Fig. 4, *P. aeruginosa* entered the logarithmic growth phase at around 8 hours of cultivation and reached a stable phase by the 18th hour.

3.5 Determination of the MIC

The MIC of berberine against *P. aeruginosa* was determined by observing the bacterial growth in test tubes. The lowest concentration of berberine that did not result in visible turbidity in the test tubes was considered the MIC. This approach aimed to identify the concentration range at which berberine does not inhibit the growth of *P. aeruginosa*.

As depicted in Fig. 5, berberine exhibited noticeable growth inhibition against the bacterium from 1280 µg/mL. However, within the concentration range below 1280 µg/mL, the final bacterial density in both treated and untreated cultures was essentially the same. Therefore, the MIC of berberine against *P. aeruginosa* was determined to be 1280 µg/mL. In subsequent experiments related to the impact on virulence factors, concentrations below 1280 µg/mL were utilized.

3.6 Toxicity Factor Assay

Biofilm inhibition assay

The pathogenicity of bacteria is influenced by the protective barrier of biofilms, which renders them more resistant to physical and chemical agents. Inhibiting biofilm formation can enhance the effectiveness of drugs against bacteria. As depicted in Fig. 6, at low berberine concentrations, there was a slight reduction in the biofilm density. However, at the concentration of 1/2 MIC, the ability to counteract biofilms was significantly enhanced, resulting in a 50.5% inhibition rate. Microscopic observations of biofilms yielded similar results. The control group formed thick cell aggregates, while the experimental group showed reduced biofilm formation and disrupted biofilm integrity. Cell aggregation and density were markedly diminished, presenting a dispersed appearance.

Pyocyanin Assay

Pyocyanin, a bluish-green toxic metabolite, is one of the virulence factors of *P. aeruginosa*. It can penetrate and disrupt the membrane structures of organisms, playing a significant role in the *P. aeruginosa* infection process^[28]. The effects of different concentrations of berberine on pyocyanin production in *P. aeruginosa* was investigated and are shown in Fig. 7. The results indicated that as the berberine concentration increased, the production of pyocyanin gradually decreased. At concentrations corresponding to 1/8 MIC, 1/4 MIC, and 1/2 MIC, berberine led to reductions in pyocyanin production of 12.8%, 33.9%, and 45.0%, respectively.

Rhamnolipid Assay

Rhamnolipid is converted into rhamnose by the hydrolysis of fatty acid chains. The content of rhamnolipid was calculated by measuring the content of rhamnose. The conversion coefficient between rhamnose and rhamnolipid was 3, i.e. 1 µg rhamnose equals 3 µg rhamnolipid. These results are depicted in Fig. 8. At 1/8 MIC concentration, the impact of berberine on rhamnolipid production was not significant. However, at concentrations of 1/4 MIC and 1/2 MIC, berberine exhibited substantial inhibition

of rhamnolipid production. The inhibitory effect was prominent at both concentrations, with reductions of 66.5% and 68.1%, respectively.

Motility Assay

Swarming motility is one of the primary modes of movement of *P. aeruginosa*, which relies on flagella. It aids the bacteria in nutrient acquisition, evading host immune responses, colonization, and biofilm formation. This process is regulated by QS in *P. aeruginosa*^[29]. Berberine inhibited the swarming motility of *P. aeruginosa*. As shown in Fig. 9, at 1/8 MIC concentration, there was a slight inhibition of swarming motility. However, on plates with a concentration of 1/2 MIC, berberine significantly suppressed the swarming motility of *P. aeruginosa*. The migration inhibition rate was 60.3%.

Molecular Docking

Molecular docking is a computational technique used to simulate the binding mode between receptor active sites and ligand molecules. It can predict the interaction patterns and binding free energies between molecules, and visualize these interactions graphically. This approach was then used to elucidate the mechanism of inhibition of QS by inhibitors^[30]. As illustrated in Fig. 10, molecular docking simulations were performed between the 3D structure of berberine and the tertiary structure of PqsA. The binding energies from 10 docking runs were calculated as follows: -6.635, -6.961, -6.863, -7.206, -6.732, -7.178, -7.610, -7.609, -7.619, and -7.198 kcal/mol, respectively. Lower binding energies indicated greater stability of the structure. The docking results between berberine and PqsA suggested a stable binding interaction between the two molecules. As shown in Table 4 – 3, berberine interacted with amino acid residues such as ILE204, TRP233, and ILE257 on PqsA.

4. Conclusion

In response to the current state of bacterial antibiotic resistance, the search for QSIs effectively suppressed QS and prevented the development of antibiotic resistance. Research on QS systems in *Pseudomonas aeruginosa* has predominantly focused on the las and rhl systems, with limited attention given to the PQS system. PqsA is a crucial target in the *Pseudomonas aeruginosa* PQS system, and screening for PqsA inhibitors can significantly contribute to understanding the QS PQS system. Affinity chromatography is an important method for screening inhibitors to determine the active substances interacting with the target. The SpyCatcher/SpyTag self-assembly system was highly suitable for directed immobilization of biomolecules due to its spontaneous and specific binding, strong covalent attachment, capable gene editing and mild reaction conditions. The immobilized target was more tolerant to the elution conditions during affinity chromatography, so it was very suitable for efficient screening in complex systems (such as natural product extracts, microbial fermentation broth, etc.).

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Yu Yi: Investigation, Conceptualization, Methodology, Writing - Review & Editing, Data curation. Ye Zhou: Investigation, Methodology, Validation, Software. Writing - Original draft preparation, Formal analysis, Visualization. Susu Lin: Investigation, Methodology, Formal analysis, Visualization. Kefan Shi: Investigation, Methodology. Jianfeng Mei: Data curation, Writing - Review & Editing. Guoqing Ying: Resources, Supervision, Project administration, Funding acquisition. Shujiang Wu: Resources, Supervision, Project administration.

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Figures

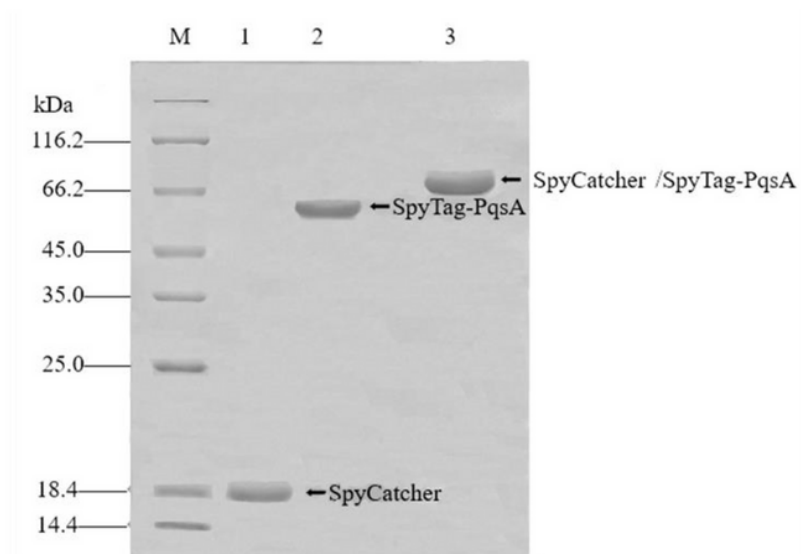


Figure 1. SDS-PAGE image of SpyCatcher and SpyTag-PqsA reaction
M: Protein marker. 1: Purified SpyCatcher, 2: Purified SpyTag-PqsA, 3: Reaction of
SpyCatcher and SpyTag-PqsA.

Figure 1

See image above for figure legend

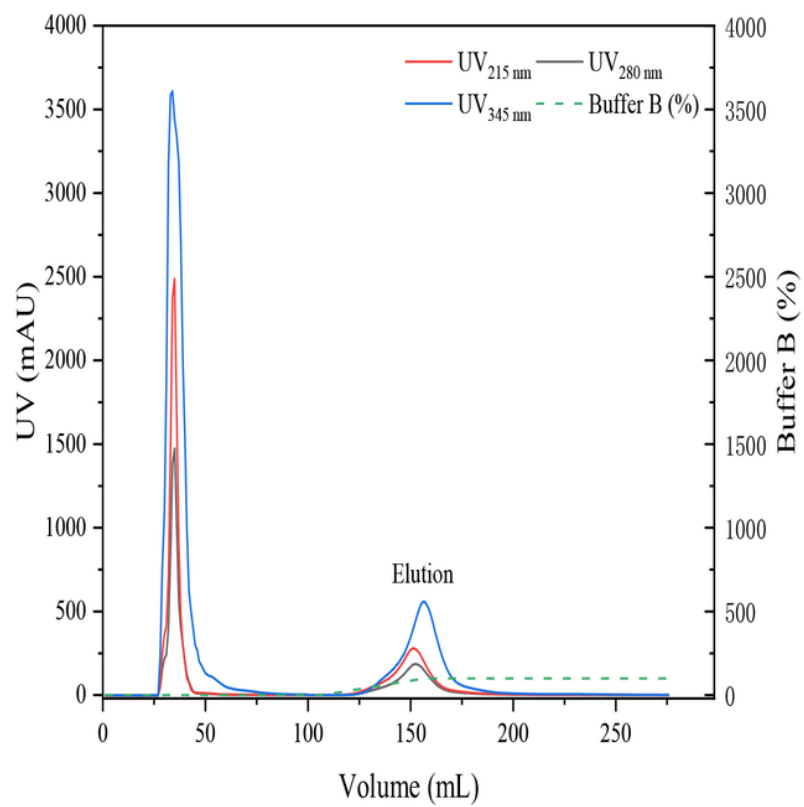


Figure 2. Affinity chromatography of the extract.

Figure 2

See image above for figure legend

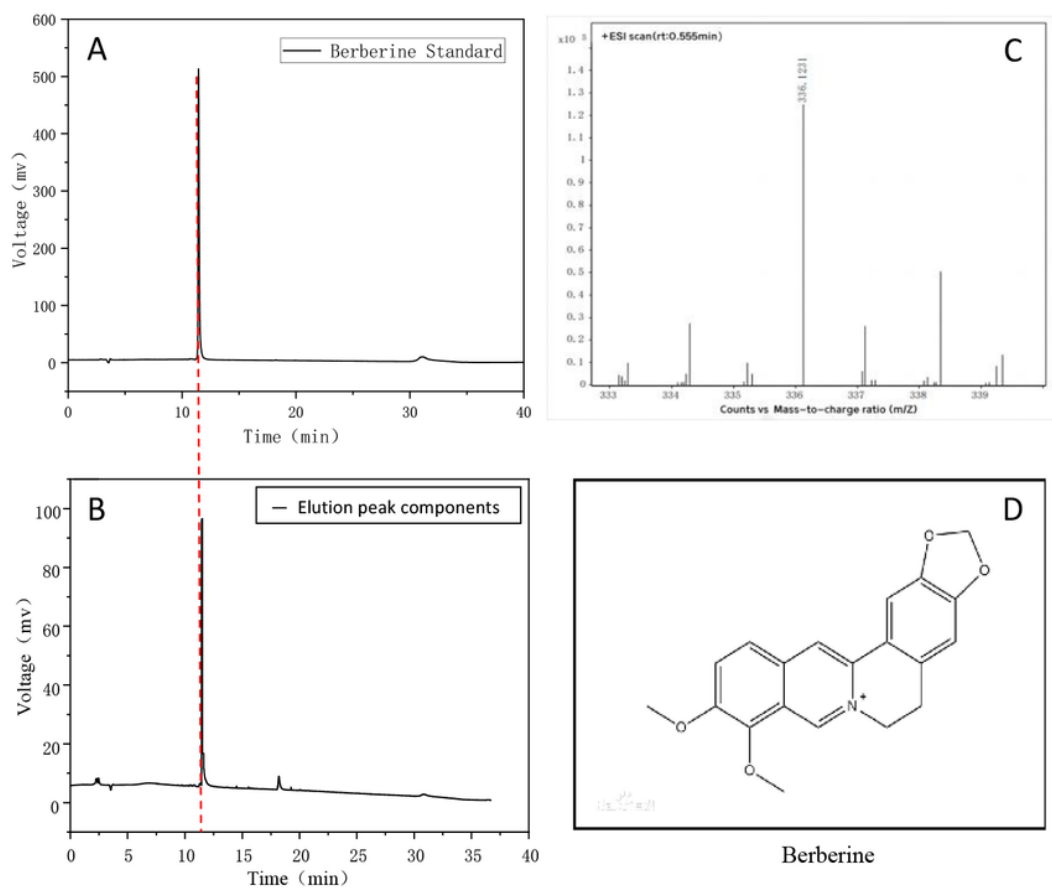


Figure 3. Analysis of the elution compounds.

Figure 3

See image above for figure legend

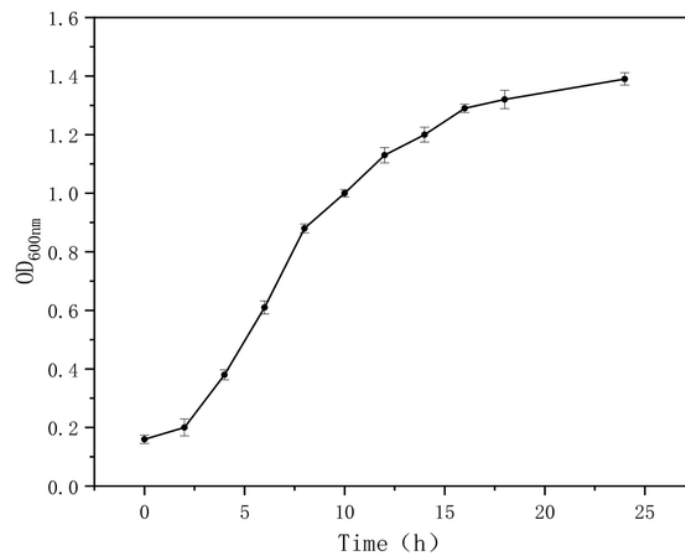


Figure 4. Growth curve of *Pseudomonas aeruginosa*.

Figure 4

See image above for figure legend

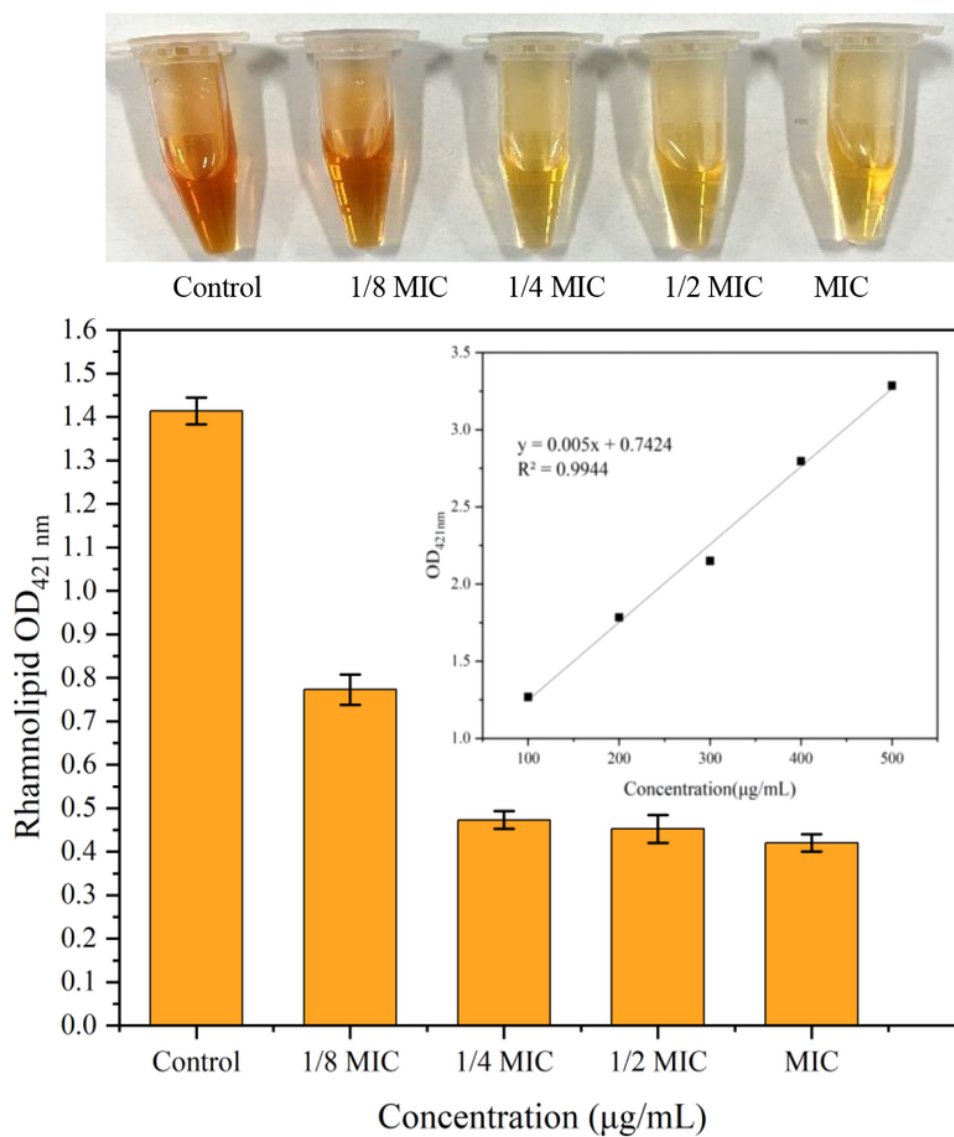


Figure 8. Effect of berberine on rhamnolipid production.

Figure 5

See image above for figure legend

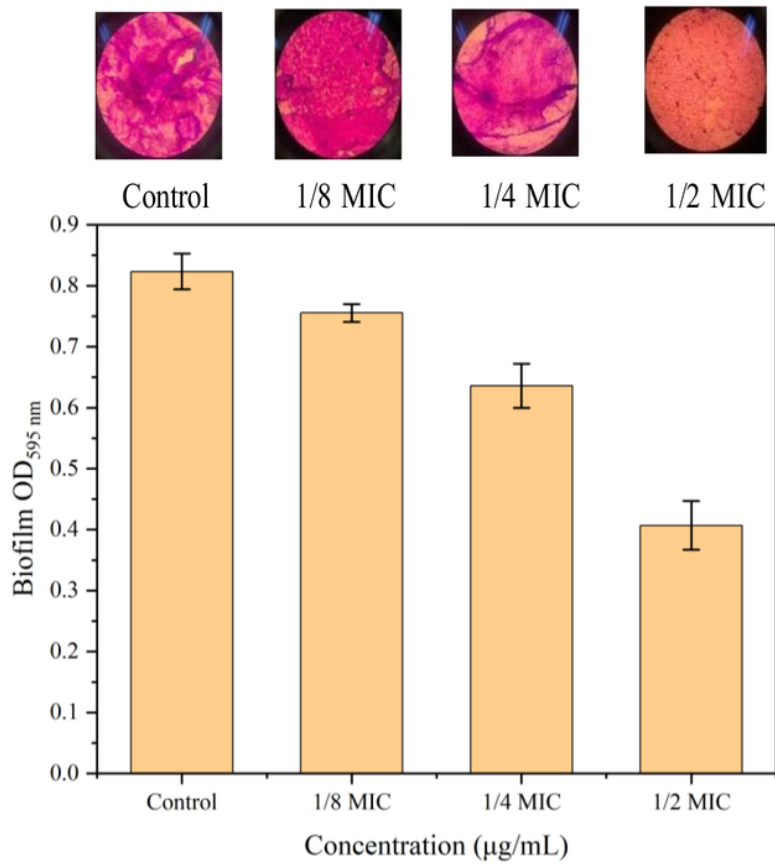


Figure 6. Inhibition of biofilm formation by berberine.

Figure 6

See image above for figure legend

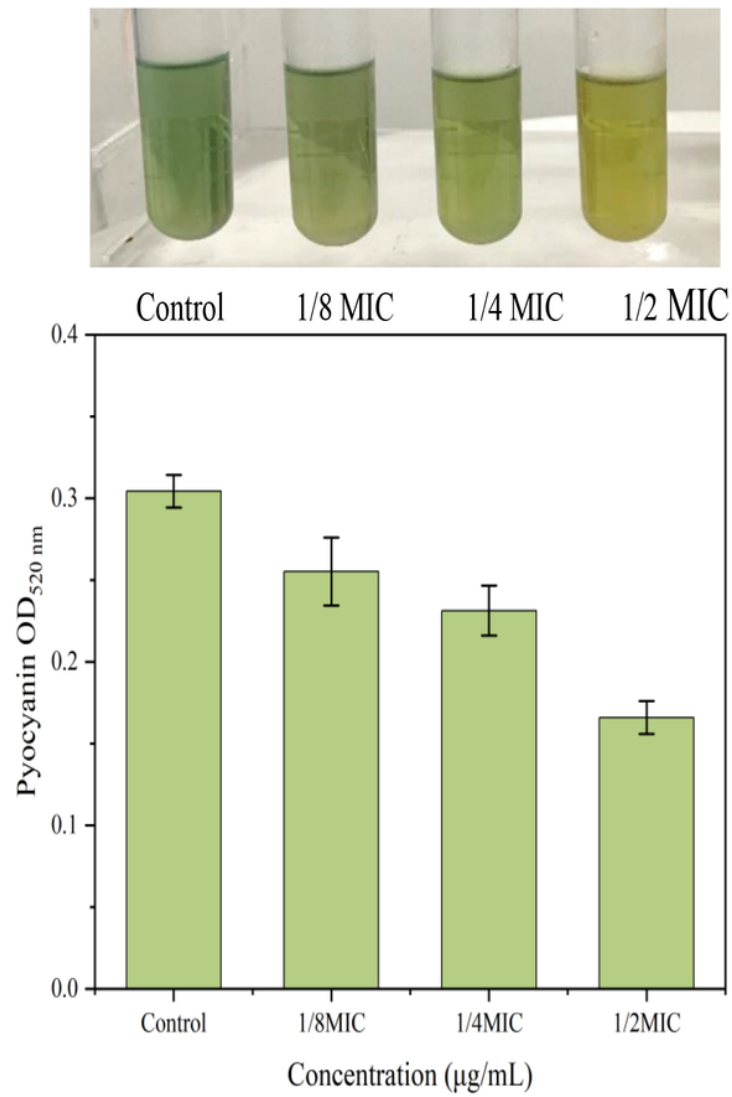


Figure 7. Production of pyocyanin.

Figure 7

See image above for figure legend

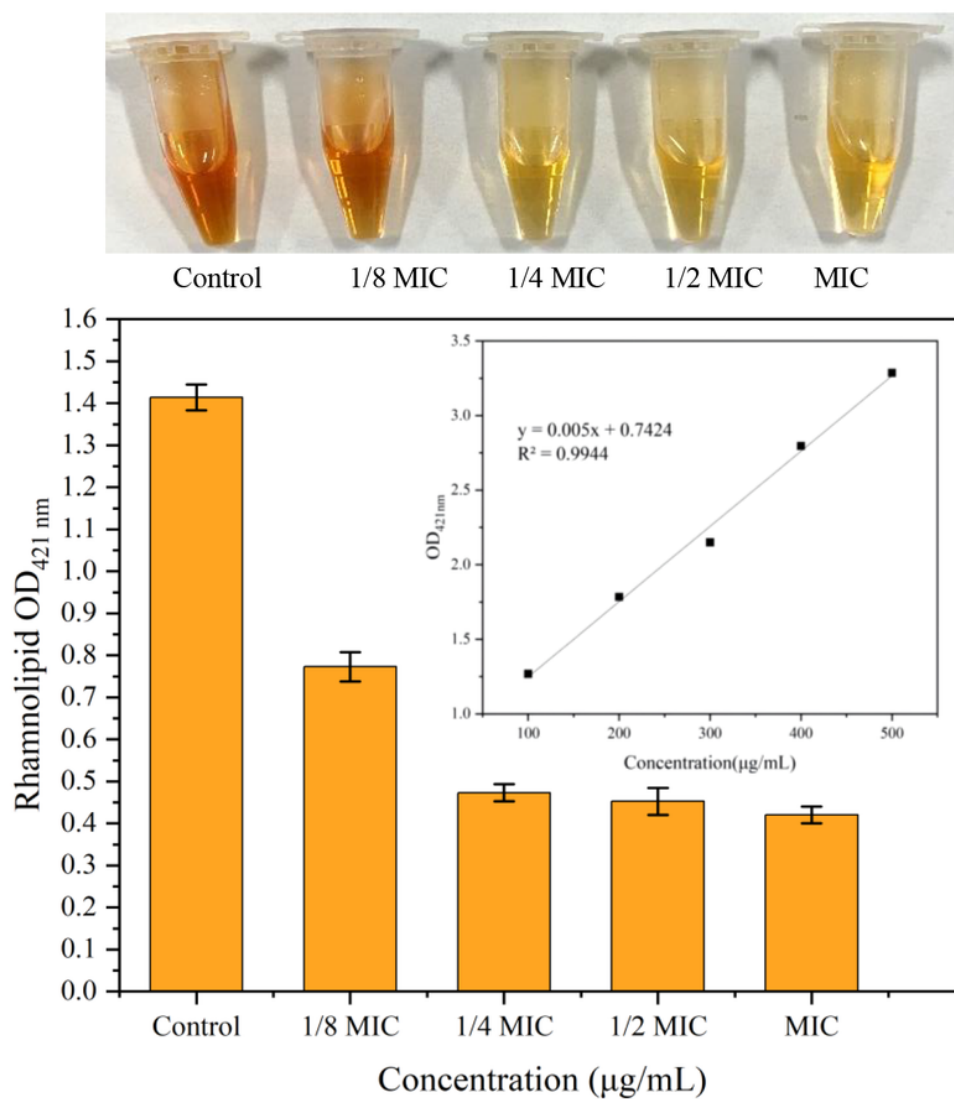


Figure 8. Effect of berberine on rhamnolipid production.

Figure 8

See image above for figure legend

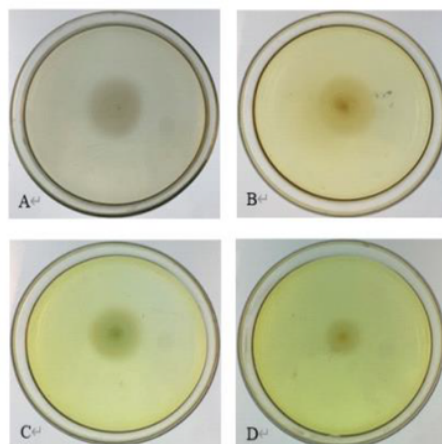


Figure 9. Swarming movement.
A-D: Control, 1/8 MIC, 1/4 MIC, 1/2 MIC.

Figure 9

See image above for figure legend

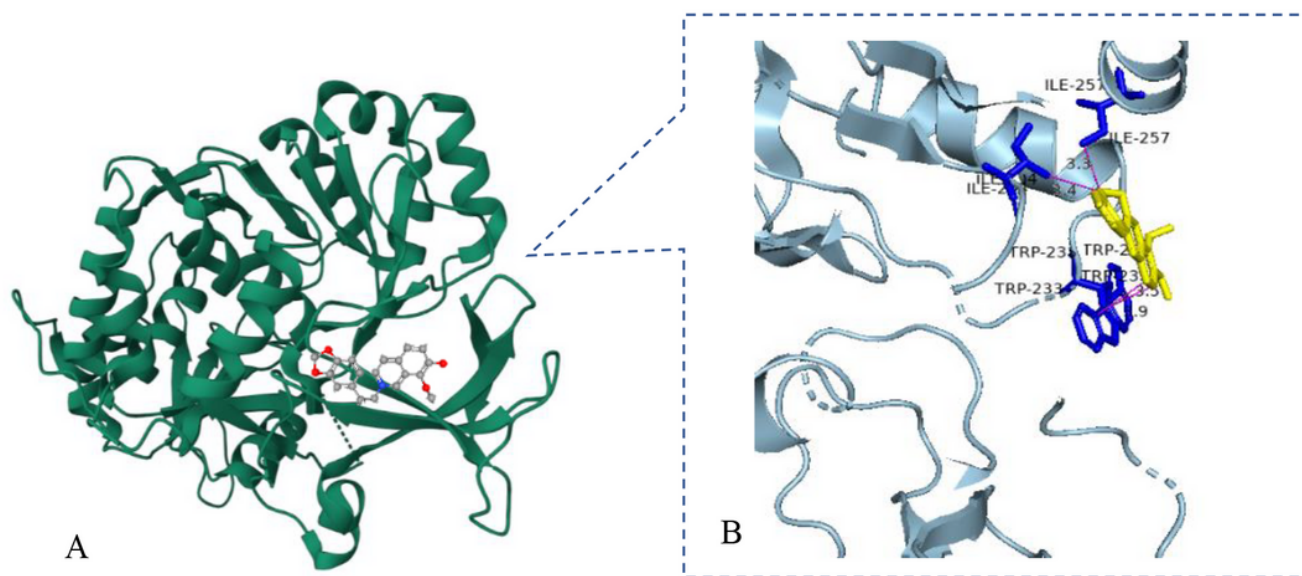


Figure 10. Molecular docking.
A: 3D structure of PqsA/ berberine
B: Docking details.

Figure 10

See image above for figure legend

Supplementary Files

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- [GraphicalAbstract.doc](#)