



New insights into the antibacterial and quorum sensing inhibition mechanism of *Artemisia argyi* leaf extracts towards *Pseudomonas aeruginosa* PAO1

Junhao Kong^{1,2,3} · Yanan Wang¹ · Kai Xia¹ · Ning Zang⁴ · Hong Zhang¹ · Xinle Liang^{1,2} 

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Abstract

This study aimed to investigate the anti-quorum sensing (QS) activity of *Artemisia argyi* leaf extracts (AALE) towards *Pseudomonas aeruginosa* PAO1 as well as the underlying molecular mechanisms. Using a biosensor *Chromobacterium violaceum* CV026, AALE were found to have anti-QS activity as AALE treatment significantly inhibited the violacein production of *C. violaceum* CV026 while produced little effect on the cell growth. Beyond that a higher dosage of AALE inhibited cell growth, sub-MIC of AALE significantly reduced the production of QS-regulated virulence factors (pyocyanin, elastase, and rhamnolipid), biofilm formation, and the swarming and swimming motility in *P. aeruginosa* PAO1 with a dosage-dependent manner. Quantitative real-time PCR (qRT-PCR) analysis did not detect the direct inhibitory effect of AALE on the expression of QS genes (*lasI*, *lasR*, *rhlI*, and *rhlR*). By iTRAQ-based quantitative proteomic analysis, 129 proteins were found to be differentially expressed upon AALE treatment, with 85 upregulated and 44 downregulated proteins, respectively. Functional enrichment analysis of the differential proteins revealed that AALE exerted anti-QS activity towards *P. aeruginosa* PAO1 by upregulating the expression of the global regulator CsrA, inducing oxidative stress, and perturbing protein homeostasis. Moreover, the inhibitory effect of AALE on the virulence of *P. aeruginosa* PAO1 was likely to be achieved by attenuating the expression of QS-regulated genes instead of QS genes. Collectively, the results of this study provide a basis for the future use of AALE as a preservative in controlling food spoilage caused by *P. aeruginosa*.

Keywords Anti-quorum sensing · *Artemisia argyi* leaf extracts · Proteomic analysis · Biofilm production · Virulence factors

Junhao Kong and Yanan Wang contributed equally to this work.

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✉ Kai Xia
xiakai333@126.com

✉ Xinle Liang
dbiot@mail.zjgsu.edu.cn

- ¹ School of Food Science and Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, China
- ² Institute of Food Biotechnology, Zhejiang Gongshang University, Hangzhou 310018, China
- ³ Institute of Tea Research, CHINA COOP, Hangzhou 310018, China
- ⁴ Medical Scientific Research Center, Guangxi Medical University, Nanning 530021, China

Introduction

Food spoilage, a major hazard usually resulting from contamination by diverse microorganisms during the food manufacturing and storage processes, is capable of posing considerable economical loss and public health risk (Riggio et al. 2019; Arunkumar et al. 2020). Among the microorganisms responsible for food spoilage, species belonging to the genus *Pseudomonas* are one dominant member (Zhang et al. 2019). *Pseudomonas aeruginosa* is a notorious and opportunistic pathogen that is associated with the food-borne disease since it can produce varied virulence factors, such as pyocyanin, rhamnolipid, and elastase (Yi et al. 2010; Abdallah et al. 2015). Hence, controlling the food spoilage caused by *P. aeruginosa* is in need of public health. However, in most cases, it is challengeable to remove *P. aeruginosa* because of its ability to build robust biofilm on the food-contact surfaces which protects it from antibacterial

agents by inducing resistance (Abdallah et al. 2014; Brindhadevi et al. 2020).

In the past years, it has been established that the production of virulence factors and biofilm in *P. aeruginosa* is regulated by a cell density-dependent system known as quorum sensing (QS) (Brindhadevi et al. 2020). The canonical QS systems identified yet in *P. aeruginosa* include Las and Rhl, among which LasI and RhlI catalyze the synthesis of QS signal N-3-oxo-dodecanoyl homoserine lactone (3-oxo-C₁₂-HSL) and butyryl-HSL (C₄-HSL), respectively, while LasR and RhlR are the cognate receptors responsible for binding signal molecules and activating the expression of multiple following genes (Kostylev et al. 2019). Las system controls the Rhl system as well as the third system using *Pseudomonas* quinolone signal (PQS) that is synthesis regulated by the cluster PqsABCDE together with PqsH as the signal molecule (Kostylev et al. 2019; Garcia-Reyes et al. 2020). PqsR binds PQS and activates the expression of multiple genes including the genes that are regulated by LasR and/or RhlR. In addition, IQS (2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde) has been proved to be a QS signal molecule in the fourth QS system (Cornelis 2020). Previous studies have shown that the repression of QS by QS inhibitors (QSIs) is able to attenuate the production of virulence factors and biofilm in *P. aeruginosa* (Zhou et al. 2019; Wang et al. 2019). Moreover, cinnamaldehyde has been successfully used in the prevention of food spoilage caused by *Pseudomonas fluorescens* (Li et al. 2018). Therefore, the inhibition of QS-controlled virulence factors production in *P. aeruginosa* by QSIs should be an alternative strategy for food preservation.

At present, myriad of compounds have been used as the QSIs, among which the crude extracts or effective components of natural and edible plants have distinct advantages as they possess less or no side effects and are able to overcome antibacterial agent resistance (Machado et al. 2020; Ahmed et al. 2019b). *Artemisia argyi* is a perennial herb belonging to the family *Asteraceae* that comprises more than 500 species (Abad et al. 2012). The leaf and leaf extracts of *A. argyi* are the well-known functional food materials in China and have been directly used as the functional flavor and colorant for hundreds of years (Song et al. 2019). *A. argyi* extracts have been proved to exhibit multitudinous biological activities, such as anti-inflammatory, antioxidant, antitumor, anticoagulant, and antibacterial activity (Ahmed et al. 2019a; Xiao et al. 2019). A recent study showed that carbon dots derived from the *A. argyi* leaves have antibacterial activity towards gram-negative bacteria (Wang et al. 2020). Moreover, the medicinal herb extracts (MHE) derived from the leaf of *A. argyi*, the root bark of *Cortex dictamni*, and the root of *Solanum melongena* are shown to be effective in inhibiting the PQS-dependent QS system in *P. aeruginosa* (Wei et al. 2020). Nevertheless, at present, underlying mechanisms

explaining the antibacterial and anti-QS activity of AALE towards *P. aeruginosa* remain elusive.

This study aimed to assess the inhibitory effect of AALE on the QS-regulated virulence factors and biofilm production in *P. aeruginosa* PAO1. Moreover, the molecular mechanisms of AALE QS inhibition were investigated at the translation level by isobaric tag for relative and absolute quantitation (iTRAQ)-dependent proteomic analysis for the first time.

Materials and methods

Strains, medium, and culture conditions

Chromobacterium violaceum CV026, a mini-Tn5 mutant derived from *C. violaceum* ATCC 31532 and deficient in the acyl-homoserine lactone (AHL) synthase CviI, was a kind gift from Prof. Chan (University of Malaya, Malaysia). *C. violaceum* CV026 was grown in LB medium (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) containing 20 µg/mL kanamycin at 28 °C, with shaking at 180 rpm. Activation of *P. aeruginosa* PAO1 (wild type) was carried out using LB medium at 37 °C, with shaking at 200 rpm. LBM medium (LB medium supplemented with 50 mM MOPS, pH 7.0), PTSB medium (50 g/L peptone, 17 g/L tryptone, 3 g/L peptone from soymeal (papain-digested for microbiology), 5 g/L NaCl, 2.5 g/L KH₂PO₄, 2.5 g/L D-glucose), GSG medium (GS medium supplemented with 20 g/L glycerol instead of 0.1 M D-glucose) (Pearson et al. 1997), LB medium, SM medium (10 g/L peptone, 2.5 g/L NaCl, 3 g/L agar), and BM medium (62 mM PBS at pH 7.0, 2 mM MgSO₄, 10 µM FeSO₄, 4 g/L glucose, 1 g/L casamino acids, and 5 g/L agar) were used in the assays of pyocyanin, elastase, rhamnolipid, biofilm formation, swimming motility, and swarming motility in *P. aeruginosa* PAO1, respectively. The media were prepared using the distilled water unless otherwise indicated. Where necessary, agar (20 g/L) was added to the liquid medium to prepare the solid medium.

AALE preparation

The extraction process was carried out as previously reported with some modifications (Ahmed et al. 2019a). The fresh *A. argyi* was purchased from local market in April, 2019 (Xihu, Hangzhou, China). The leaves were air-dried at room temperature (~28 °C) and ground to powder by an electric blender. Powder of 2000 g was percolated with 10,000 mL 95% (v/v) ethanol for 48 h (agitation for 3 times every 12 h). Then the leach liquor was harvested by suction filtration and stored at 4 °C, while the residue was used for extraction again. Afterwards, activated carbon was added to the pooled leach liquor to adsorb the pigment for 12 h.

The colorless leach liquor was harvested by suction filtration using a 0.45 µm filter paper, followed by concentrating on a rotary evaporator (RV 10, IKA, Germany) at 40 °C. The concentrated extracts were further dried by lyophilization and re-dissolved using ultrapure water to make a stock solution of 100 mg/mL. The stock solution was stored at −20 °C before use.

Minimal inhibitory concentration determination

The minimal inhibitory concentration (MIC) of *P. aeruginosa* PAO1 and *C. violaceum* CV026 towards AALE was determined as previously described (Wang et al. 2019). Briefly, the exponential-phase culture ($OD_{600} = 0.6$) was diluted by 1:100 into 200 µL fresh medium containing diluted AALE solution (0.1–50 mg/mL) pre-prepared in a 96-well microtiter plate to correspond with a cell density of $\sim 2 \times 10^5$ CFU/mL. The plate was incubated for 24 h. The MIC was determined as the lowest AALE concentration that inhibited visible cell growth. All experiments were conducted with three biological replicates.

Violacein production assay

Chromobacterium violaceum CV026 was used to detect the anti-QS activity of the *A. argyi* leaf and AALE. The experiments were performed according to previous reports (Li et al. 2018; McLean et al. 2004), with some modifications. In soft agar overlay protocol, the dry *A. argyi* leaf was first washed using the 75% ethanol and sterilized water (each for three times), respectively. Then, the leaf was placed on the surface of pre-prepared solid LB medium (10 mL), followed by adding 15 mL mixture composed of the *C. violaceum* CV026 overnight culture (1.5 mL, $OD_{600} = 0.8 \pm 0.05$), kanamycin with a final concentration of 20 µg/mL, C₆-HSL (Sigma-Aldrich, Shanghai, China) with a final concentration of 40 µM, and LB agar (13.5 mL). In Oxford cup assay, the Oxford cups (Oxford cup is a stainless cylinder with an outer diameter of 7.8 ± 0.1 mm, inner diameter of 6.0 ± 0.1 mm, and height of 10.0 ± 0.1 mm) (Yi et al. 2010) were placed to the surface of pre-made LB solid medium as described above, followed by adding 100 µL AALE with different concentrations. After the medium had solidified, the plates were incubated at 28 °C for 20 h. The sterilized water and physiological saline (0.85%, w/v) were used as the control group. The inhibition on violacein production was determined by observing the color-changing zone.

To analyze violacein production quantitatively, a 16-h overnight culture was diluted by 1:100 into 5 mL fresh LB medium containing either 50, 25, 12.5, 6.3, 3.2, 1.6, or 0.8 mg/mL AALE. After incubation for 18 h, cells were harvested by centrifugation (12,000×g, 5 min) and washed twice using fresh LB medium, followed by adding 800 µL

ethanol to suspend the cells. Then, the cells were lysed by sonication. The supernatant was collected by centrifugation (12,000×g, 5 min) and used for the detection of OD₅₇₅. Cell growth was determined by measuring OD₆₀₀ using a microtiter plate reader (Victor™X3, PerkinElmer, Waltham, USA). All experiments were performed with three biological replicates.

Virulence factors production assay

The assays of virulence factors including pyocyanin, elastase, and rhamnolipid were carried out as previously reported (Ahmed et al. 2019b; Banerjee et al. 2017; Pearson et al. 1997), with some modifications. A 16-h overnight culture of *P. aeruginosa* PAO1 was diluted by 1:100 into the fresh either LBM (5 mL), PTSB (5 mL), or GSG (100 mL) medium containing AALE with different concentrations. After incubation for 18 h at 37 °C with shaking at 200 rpm, the supernatant was collected by centrifugation (12,000×g, 5 min) and filter sterilized for following assays. The pyocyanin concentration was determined by adding 3 mL chloroform to 3.3 mL supernatant and vortexed until the liquid color changed to greenish blue. Then, the sample mixtures were centrifuged (12,000×g, 10 min) and the lower organic layer was harvested and transferred to a new tube containing 0.8 mL HCl (0.2 M), followed by shaking until the color changed to red. Afterwards, the mixture was centrifuged and the upper layer was taken out and used for detection at OD₅₂₀. The concentration of pyocyanin (µg/mL) was determined by multiplying the value of OD₅₂₀ by 17.072 (Zhang et al. 2020). The elastase production was determined by adding 0.9 mL reaction buffer (1 mM CaCl₂, 100 mM Tris buffer, 5 mg/mL elastin-Congo red, pH 7.2) to 0.1 mL supernatant, followed by incubating for 3 h at 37 °C with shaking at 200 rpm. Then, the reaction was terminated by adding 0.1 mL EDTA (0.12 M). Insoluble substrate was removed by centrifugation, and the supernatant was used for detection at OD₄₉₅. Rhamnolipid assay was performed by orcinol method. Shortly, the culture supernatant of *P. aeruginosa* PAO1 grown in the GSG medium was filtered through a 0.22 µm filter membrane and subjected to a successive extraction process for three times using an equal volume of diethyl ether (56 mL). The pooled ether extracts were extracted once using 20 mM HCl, and the ether phase was evaporated to dryness by lyophilization. The extract was dissolved in 5 mL of the NaHCO₃ buffer (0.05 M) and stored at 4 °C before use. Then, 1 mL of the rhamnolipid extract was added to 4 mL solution comprising 0.18% orcinol (w/v) in 53% (v/v) H₂SO₄, followed by heating for 15 min in boiling water and cooling at room temperature. The absorbance of the solution was measured at OD₄₂₁. The rhamnose content of each sample was determined by comparing to the standard curves delineated using rhamnose. Rhamnolipid

was determined by the relation that 1.0 mg of rhamnose corresponds to 2.5 mg of rhamnolipid (Pearson et al. 1997). All experiments were performed with three biological replicates.

Biofilm formation assay

The assay of biofilm formation was carried out as previously reported with some modifications (Xu et al. 2020). Briefly, a 16-h overnight culture of *P. aeruginosa* PAO1 was diluted by 1:100 into 5 mL fresh LB medium comprising AALE with different concentrations contained in a 24-well polystyrene culture plate (final OD₆₀₀ of ~0.04), followed by incubating at 37 °C without shaking for 24 h. The adhered biofilm was quantified by staining the cells with 1% (w/v) crystal violet solution for 30 min at room temperature. The excess crystal violet was removed by washing the wells three times with distilled water. Crystal violet bound to the adhered cells was solubilized in 1 mL of 95% (v/v) ethanol and quantified by measuring absorbance at a wavelength of 590 nm using the microtiter plate reader (VictorTMX3, PerkinElmer, Waltham, USA).

For biofilm assay using a differential interference microscope (Olympus U-LH100-3, Shinjuku, Japan), the sterilized coverslips were added to the well after inoculation as described above. After incubation for 24 h, the coverslips were taken out and washed gently using PBS buffer (0.15 M, pH 7.2) to remove the planktonic cells. The biofilm formation of each tested group was captured using the microscope. All experiments were performed with three biological replicates.

Swimming and swarming motility assays

Swimming and swarming motility assays were carried out as previously reported (Li et al. 2018), with minor modifications. Briefly, a 16-h overnight culture (5 µL) of *P. aeruginosa* PAO1 was inoculated at the center of SM medium and BM solid medium containing 12.5, 6.3, 3.2, 1.6, and 0.8 mg/mL AALE, respectively. The plates were incubated at 37 °C without shaking for 24 h.

RNA extraction and quantitative transcriptional analysis

RNA extraction and quantitative real-time PCR (qRT-PCR) were performed as previously described (Xia et al. 2016). Briefly, a 16-h overnight culture of *P. aeruginosa* PAO1 was diluted by 1:100 into 30 mL fresh LB medium containing AALE with a final concentration of 6.3 mg/mL. After incubation for 18 h at 37 °C with shaking at 200 rpm, the cells were harvested by centrifugation (12,000×g, 5 min). RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). Then,

approximately 2 µg of RNA were reverse transcribed using the PrimeScriptTMII 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The qRT-PCR analysis was performed using the SYBR Premix Ex TaqTM II Kit (TaKaRa, Dalian, China) with a final volume of 20 µL in each reaction system. The primers used for qRT-PCR were presented in Supplemental Table S1. The data were analyzed by the $\Delta\Delta C_t$ method using 16S rRNA as an internal reference. The relative fold change was calculated from $2^{-\Delta\Delta C_t}$, in which, $\Delta C_t = C_t$ target gene – C_t internal reference gene, $\Delta\Delta C_t = C_t$ sample – C_t control.

iTRAQ-dependent proteomic analysis

The proteomic analysis was executed strictly as previously described (the detailed work flow was shown in ref. Xia et al. 2016), except for the sample preparation. In short, a 16-h overnight culture of *P. aeruginosa* PAO1 was diluted by 1:100 into 120 mL fresh LB medium either containing AALE with a final concentration of 6.3 mg/mL or without AALE in a 500 mL Erlenmeyer flask (the AALE were added at the initial growth due to that it had been confirmed that AALE of a concentration below 6.3 mg/mL had no influence on the time needed for the cells to enter into the exponential-growth phase). After incubation for 18 h at 37 °C with shaking at 200 rpm, the cells were harvested by centrifugation (12,000×g, 5 min). Cell samples harvested from three independent cultures without AALE (named as SX1) or with AALE of 6.3 mg/mL (named as SX2) were used for the following proteomic analysis. After protein extraction and digestion, SX1 and SX2 were labeled using the iTRAQ reagents 4-plex kit (AB Sciex), among which iTRAQ reagents 114 and 115 were used to coordinately label the peptides from the control SX1 while the iTRAQ reagents 116 and 117 were used to coordinately label the peptides from the SX2. The original data files gathered by the MS in wiff format were processed with the Protein Pilot Software v4.5 (AB SCIEX, USA) against the *P. aeruginosa* protein database.

The false discovery rate (FDR) was calculated as the number of false positive matches divided by the number of total matches. Peptides with a global FDR value below 1% were selected for the subsequent analyses. Upregulation was defined as an abundance (mean of 116 and 117: mean of 114 and 115) of at least 1.5, while downregulation was defined as an abundance below 0.67 ($p < 0.05$). To define the functional subcategories and metabolic processes of the differentially expressed proteins (DEPs), the Gene Ontology (GO) annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were carried out by Goatools (Klopfenstein et al. 2018) and KOBAS (Xie et al. 2011), respectively. In addition, DEPs related to the certain biological function defined by PseudoCAP were analyzed.

Statistical analysis

All statistical analyses were performed using the Origin software (version 9.0) (OriginLab, Northampton, MA, USA). Where appropriate, the data were analyzed using the Student's *t* test and a one-way analysis of variance (ANOVA) with a Bonferroni's multiple-comparison test. Differences were considered statistically significant at $p < 0.05$. Values were shown as the mean of three biological replicates $\pm$ standard deviation.

Results

The minimal inhibitory concentration of AALE

The MIC of *C. violaceum* CV026 and *P. aeruginosa* PAO1 to AALE was determined. AALE showed antibacterial activity against the *C. violaceum* CV026 and *P. aeruginosa* PAO1 with MIC values of 30 and 35 mg/mL, respectively. Taking

this result into consideration, the sub-MIC values of AALE were mainly used in the QS inhibition tests.

The inhibitory effect of AALE on violacein production by *C. violaceum* CV026

The anti-QS activity of AALE was assessed according to its ability to inhibit the violacein production of *C. violaceum* CV026 (Duran et al. 2016). The result showed that *A. argyi* leaf was able to block violacein production since a clear inhibition region occurred near the leaf (Fig. 1a), indicating that *A. argyi* leaf had compounds effective in attenuating QS. This was further supported by the Oxford cup assay where AALE of 3.2 mg/mL were capable of inhibiting the violacein production (Fig. 1b, No. 3), while such case was not found in the control group in which either water or physiological saline was added (Fig. 1b, No. 4 and 5). Moreover, the most significant inhibitory effect was observed when AALE of 12.5 mg/mL was used (Fig. 1b, No. 1), suggesting that the inhibitory effect of AALE on the violacein production of *C.*

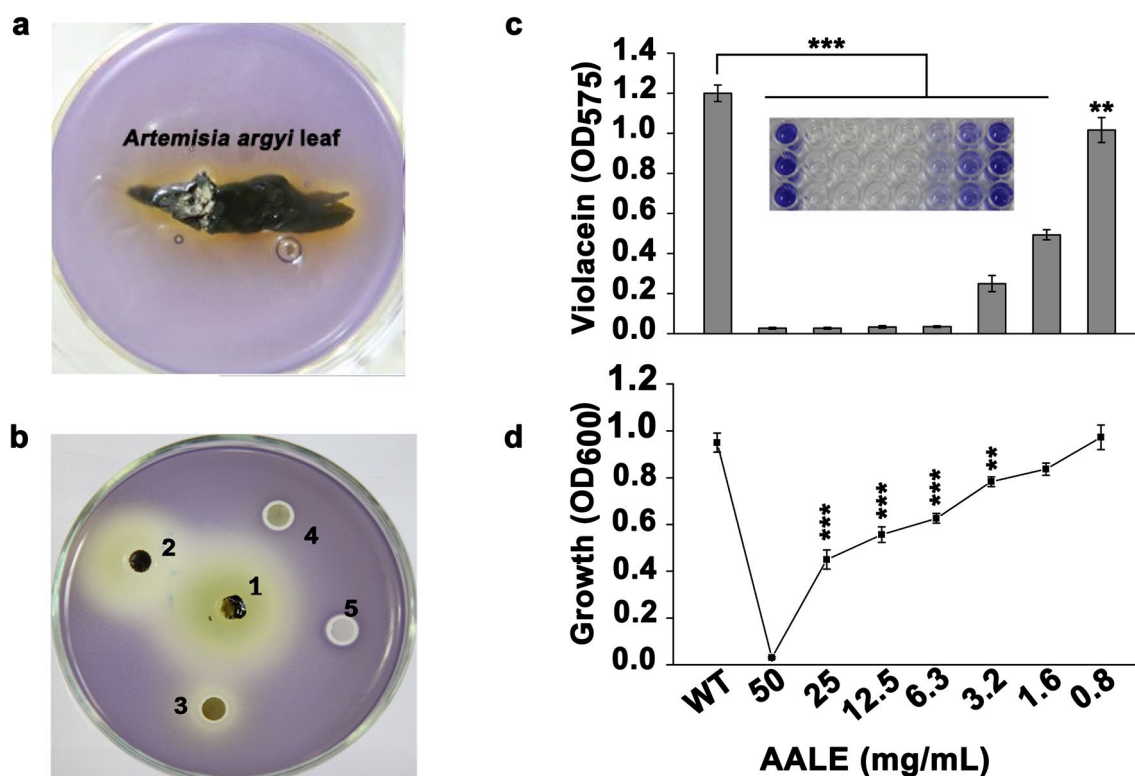


Fig. 1 Assay of the violacein production by *C. violaceum* CV026. **a** Inhibitory effect of *A. argyi* leaf on the violacein production. **b** Inhibitory effect of AALE on the violacein production. Number 1, 2, and 3 indicate that AALE with a final concentration of 12.5, 6.3, and 3.2 mg/mL were added, respectively; number 4 and 5 indicate the water and physiological saline were added, respectively. **c** Quantification of violacein content in the presence of AALE with different

concentrations ranging from 0.8 to 50 mg/mL. **d** Influence of AALE with different concentrations on the cell growth after incubation for 18 h. WT indicating without treatment is denoted as the control group. Data obtained from three biological replicates were shown as mean $\pm$ standard deviation. Stars indicate significant differences (** $p < 0.01$; *** $p < 0.001$)

violaceum CV026 was dosage-dependent. This speculation was further supported by the violacein quantitative assay wherein the violacein production of *C. violaceum* CV026 was completely inhibited when exposed to the AALE with a final concentration higher than 6.3 mg/mL, while the production of violacein was restored steadily as the concentration of AALE decreased (Fig. 1c). In addition, AALE with a relative high concentration (> 3.2 mg/mL) had inhibitory effect on the cell growth (Fig. 1d).

The inhibitory effect of AALE on QS-controlled virulence factors production by *P. aeruginosa* PAO1

As shown in Fig. 2a, AALE treatment significantly reduced the pyocyanin production of *P. aeruginosa* PAO1 with a manner of concentration-dependent on all tested concentrations except for 0.8 mg/mL, compared with that observed in the control group (WT). This kind of inhibition was not ascribed to the antibacterial activity of AALE since AALE with a final concentration below 25 mg/mL had no significant inhibitory effect on the cell

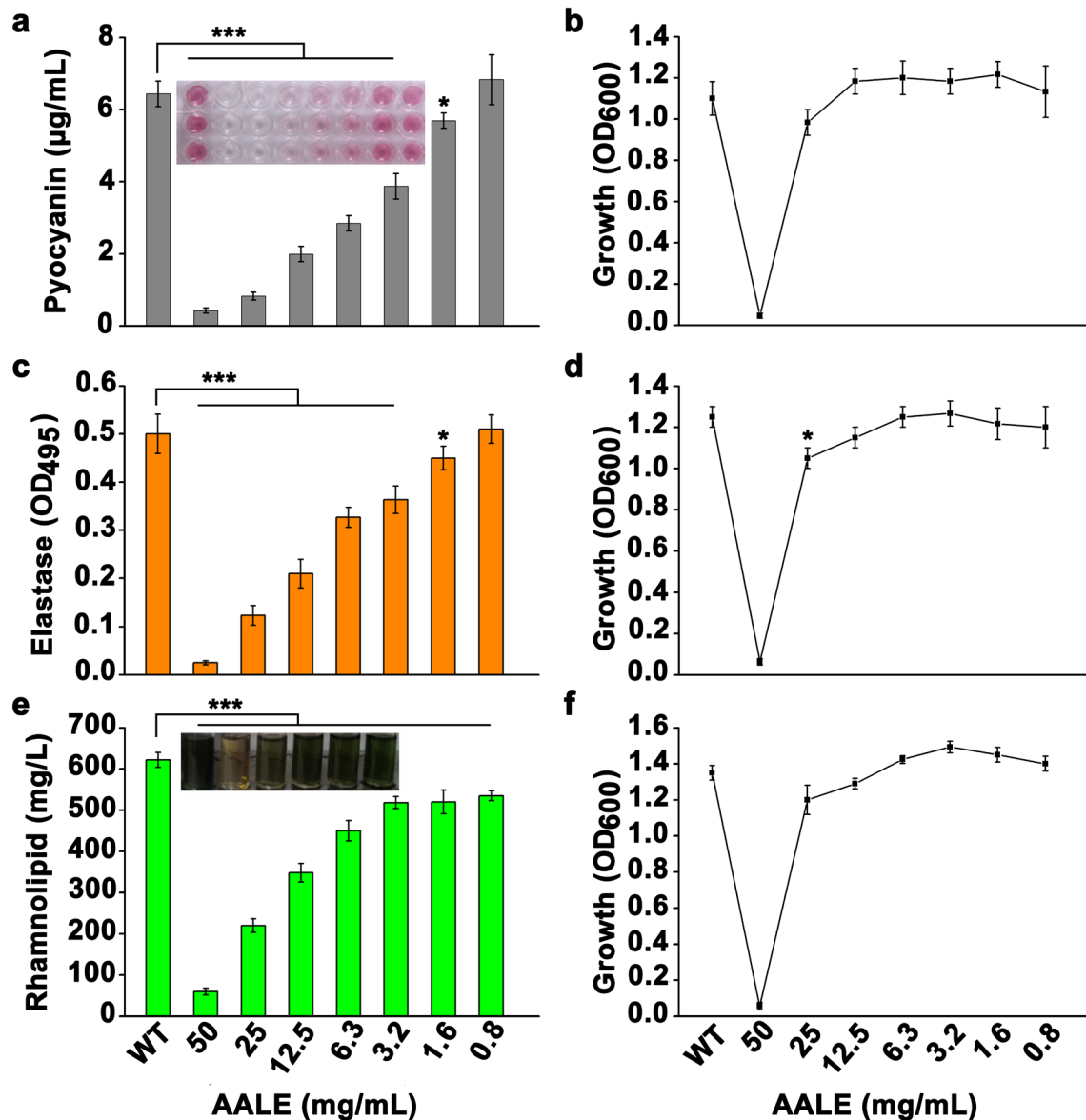


Fig. 2 Assay of the QS-controlled virulence factors production by *P. aeruginosa* PAO1. Inhibitory effect of AALE on the production of pyocyanin (a), elastase (c), and rhamnolipid (e). The cell growth of *P. aeruginosa* PAO1 corresponding to the assays of pyocyanin, elastase, and rhamnolipid after an incubation of 18 h in the presence of AALE

with different concentrations (b, d, f). WT indicates without treatment. Data obtained from three biological replicates were shown as mean $\pm$ standard deviation. Stars indicate significant differences (* $p < 0.05$; *** $p < 0.001$)

growth (Fig. 2b), suggesting the anti-QS activity of AALE towards *P. aeruginosa* PAO1. Similarly, AALE treatment significantly inhibited the elastase production while did not influence the cell growth in most cases (Fig. 2c, d). In addition, the production of rhamnolipid in *P. aeruginosa* PAO1 was decreased by AALE treatment on all tested concentrations, although the growth of *P. aeruginosa* PAO1 was not significantly influenced in the presence of AALE (Fig. 2e, f).

The inhibitory effect of AALE on the swimming and swarming motility of *P. aeruginosa* PAO1

AALE weakened the swarming motility of *P. aeruginosa* PAO1 on all tested concentrations compared with that observed in the control group, among which AALE of 12.5 mg/mL had the most significant inhibitory effect (Fig. 3a). Similarly, AALE significantly weakened the swimming motility of *P. aeruginosa* PAO1 in case of that its concentration higher than 3.2 mg/mL (Fig. 3b).

The inhibitory effect of AALE on biofilm formation by *P. aeruginosa* PAO1

Crystal violet assay showed that the biofilm formation of *P. aeruginosa* PAO1 was significantly inhibited by the presence of AALE in a concentration-dependent manner compared with that of the control group, with the value of OD₅₉₀ decreased from 0.36 to 0.12 (Fig. 4a). The maximum inhibitory effect was achieved by AALE with a final concentration higher than 12.5 mg/mL, while the minimum inhibitory effect happened upon the use of AALE with a final concentration of 0.8 mg/mL. The inhibition of biofilm formation might result from the influence of AALE on the cell growth. To clarify this confusion, the growth of *P. aeruginosa* PAO1 in the presence of AALE with a final concentration of 6.3 or 3.2 mg/mL was monitored. The results showed that AALE posed no significant influence on the growth of *P. aeruginosa* PAO1 compared with that observed in the control group (Fig. 4b), confirming the anti-QS activity of AALE.

Beyond the crystal violet staining assay, the in situ morphology of biofilm was also captured by a differential interference microscope (Fig. 4c). Compared with the control group (CC) in which no biofilm was observed, the *P. aeruginosa* PAO1 was able to form thick biofilm after a 24-h incubation in the absence of AALE (WT). However, the addition of AALE significantly attenuated the biofilm formation and produced an obviously thinned sheet-like biofilm, which supported the results obtained by the crystal violet assay. Collectively, these findings suggest that AALE are effective in blocking the biofilm formation of *P. aeruginosa* PAO1.

The inhibitory effect of AALE on the expression of QS genes in *P. aeruginosa* PAO1

It was estimated that the inhibitory effect of AALE on the production of virulence factors and biofilm in *P. aeruginosa* PAO1 might be achieved, in part, by perturbing the expression of QS genes. To authenticate this, the expression of *lasI*, *lasR*, *rhlI*, and *rhlR* in response to AALE of 6.3 mg/mL was examined by qRT-PCR analysis. As shown in Fig. 5, it was unexpected that the expression of these genes was not significantly affected under the AALE treatment compared with that observed in the control group (WT). This indicates that the inhibitory effect of AALE towards QS-controlled virulence factors is not executed by directly repressing the expression of QS genes.

Global changes in the proteome of *P. aeruginosa* PAO1 upon AALE treatment

To clarify the underlying mechanisms deciphering how AALE attenuate the QS-controlled virulence factors and biofilm production of *P. aeruginosa* PAO1, an iTRAQ-based proteomic analysis was performed. The results showed that a total of 2,850 proteins were identified, among which 129 proteins were observed to be differentially expressed in SX2 compared with that in SX1, with 85 upregulated and 44 downregulated proteins (Supplemental Table S2). It was worth noting that the proteins encoded by QS genes were not found to be differentially expressed, which was in line with the data obtained by qRT-PCR analysis. GO enrichment analysis showed that most of the DEPs locate in the region of cytoplasm, cytosol, and ribosome (Fig. 6a). The DEPs were mainly involved in the biological processes like protein folding, translation, cell redox homeostasis, oxidation–reduction process, and amino acid metabolic process (Fig. 6b). Moreover, the DEPs possessed hydrolase activity, oxidoreductase activity, transferase activity, DNA/RNA binding, and catalytic activity (Fig. 6c). KEGG analysis presented that the DEPs mainly participated in amino acid metabolism, chaperones and folding catalysts, ABC transporters or transporters, glycolysis/gluconeogenesis, ribosome, and nucleotide metabolism (Fig. 6d).

Differential proteins related to secreted factors

A significant downregulation was observed in the expression of flagellin (FliC), motility protein (FimV), glycosyltransferase (PslC), acyl carrier protein (AcpP), putative thioesterase (PA2411), chitinase (ChiC), LasA protease, elastase LasB, and phenazine biosynthesis protein (PhzB2) that is involved in the pyocyanin biosynthesis in SX2 compared with that in SX1 (Fig. 7). Notably, these proteins are directly responsible for the production of virulence factors,

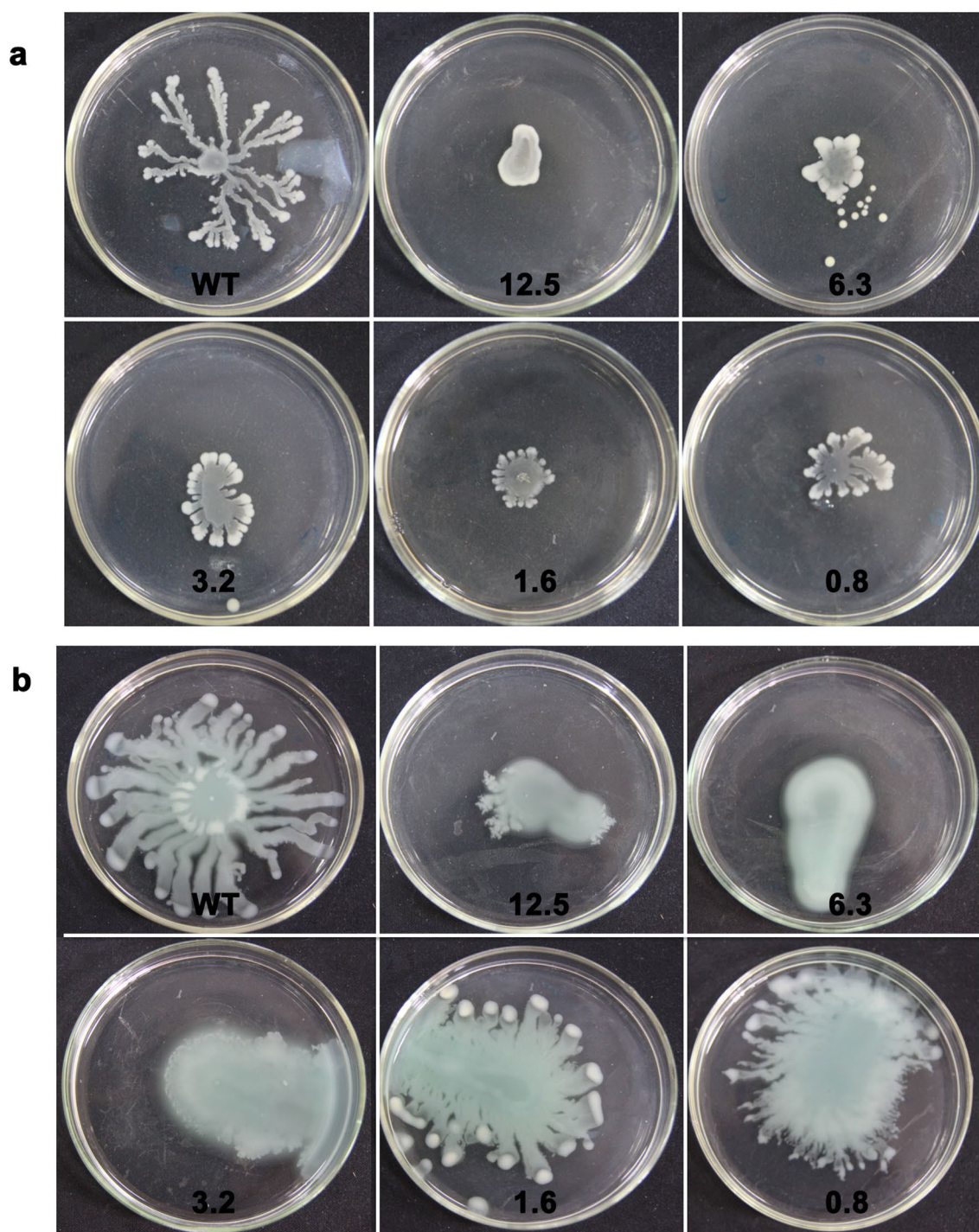


Fig. 3 Swarming (a) and swimming motility (b) of *P. aeruginosa* PAO1 in the presence of AALE with different concentrations ranging from 0.8 to 12.5 mg/mL

biofilm formation, and the cell motility in *P. aeruginosa* PAO1 (Banerjee et al. 2017; Tan et al. 2013). Thus, their downregulation supported the decreased production of pyocyanin, elastase, and rhamnolipid as well as the decreased cell mobility shown in Figs. 2 and 3.

Differential proteins related to cell envelope elements

AALE treatment reduced the expression of outer membrane protein (OmpA), amino acid transporter (PA5153),

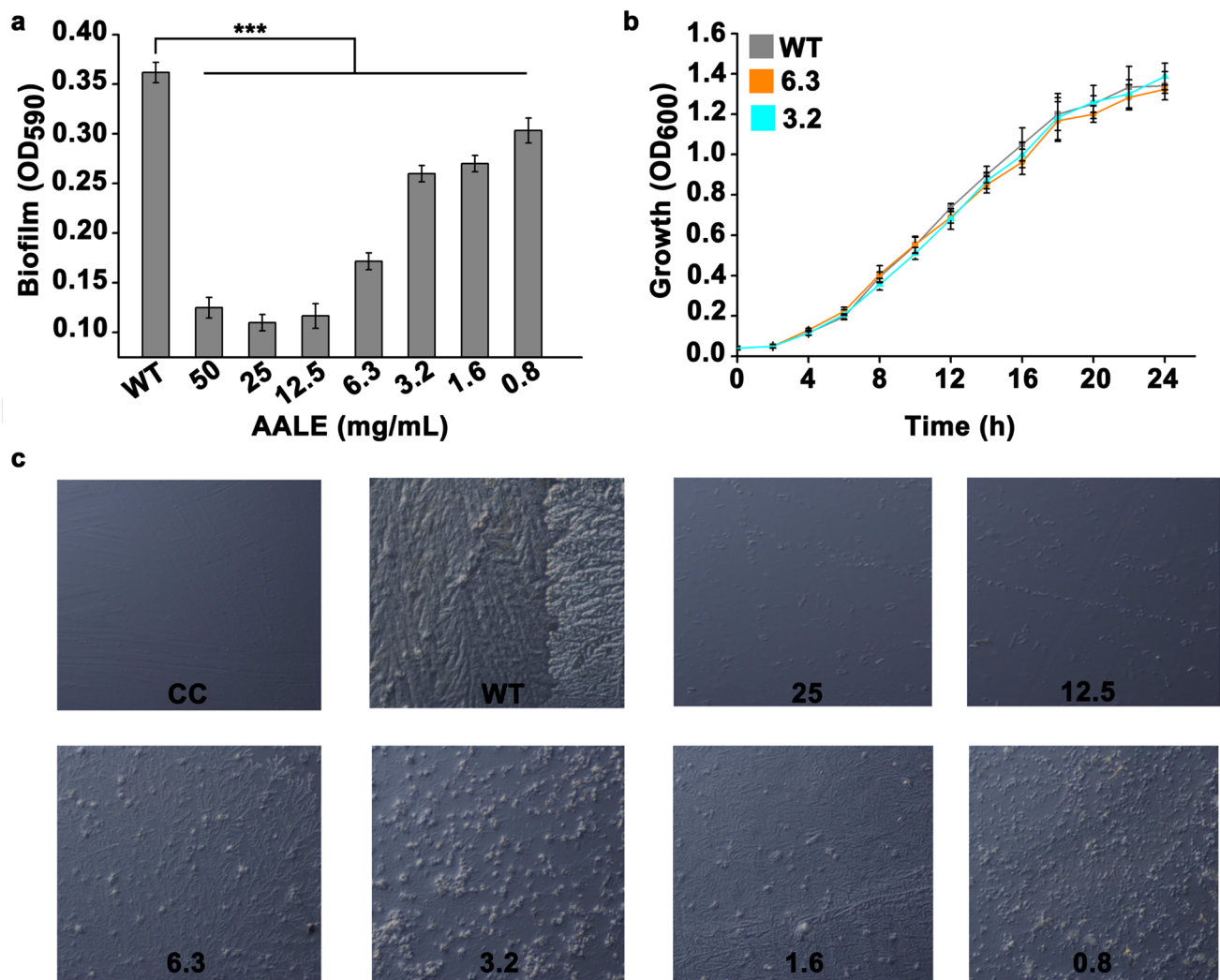


Fig. 4 Assay of the biofilm formation by *P. aeruginosa* PAO1 in the presence of AALE. **a** Effect of AALE on the biofilm formation quantified by crystal violet assay. **b** The cell growth of *P. aeruginosa* PAO1 in the presence of AALE with a final concentration of 6.3 and 3.2 mg/mL. **c** Effect of AALE on the biofilm formation imaged by

a differential interference microscope. CC indicates the control coverslip. Data obtained from three biological replicates were shown as mean $\pm$ standard deviation. Stars indicate significant differences (***) $p < 0.001$

polyamine transporter (AgtB and SpuD), and porin OprF (Fig. 7), while induced the expression of D-alanine-D-alanine ligase (Ddl) that is involved in the pathways of peptidoglycan biosynthesis, ferric uptake regulation protein (Fur), ferric iron-binding periplasmic protein (HitA), and the histidine kinase of two-component system (TCS) (CheA and PA1458). These findings suggest that AALE inhibit the QS-controlled virulence factors production by interfering with nutrients uptake and have role in impacting cell envelope stability.

Differential proteins involved in oxidative stress response

Pseudomonas aeruginosa is able to defend the oxidative stress derived from the accumulation of reactive oxygen species (ROS) by mediating the expression of antioxidant enzymes like superoxide dismutase, peroxidase, and catalase (Zhang et al. 2020). Interestingly, AALE treatment significantly inhibited the expression of chloroperoxidase (Cpo), catalase (KatE), thioredoxin (TrxB2), and dihydrolipoyl

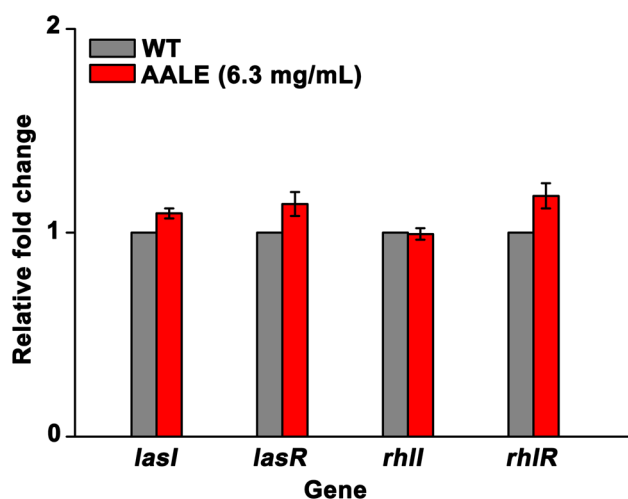


Fig. 5 The effect of AALE treatment on the expression of QS genes. The relative expression level of *lasI*, *lasR*, *rhII*, and *rhIR* was determined by qRT-PCR using the 16S rRNA gene as an internal reference. Data obtained from three biological replicates were shown as mean $\pm$ standard deviation. WT indicates without treatment

dehydrogenase (IpdG and IpdV) while induced the expression of superoxide dismutase (SodB), which appeared to increase the accumulation of H_2O_2 that is responsible for inducing oxidative stress (Fig. 7). In addition, the upregulated expression of bacterioferritin (Bfr and PA4880) that is

involved in the iron storage indicated that AALE had role in perturbing iron homeostasis. The imbalance of iron concentration also has contribution to the induction of oxidative stress (Sethupathy et al. 2016). Collectively, these results suggest that AALE attenuate the virulence factors and bio-film production of *P. aeruginosa* PAO1 by affecting iron homeostasis and the oxidative stress response.

Differential proteins involved in citrate cycle and amino acid metabolism

Functional enrichment analysis of the DEPs suggested that cells activated the citrate cycle to withstand the stress caused by AALE as the expression of fumarate hydratase class II (FumC), aconitate hydratase (AconA), and succinate-CoA ligase (SucD) was significantly upregulated upon AALE treatment (Fig. 7). In addition, almost all of the proteins involved in the metabolism of cysteine (PA0400, cystathionine gamma-lyase; CysM, cysteine synthase), histidine (HutU, urocanate hydratase), arginine (ArgJ, arginine biosynthesis bifunctional protein; ArgC, *N*-acetyl-gamma-glutamyl-phosphate reductase), valine (PA0747, probable aldehyde dehydrogenase), isoleucine (IlvH, acetolactate synthase isozyme III; IlvE, branched-chain amino acid aminotransferase), glutamate (GdhA, glutamate dehydrogenase), and glycine (GcvT2, glycine cleavage system protein T2) were significantly upregulated in SX2 compared with that in SX1.

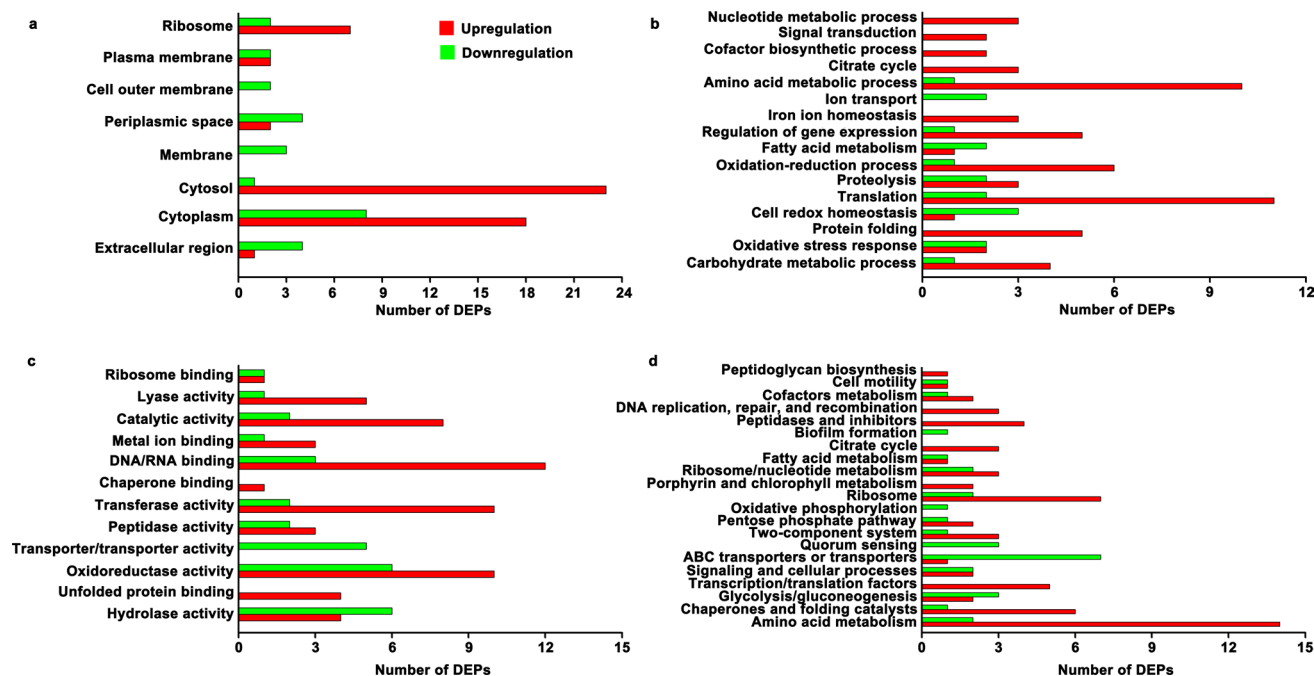


Fig. 6 GO and KEGG enrichment cluster analyses of the differentially expressed proteins (DEPs). The proteins relevant to cellular component (a), biological process (b), and molecular function (c). d

KEGG pathway cluster of the differential proteins. The detailed information of these proteins were shown in Supplemental Table S2

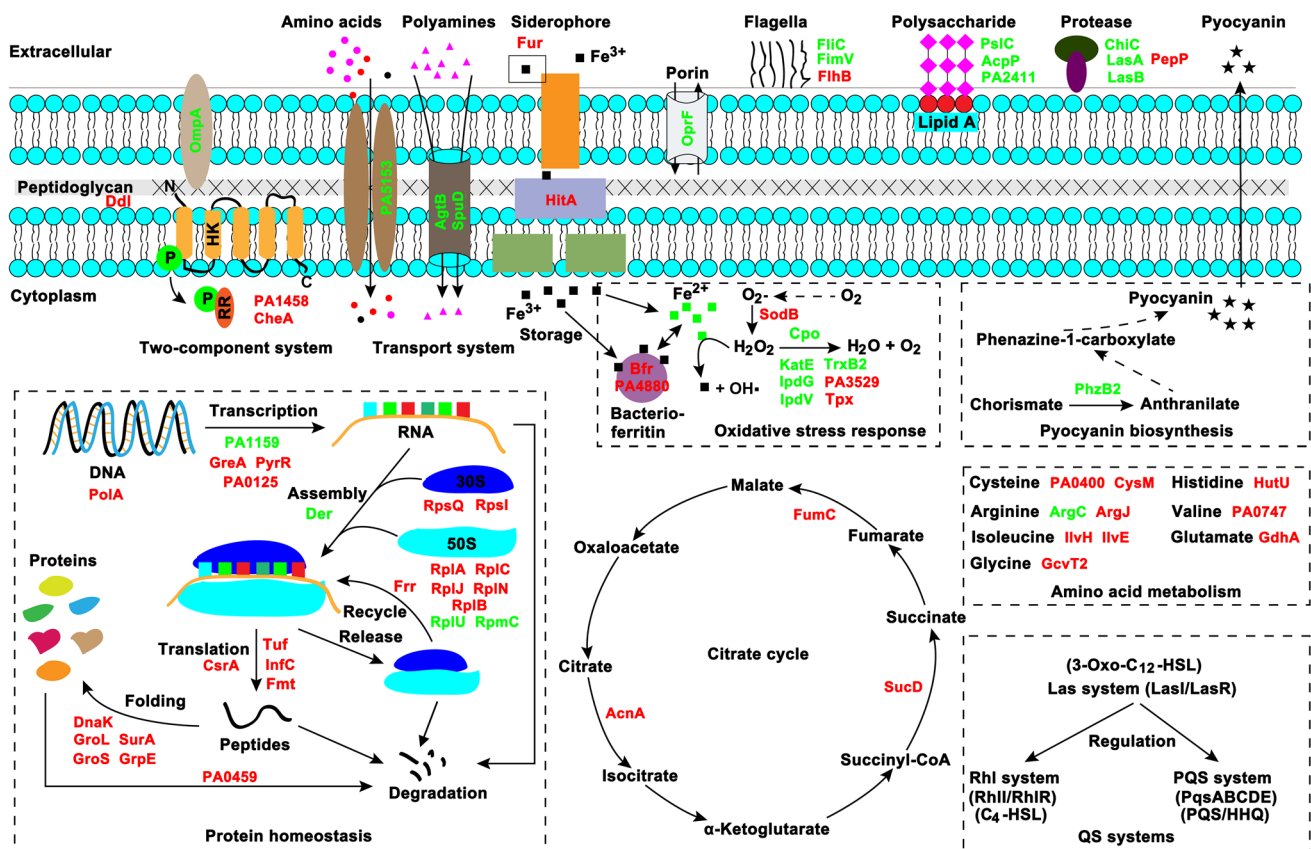


Fig. 7 Outline of the DEPs in *P. aeruginosa* PAO1 upon AALE treatment. The upregulated and downregulated proteins included in different biological processes were denoted as red and green texts,

respectively. Dashed arrows indicate multiple steps. The detailed information of these proteins were presented in Supplemental Table S2

Differential proteins involved in protein homeostasis

It was interesting that the DEPs involved in the protein homeostasis were the most abundant in SX2 compared with that in SX1. The upregulation of transcription elongation factor GreA, bifunctional protein PyrR, 30S ribosomal proteins (RpsQ and RpsL), 50S ribosomal proteins (e.g., RplA, RplC, and RplB), elongation factor Tu (Tuf), translation initiation factor IF-3 (InfC), and ribosome-recycling factors (Frr) seemed to enhance the protein synthesis (Fig. 7). The upregulation of molecular chaperones (e.g., DnaK, GroL, and GroS) combined with the upregulation of ClpA/B protease ATP binding subunit (PA0459) indicated that unfolding or dysfunctional proteins increased under AALE treatment. On the other hand, the downregulation of GTPase Der and 50S ribosomal proteins (RplU and RpmC) together with the upregulation of translational repressor CsrA were also observed, indicating a complex regulation of protein synthesis in *P. aeruginosa* PAO1 upon the AALE treatment.

Discussion

AALE exert dual antibacterial and anti-QS activity in a dosage-dependent manner

Artemisia argyi, as a well-known edible functional plant, has been utilized in Chinese traditional foods for hundreds of years due to its anti-inflammatory and antioxidant activity (Xiao et al. 2019). However, at present, the antibacterial and anti-QS activity of *A. argyi* extracts have not been fully understood. In this study, AALE were proved to have antibacterial and anti-QS activity in a dosage-dependent manner towards *C. violaceum* CV026 and *P. aeruginosa* PAO1. First, AALE of a relative high concentration (> 50 mg/mL) were able to absolutely inhibit the cell growth. Second, AALE of a lower concentration (even 0.8 mg/mL) were capable of attenuating the violacein production of *C. violaceum* CV026 and the virulence factors production and bio-film formation of *P. aeruginosa* PAO1 without influencing the cell growth. These results are in agreement with previous findings, wherein the extracts of star anise can inhibit the

biofilm formation of foodborne bacteria in a dose-dependent manner and the *Calpurnia aurea* extracts inhibit the violacein production of *Chromobacterium violaceum* ATCC 12472 in a dose-dependent manner (Cosa et al. 2020; Rahman et al. 2017). Thus, the results of this study shed light on the application of AALE as a QSI. Whereas the successful application of QSIs as safety food additives in controlling food spoilage (Sorensen et al. 2010; Li et al. 2018), the results of this study further suggest the potential use of AALE in food industry to prevent food spoilage caused by *P. aeruginosa* contamination. Nevertheless, the components of the AALE were not analyzed and characterized in this study. The active chemicals of AALE responsible for the anti-QS activity need future investigation.

AALE have no direct inhibitory effect on the expression of QS genes

Knowing the inhibitory effect of AALE on the virulence factors production of *P. aeruginosa* PAO1, the underlying molecular mechanisms were further investigated. There is growing evidences showing that the inhibitory effect of natural plant-derived compounds on the QS-regulated virulence in *P. aeruginosa* is usually achieved by three ways: inhibition of AHLs synthesis by repressing the expression of QS genes, degradation of AHLs, and interference with the binding of receptor and AHLs (Machado et al. 2020). The results of this study suggest that AALE have no direct inhibitory effect on the expression of QS genes including *lasI*, *lasR*, *rhlI*, and *rhlR* both at the transcription and translation level. Moreover, the proteins involved in the synthesis of other QS signals (e.g., PQS and IQS) were not found to be differentially expressed upon AALE treatment (Fig. 7 and Supplemental Table S2). These appear to contradict the previous finding that the MHE derived from the leaf of *A. argyi*, the root bark of *C. dictamnii*, and the root of *S. melongena* are effective in attenuating the hierarchy QS systems of *P. aeruginosa* (Las, Rhl, and PQS) at the transcriptional level (Wei et al. 2020). This discrepancy may result from that the effective compounds that are able to inhibit the expression of QS genes are extracted from another two medical herbs *C. dictamnii* and *S. melongena* instead of *A. argyi*. Hence, the anti-QS activity of AALE may be achieved by other mechanisms. Previous study showed that cinnamaldehyde extracted from the cinnamon is not able to interfere with the production of AHLs by *P. fluorescens* but exerts anti-QS activity by working as AHLs antagonists in preventing the binding of AHLs to LuxR (Li et al. 2018). The flavonoid fraction of *Psidium guajava* does not inhibit AHLs synthesis but interfere with the signal response, thereby repressing the virulence factors production of *P. aeruginosa* (Vasavi et al. 2014). In addition, it has been proved that green tea polyphenols at sub-inhibitory concentrations can cause the degradation of

AI-2 molecules of *Shewanella baltica* QS system and inhibit the biofilm formation as well as the cell motility (Zhu et al. 2015). Thus, in view of the complex composition of AALE, it is possible that AALE exert anti-QS activity towards *P. aeruginosa* PAO1 by playing as AHLs antagonists or disturbing signal response.

It is worth noting that the expression of CsrA (carbon storage regulator) was significantly upregulated upon AALE treatment. CsrA, a homologue of RsmA (repressor of secondary metabolism), is a well-known global posttranscriptional regulator critical for the control of over 500 genes expression, including the genes related to the virulence factors and biofilm production of *P. aeruginosa* in response to extracellular stimuli (Gebhardt et al. 2020; Brencic and Lory 2009). It has been proved that the overexpression of *rsmA* leads to the decreased production of elastase and pyocyanin in *P. aeruginosa* by negatively controlling the expression of *lasI* and *rhlI* (Pessi et al. 2001). In addition, overexpressing *rsmA* from *P. aeruginosa* in *Pseudomonas syringae* significantly inhibited the production of protease and pyoverdine as well as the swarming activity and biofilm formation in the latter (Kong et al. 2012). Interestingly, the decreased production of virulence factors and biofilm formation as well as the upregulated level of CsrA were found upon AALE treatment, while the expression of *lasI* and *rhlI* was not observed to be downregulated, presumably because the negative effect of CsrA is counteracted by other regulators during the stationary-phase growth. Collectively, these results suggest that the QS inhibition of AALE is attributed to its complex composition and multiple targets, among which the CsrA may be one important target.

AALE inhibit the virulence of *P. aeruginosa* PAO1 by a combination of multiplex regulations

Among the diverse activities of QSI towards the QS-controlled virulence factors and biofilm production in *P. aeruginosa*, the anti-QS activity resulting from the accumulation of ROS that is capable of inducing oxidative stress is one important way. Curcumin and hordenine have been proved to be able to attenuate the QS-regulated virulence of *P. aeruginosa* PAO1 by affecting oxidative stress response (Sethupathy et al. 2016; Zhou et al. 2019). *A. argyi* essential oils (AAEO) have been reported to exert antibacterial effect on *Candida albicans* SC5314 by facilitating intracellular ROS accumulation and mitochondria damage (Shi et al. 2017). Here, the results of this study suggest that AALE treatment induces oxidative stress by increasing the accumulation of H₂O₂ and interfering with iron homeostasis (Fig. 7). It has been proved that the exposure of *P. aeruginosa* to H₂O₂ results in the decreased production of protease, elastase, and pyocyanin (Garcia-Contreras et al. 2015). In addition, H₂O₂ of a low concentration (1 µM) is able to induce the

conformational change of LasR by modifying the Cys79 of LasR, thereby perturbing the binding of 3-oxo-C₁₂-HSL to LasR and inhibiting the virulence factors production (Deng et al. 2013). Therefore, it is possible that the inhibition of AALE on the QS-regulated virulence and biofilm formation in *P. aeruginosa* PAO1 is carried out by interfering with the binding of AHLs and LasR receptor via increasing H₂O₂ level.

The increased oxidative stress will inevitably affect the protein synthesis and function since oxidative damage will result in protein misfolding and fragmentation (Yan et al. 2018). This case occurred in *P. aeruginosa* PAO1 by the observation that the proteins involved in the synthesis of branched amino acids were overexpressed. Previous studies showed that the increased level of branched amino acids is an indication of destructed protein synthesis upon QSI treatment (Zhou et al. 2019; Chen et al. 2017). In addition, most of the proteins related to the pathways of protein synthesis were found to be significantly upregulated upon AALE treatment, which further suggests the urgent need of protein synthesis resulting from the protein damage. The excessive synthesis of proteins responsible for the oxidative stress response may influence indirectly on the synthesis of proteins directly involved in the biological processes important for virulence factors and biofilm production.

Finally, it is interesting to find that the expression of lysine-arginine-ornithine-histidine-octopine transport protein (PA5153) and polyamine transporter (AgtB and SpuD) was repressed upon AALE treatment (Fig. 7). The down-regulation of PA5153 should reduce the uptake of lysine, arginine, ornithine, histidine, and octopine to some extent, which may affect the protein synthesis. Polyamines such as spermidine and cadaverine are important carbon source and nitrogen source in *P. aeruginosa* (Yao et al. 2011). At present, the physiological role of polyamines is not fully understood. If have, a recent study showed that spermidine located on the membrane surface has role in protecting cell from H₂O₂ treatment (Johnson et al. 2012). Thus, it is possible, although not established, that the changes of polyamines may contribute to the oxidative stress caused by AALE treatment.

In conclusion, the present study investigated the antibacterial and anti-QS activity of AALE towards the food-borne bacteria *P. aeruginosa* PAO1. AALE with a sub-MIC are able to attenuate the virulence factors and biofilm production of *P. aeruginosa* PAO1, while this inhibitory effect is not achieved directly by repressing the expression of QS genes. The results of quantitative proteomic analysis suggest that AALE exert anti-QS activity by upregulating the expression of the global regulator CsrA, inducing oxidative stress, perturbing protein homeostasis, and affecting the expression of proteins related to the synthesis of virulence factors. Collectively, these results provide a basis for the use of AALE

as a preservative in controlling food spoilage caused by *P. aeruginosa*.

Author contributions JK and YW conducted lab work and data analysis. KX interpreted the data, designed the experiments, and drafted the manuscript. ZZ and HZ conducted data analysis. XL conceived of the study, performed data review, and contributed to the paper writing. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This paper does not contain any studies with human participants or animals.

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