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ORIGINAL RESEARCH PAPER

Equisetin as potential quorum sensing inhibitor of *Pseudomonas aeruginosa*

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Abstract
Objective To screen for the quorum-sensing (QS) inhibitors from marine-derived fungi and evaluate their anti-QS properties in *Pseudomonas aeruginosa*.
Results QS inhibitory activity was found in secondary metabolites of a marine fungus *Fusarium* sp. Z10 using *P. aeruginosa* QSIS-*lasI* biosensor. The major active compound of this fungus was isolated by HPLC and identified as equisetin. Subinhibitory concentration of equisetin could inhibit the formation of biofilm, swarming motility, and the production of virulence factors in *P. aeruginosa*. The inhibition of *las*, *PQS*, and *rhl* system by equisetin were determined using *Escherichia coli* MG4/pKDT17, *E.coli* pEAL08-2, and *E.coli* pDSY, respectively. Real–time RT-PCR assays showed that equisetin could down-regulate the mRNA expression of QS-related genes.
Conclusions Equisetin proved its potential as an inhibitor against *P. aeruginosa* QS system and might also serve as precursor compound in development of novel therapeutics for infectious diseases by optimal design of structures.

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium and is ubiquitous in the environment. It is a common nosocomial pathogen responsible for a high incidence of acute and chronic infection. The infections caused by *P. aeruginosa* are difficult to eradicate because of the production of biofilms (Tambyah et al. 2011). In addition, *P. aeruginosa* easily develops resistance to most conventional antibiotics. Therefore, the development of novel treatment strategy is imperative.

Quorum sensing (QS), a bacterial intercellular communication mechanism, plays a key role in regulating the expression of virulence genes and biofilm formation. Quorum-sensing inhibitors (QSIs) could be new therapeutic agents for bacterial infection, they inhibit or attenuate pathogenicity without effecting bacterial growth. Halogenated furanones produced by *Delisea pulchra* inhibited QS-mediated activities in bacteria by competing with cognate acylhomoserine lactone (AHL) signals for their receptor site (Manefield et al. 2001). Penicillic acid and patulin are secondary metabolites of *Penicillium* spp. and inhibit the QS system by targeting signal molecule receptor proteins (Rasmussen et al. 2005). Unfortunately, most QSIs are unsuitable for use in humans due to their toxicity, high reactivity, and instability.

Marine-derived fungi secondary metabolites are possible rich sources of structurally novel and biologically active secondary metabolites. In the present study, we have focused on screening and investigating QSIs isolated from marine fungi. This is the first study reporting that equisetin can be isolated from a marine fungus, *Fusarium* sp. Z10, which shows anti-QS properties against *P. aeruginosa*.

Materials and methods

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Unless otherwise stated, all of the strains were grown in LB medium. An aliquot (50 µl) of overnight culture was inoculated into 5 ml medium, and the bacterial cultures were incubated at 37 °C.

Isolation of marine fungi and preparation of the crude extracts

Fungal strains were isolated from seawater samples collected from Iiaozhou Bay, China. The fungi were grown on Peter medium (0.3% yeast extract, 0.3% maltose, 0.5% tryptone, 2% glucose, 2.44% sea salt, and 1.5% agar, pH 7.3) at 30 °C for 7–10 days. The Solid-state fermentation products were extracted with ethyl acetate. The extract was redissolved in methanol (50 mg/ml) and filtered using 0.22 µM membranes before screening.

Screening for QSIs and purification of active compound

The screening assay using *P. aeruginosa* QSIS-*lasI* biosensors was performed as described previously (Wang et al. 2011). The active compounds from crude extracts were isolated by preparative HPLC. Samples were fractionated on a C18 reverse chromatographic column at 2.5 ml/min and the following H_2O/CH_3CN solvent gradients: a linear gradient 10–70% CH_3CN/H_2O for 15 min; isocratic at 100% for another 5 min; and 10% CH_3CN/H_2O in 5 min. Anti-QS activities of the pure compounds were detected by QSIS-*lasI* biosensors.

Growth curve analysis

P. aeruginosa PAO1 were cultured overnight, diluted to $OD_{600} \approx 0.05$, and then transferred into 96-well plates with equisetin at different concentrations. Bacterial cultures were incubated for 24 h under shaking at 130 g, and was measured at 600 nm during incubation.

Effects of equisetin on virulence factor production and biofilm formation

P. aeruginosa PAO1 strains were cultivated for 2.5 h, and then incubated with and without equisetin for another 6 h. Pyocyanin production was measured at 520 nm in acidic solution (Vasavi et al. 2016). Elastolytic activity was assessed with the elastin Congo red assay and measured at 490 nm (Musthafa et al. 2011). Rhamnolipid production was quantified with the orcinol method and measured at 420 nm (Rathinam et al. 2017). Biofilm formation was determined by crystal violet staining and measured at 590 nm (Vasavi et al. 2016).

Swarming motility assays

The effect of equisetin on the *P. aeruginosa* PAO1 swarming phenotype was tested as described previously (Gutierrez et al. 2013). Swarming agar consisted of M9 salt medium supplemented with 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1.1 mM glucose, 0.5% casamino acids, and 0.5% agarose. Overnight cultures of *P. aeruginosa* PAO1 (2 µL) were point inoculated on the swarm agar plates and incubated for 24 h.

Effects of equisetin on *lasB*, *pqsA,* and *rhl* transcriptional activity in *E. coli*

The effects of equisetin on the β-galactosidase luminescence of *E. coli* MG4/pKDT17, *E. coli* pEAL08-2 (Cugini et al. 2007; Pearson et al. 1994), and *E. coli* pDSY were inspected as described previously. PKDT17, pEAL08-2, and pDSY produce transcriptional activator LasR with *lasB* promoter, PqsR with *PqsA* promoter, and RhlR with *rhlA* promoter driving *lacZ* expression, respectively. The β-galactosidase activities were determined using the β-gal Assay Kit.

Real-time RT-PCR

P. aeruginosa PAO1 were cultivated for 2.5 h, and then incubated in the presence and absence of equisetin for another 8 h. RNA was isolated by a RNA isolation kit. cDNA was synthesized from 1 µg of RNA using high-capacity RNA-to-cDNA master mix. All primers for real-time PCR are listed in supplementary Table 2. Amplification was performed with SYBR-Green master real-time PCR mix kit. Forty cycles were run, and the gene *rpsl* was used as a reference gene for normalizing gene expression.

Statistical analysis

All experiments were repeated in triplicate, and all data from three independent experiments were expressed as mean ± SD. Graphpad Prism 5 software was used for statistical calculations.

Results

Screening of fungal extracts and identification of active compound

A total of 36 strains of fungi were isolated from seawater samples, and their extracts were screened for QS inhibitory activity. The strain Z10 proved a QSI-producing fungus by QSIS-*lasI* selector (data not shown). Its 18S rDNA sequences had been submitted to the GenBank (NCBI) (Accession number MF 973,465). Based on sequence analysis, the strain was identified and named as *Fusarium* sp. Z10 (data not shown).

A bioactive compound (24.6 mg) was isolated from 250 ml *Fusarium* sp. Z10 culture by preparative HPLC at a retention time of 22.6 min (Supplementary Fig. 1), and its purity was more than 97%. The spectral data (Electrospray Mass Spectrometry, ^{1}H-NMR and ^{13}C-NMR) revealed the active compound was equisetin (Ratnaweera et al. 2015) (Supplementary Fig. 2 and Fig. 3, Supplementary Table 3). The structure of equisetin was shown in Supplementary Fig. 4.

QSIS-*lasI* selector strain harbors a QS-controlled *sacB* gene that causes cell death when expressed in the presence of signal molecules and sucrose. The rescued effect of QSIs was signaled by the growth of the QSIS-*lasI* biosensor was manifested by a red zone. According to the QSIS-*lasI* agar well-diffusion assay, equisetin showed anti-QS activity with a concentration-dependent effect (Fig. 1a).

Effect of equisetin on *P. aeruginosa* PAO1 Growth

Most of QSIs could inhibit QS and QS-controlled virulence phenotypes at noninhibitory concentrations to avoid imposing harsh or direct selective pressure like antibiotics. The assay of virulence phenotypes

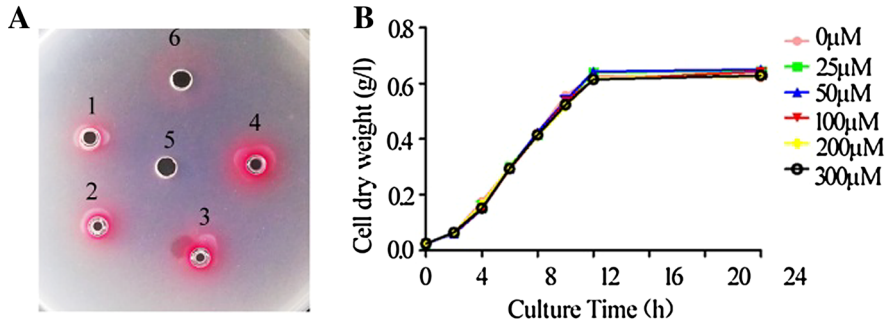

Fig. 1 **a** QSIS-*lasI* diffusion assay of equisetin. 20 μM (1), 40 μM (2), 80 μM (3), 100 μM (4) of equisetin were tested. C30 (6) was taken as the positive control at 25 μM (Hentzer et al. 2003). To calibrate this screening system, methanol (5) was used as the negative control at 5 μl. **b** Growth curve analysis. *P. aeruginosa* PAO1 were grown in the presence of 0, 25, 50, 100, 200, and 300 μM of equisetin. The optical density (OD) units were converted to dry cell weight (DCW) by an OD–DCW correlation: 1 OD 600 = 0.51 g DCW/l (Zhou et al. 2013)

should be performed at subinhibitory concentration. We investigated the effect of equisetin on planktonic growth of PAO1. Growth curve analysis confirmed that PAO1 growth was unaffected by equisetin, ranging from 15 to 300 μM (Fig. 1b).

Equisetin inhibit the production of virulence factor, biofilm formation, and swarming motility

P. aeruginosa can synthesize a variety of virulence factors and form biofilm, which were regulated by the QS system. The productions of pyocyanin, rhamnolipid, and elastase in PAO1 were decreased by 60.4, 56.1, and 46.4% under 300 μM equisetin, respectively (Fig. 2a, b, c). Equisetin could attenuate biofilm formation by 58.3% in PAO1 at 300 μM (Fig. 2d).

Flagella-mediated swarming motility is a mode of bacterial migration that is controlled by QS system on the surface of semisolid media. Bacteria can perform this coordinated manner to adapt to environmental changes. Swarming motility of PAO1 treated with 300 μM equisetin was reduced compared to untreated culture (Fig. 2e).

Equisetin inhibit *las*, *PQS*, and *rhl* systems

P. aeruginosa has multiple QS systems that interact closely with each another. To exclude the influence of other QS systems and examine the specific effect of QSIs on a single system, we used *E. coli* MG4/pKDT17, *E.coli* pEAL08-2, and *E.coli* pDSY as the monitor strain for *las*, *PQS,* and *rhl* systems, respectively. Equisetin decreased the *β*-galactosidase luminescence in pKDT17, pEAL08-2, and pDSY by up to 45.9, 60.1, and 30.1% at 300 μM, respectively (Fig. 3a, b, c).

The mRNAs of several QS-regulated genes were detected by quantitative RT-PCR because of their key role in virulence expression. As shown in Fig. 4, the expression of *lasB*, *lasI*, *lasR* were downregulated by 58.9, 16.1, 24.2%, respectively; the expressions of *pqsA* and *pqsR* were downregulated by 67.9 and 77.1%, respectively; additionally, the expressions of *rhlA*, *rhlI, and rhlR* were downregulated by 31.7, 23.4, and 36.2% at 300 μM, respectively.

Discussion

In order to cope with bacterial infections, the research of QS inhibitors is an emerging strategy that aims at developing new compounds to attenuate the production of virulence factors in pathogenic bacteria without affecting bacterial growth. Equisetin was first isolated from a terrestrial fungus *Fusarium equiseti* NRRL 5537 (Burmeister et al. 1974), and belongs to tetramate-containing natural products. These kinds of compounds usually possess antimicrobial properties. Equisetin inhibited the growth of Gram-positive bacteria and HIV-1 integrase activity (Ratnaweera et al. 2015; Singh et al. 1998). However, it showed no activity against Gram-negative bacteria, *P. aeruginosa*, and its anti-QS properties had not yet been examined (Ratnaweera et al. 2015). Equisetin was isolated from secondary metabolites of marine fungus

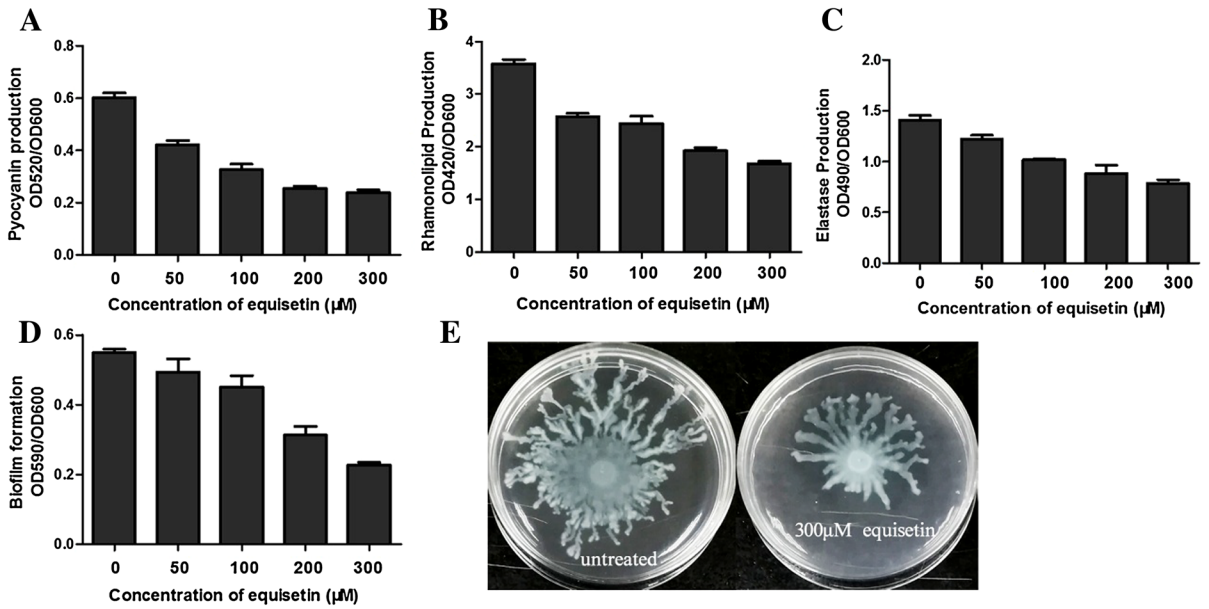

Fig. 2 Effects of equisetin on virulence factor, biofilm formation, and swarming motility of PAO1. The effects of equisetin on pyocyanin production **a**, rhamnolipid production **b**, elastase activity **c**, biofilm formation **d,** and swarming motility **e** of *P. aeruginosa* PAO1

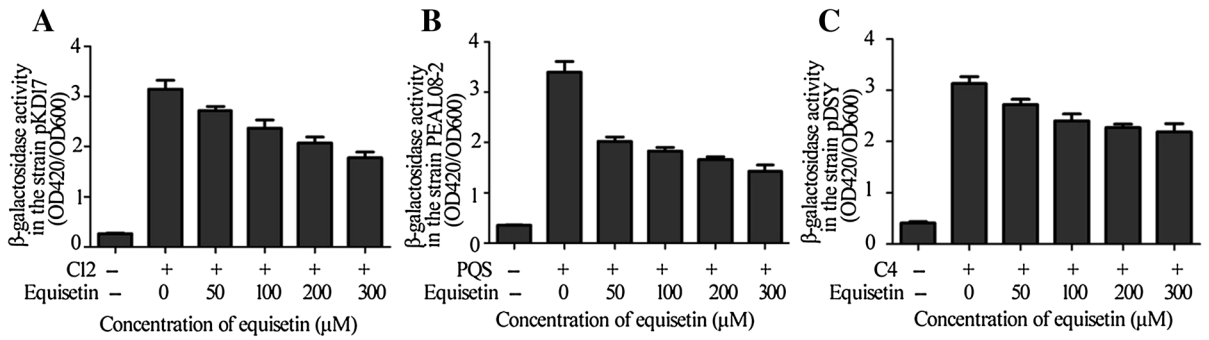

Fig. 3 Effects of equisetin on *las, PQS*, and *rhl* system and the expression of the QS-related genes. *β*-galactosidase activity was measured in the *E. coli* MG4/pKDT17 **a**, *E. coli* pEAL08-2 **b,** and *E.coli* pDSY **c**

Fusarium sp. Z10 in this study. This is the first study to reveal the anti-QS properties of equisetin.

The hierarchy QS network of *P. aeruginosa* consists of at least four interconnected systems, including *las*, *rhl, PQS* and *IQS* systems (Lee and Zhang 2015). The *las* and *rhl* systems are composed of AHL signal molecules synthases, LasI and RhlI, and their cognate transcriptional regulators, LasR and RhlR. The *PQS* system is composed of a transcriptional regulator PqsR, and two operons (*phnAB* and *pqsABCDE*) required for the biosynthesis of *Pseudomonas* quinoline signals (Dekimpe and Deziel 2009). Elastase, pyocyanin, rhamnolipid, and swarming motility of *P. aeruginosa* are mainly controlled by *las*, *PQS*, *rhl, and rhl* signaling systems, respectively. Our data showed that equisetin inhibited *las* system, while both elastase of PAO1 and transcriptional activation of *lasB* in *E. coli* MG4/pKDT17 decreased. Similarly, equisetin attenuated both the pyocyanin production of PAO1 and the transcriptional activation of *PqsA* in *E. coli* pEAL08-2 by inhibiting *PQS* system. The declines in the production of rhamnolipid, inhibition of swarming motility, and transcriptional activation of *rhlA* in *E. coli* pDSY indicated that equisetin inhibited *rhl* system. These findings were confirmed by RT-PCR assays of QS-

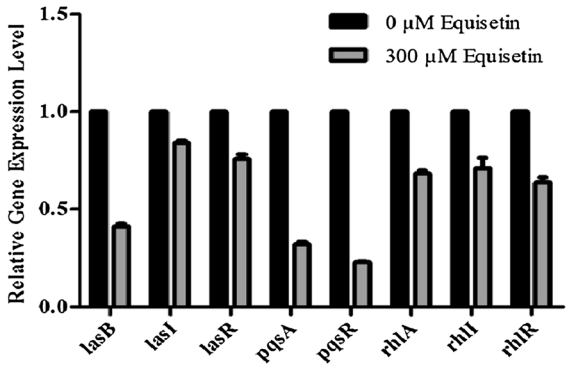

Fig. 4 Effect of equisetin on the mRNA of QS-regulated genes assessed by real-time RT-PCR. The housekeeping gene *rpsl* was used to normalize the gene expression data

related gene. Also, the *PQS* system was the most significantly inhibited by equisetin among the three QS systems.

In conclusion, as a QSI, equisetin could attenuate QS-regulated virulence phenotypes in *P. aeruginosa* without affecting the growth of bacteria. Moreover, it could also serve as a leading compound for the treatment of *P. aeruginosa* infections. It is possible to optimize the structure of equisetin to improve its anti-QS activity in the future.

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Supporting information Supplementary Table 1—Bacterial strains and plasmids.

Supplementary Table 2—The primer sequence of RT-PCR.

Supplementary Table 3—13C NMR data of equisetin.

Supplementary Fig. 1—HPLC analysis.

Supplementary Fig. 2—ESIMS data of Equisetin. Supplementary Fig. 3—1H-NMR and 13C-NMR data of Equisetin.

Supplementary Fig. 4—Chemical structure of equisetin.

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