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Inhibition of Quorum Sensing-Associated Virulence Factors in *Pseudomonas aeruginosa* PAO1 by *Folium artemisiae argyi* Extract

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There is an urgent need for developing new antibiotics and novel anti-infective strategies due to the emergence of multidrug-resistant pathogens. Conventional antibiotics targeting bacterial viability exert a selective pressure on pathogens and inherently induce the rise of antibiotic resistance. Quorum sensing (QS) is a bacterial cell–cell signaling system which plays important roles in bacterial pathogenicity. Inhibitors of QS or pathogenicity represent promising new drug candidates that less likely cause drug resistance. Chinese herbs have long been used to treat infectious diseases, and represent a potentially rich resource for new antimicrobials. In this study, we investigated 20 Chinese herbs known for Qing Re Jie Du function (i.e. treating symptoms resembling infections) using QS and virulence reporters based on promoters-*luxCDABE* fusions. Antimicrobial activity against *Pseudomonas aeruginosa* PAO1 (PA), *Staphylococcus aureus* methicillin resistant strain (MRSA), and *Escherichia coli* has been revealed in some of the herbs. More importantly, our data demonstrated that some of the herbs inhibited virulence factor gene expression in PA without affecting its growth. Specially, the extract of Folium artemisiae argyi significantly inhibited the expression of a range of important virulence factors associated with QS system. Pyocyanin production and swarming ability of PAO1 were significantly reduced by the extract. It is clear that the herbs traditionally used in Chinese medicine for treating infectious diseases seem to function through inhibiting both bacterial viability and virulence, representing a promising source for new anti-infective development.

Key words: *Pseudomonas aeruginosa*, Virulence factors, Quorum sensing, *Folium artemisiae argyi*.

Since the introduction of penicillin, antibiotic therapy is the most commonly-used strategy to control infections of pathogens. However, it leads to another problem, the generation of antibiotic resistant bacteria which has became a serious threat to human health and limits our ability to treat infectious diseases. It is clear that we are in a race to develop new antimicrobials to supplement our dwindling antibiotic arsenal for combating the growing emergence of antibiotic resistant strains.

Conventional antibiotics focus on therapeutics that target in *vitro* viability, which exert serious selective pressure on pathogenic bacteria and induce serious antibiotic resistance. Rather than focusing on therapeutics that target in *vitro* viability, an alternative approach is to target functions essential for infection, such as virulence factors required to cause host damage and diseaseÿwhich has several potential advantages including expanding the repertoire of bacterial targets, preserving the host endogenous

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microbiome, and exerting less selective pressure, which may result in decreased antibiotic resistance[1].

Pseudomonas aeruginosa (PA) is a prevalent opportunistic human pathogen associated with various acute and chronic infections in human sespecially those who are immuno-compromised or suffering other chronic diseases[2]. *P. aeruginosa*is one of the most common nosocomial pathogens, and is responsible for the majority of morbidity and mortality of patients with cystic fibrosis. The ecological success of this opportunistic bacterium can be attributed not only to its broad metabolic versatility, but also to its well-regulated release of virulence factors.

Quorum sensing (QS) is a gene-regulatory mechanism in response to changes in bacterial cell density depending on these autoinducers. Extensive studies have shown that QS regulates virulence productions and coordinates population behaviors. *P. aeruginosa* possesses two intertwined acyl-homoserine lactone (HSL) based QS systems: *las* and *rhl* systems. The transcriptional regulators LasR and RhlR and the cognate autoinducers, N-(3-oxododecanoyl)-L-HSL (3-oxo-C12-HSL) and N-butyryl-L-HSL (C4-HSL), constitute the *las* and *rhl* system[3].

Traditional Chinese medicines (TCM) have been effectively used to treat infectious diseases for thousands of years in China. Many components from TCMs have been identified as effective in the treatment of various inflammatory diseases such as gastritis, stomatitis, dermatitis, and pneumonia[4]. They represent a rich resource for antibacterial compound exploration. Folium artemisiae argyi, referred to Compositae is known for the diverse functions for treatment of human diseases such as urinary tract infection, skin infection and carbuncle. However, the mechanism for the infection treatment remains unclear.

In this study, antimicrobial activity in these herbs was investigated against several pathogens including PA, *Staphylococcus aureus*ÿmethecilin resistant strain (MRSA), and *Escherichae coli*, and significant antimicrobial activities were revealed in some of the herbal extracts. More importantly, we revealed that some of the Chinese herbs could inhibit *P. aeruginosa* virulence factors while had no influence on cell viability. Specially, we found that crude extract of the Chinese herb Folium artemisiae argyi could significantly inhibit the expression of virulence factors associated with QS system and reduced the pyocyanin production and swarming ability of *P. aeruginosa* PAO1.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and derivatives were routinely grown at 37°C on LB (Luria-Bertani) or BHI (Brain-Heart Infusion) agar plates or in LB broth with shaking at 200 rpm. Tetracycline (Tet) and Vancomycin (Van) (Amresco, USA).

Plant extraction

The plant materials were obtained from local store in (Yikang, China). The air-dried plant materials were boiled slightly with 70% ethanol firstly and then distilled water (plant material weight to solvent volume ratio was 1/5-1/10) for 1.5-2h. The crude ethanolic extract and aqueous extract of each plant was filtered using filter paper (Shuangquan, China). The extract was collected by centrifugation at room temperature. Then the extracts were evaporated under vacuum at 40 °C using a rotary vacuum evaporator (Buchi, Switzerland). The concentrated extracts were dried into powder and conserved in -20°C.Aqueous extract was dissolved in deionized water and ethanol extract was dissolved in 50% methanol to obtain desired dilutions for testing and filter-sterilized using 0.22ìm (pore size) Iwaki filter disks.

Double Agar diffusion assay

Double Agar diffusion assay was performed to detect the anti-virulence activity and antimicrobial activity of the different herbal extracts. Bacterial growth inhibition would result in a clear halo around the discs, while the effect of plant extract on virulence gene's expression was quantified by the light levels**.** The reporter strains (as listed in Table 1) were incubated in LB liquid medium at 37°C overnight. The upper layer medium (LB medium with 0.7% agar), cooled to 40°C was mixed with 100ìl of the overnight cultures grown overnight in LB, adjusted to OD_{600} nm = 0.1, and spread on the lower layer medium (LB medium with 1% agar) previously prepared. Discs used (6 mm diameter) were made of sterile filter paper

(Shuangquan, China) and were impregnated with the 10ìl serial dilutions of extract. Discs loaded with sterile water or 50% methanol alone was used as control. The plates were incubated at 37°C overnight and imaging was performed using the LAS300 imaging system (Fuji Corp.).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
E. coli		
DH10B	F⁻mcrA Δ (*mrr-hsd*RMS-*mcrBC*) 80d*lacZ* Δ M15 Δ *lacX*74 *deoR recA1 endA1 araD139* Δ (*ara leu*) 7697*galUgalK*λ⁻*rpsLnupG*	Invitrogen
Staphylococcus aureus		
MRSA	methecilin resistant strain	This lab
P. aeruginosa		
PAO1	Wild type	This lab
Plasmids		
CTX6.1	Integration plasmid origins of plasmid mini-CTX-*lux*; Tc^r	This lab
pkD-*phzA1*	pMS402 containing *phzA1*promoter region; Kn^r, Tmp^r	[5]
pkD-*phzA2*	pMS402 containing *phzA2* promoter region; Kn^r, Tmp^r	[5]
pkD-*lasI*	pMS402 containing *lasI* promoter region; Kn^r, Tmp^r	[6]
pkD-*lasR*	pMS402 containing *lasR* promoter region; Kn^r, Tmp^r	[6]
pkD-*rhlR*	pMS402 containing *rhlR* promoter region; Kn^r, Tmp^r	[6]
pkD-*rhlI*	pMS402 containing *rhlI* promoter region; Kn^r, Tmp^r	[6]
pkD-*pilG*	pMS402 containing *pilG* promoter region ; Kn^r, Tmp^r	[5]
pkD-*fliC*	pMS402 containing *fliC* promoter region; Kn^r, Tmp^r	[5]
pkD-*oprH*	pMS402 containing *oprH* promoter region; Kn^r, Tmp^r	[5]
pkD-*exoS*	pMS402 containing *exoS* promoter region; Kn^r, Tmp^r	[5]
pkD-*migA*	pMS402 containing *migA* promoter region; Kn^r, Tmp^r	[5]
pkD-*exoT*	pMS402 containing *exoT* promoter region; Kn^r, Tmp^r	[5]

Table 2. Twenty Chinese herbal medicines used in study

The names of herbs	Source plant /animal (Latin name)
Cortex Moutan	*Paeonia suffruticosa* Andr.
Rhizoma Paridis Chinensis	*Paris polyphylla* Smith var. chinensis (Franch.) Hara
Folium Artemisiae Argyi	*Artemisia argyi* Levl. et Vant
Radix Sanguisorbae	*Sanguisorba officinalis* L.
Radix Paeoniae Rubra	*Paeonia lactiflora* Pall.
Radix Isatidis	*Isatis indigotica* Fort.
Caulis Sargentodoxae	*Sargentodoxa cuneata* (Oliv.) Rehd.et Wils.
Rhizoma Belamcandae	*Belamcanda chinensis* (L.) DC.
Radix Stellariae	*Stellaria dichotoma* L. var. lanceolata Bge.
Radix Lithospermi	Lithospermum erythrorhizon Sieb. et Zucc.
Semen Cassiae	*Cassia obtusifolia* L.
Fructus Quisqualis	*Quisqualis indica* L.
Fructus Bruceae	*Brucea javanica* (L.) Merr.
Fructus Cnidii	*Cnidium monnieri* (L.) Cuss.
Herba Portulacae Herba	*Portulaca oleracea* L.
Herba Agrimoniae	*Agrimonia Pilosa* Ledeb.
Herba Lobeliae Chinensis	*Lobelia chinensis* lour.
Herba Scutellariae Barbatae	*Scutellaria barbata* D. Don
Herba Solani Nigri	*Solanum nigrum* L.
Flos Lonicerae Japonicae	*Lonicera japonica* Thunb.

Expression monitoring assay in liqud medium

The *luxCDABE*-based reporter fusions were integrated into chromosome of PAO1 and used to measure gene expression levels in liquid cultures (in counts per second) (c.p.s.) in a Victor[3] multilabel plate reader (Perkin-Elmer)[7]. The reporter strains were cultivated in LB medium and the overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 and cultivated for two additional hours before being used as inoculants. The cultures were inoculated into parallel wells on a 96-well black plate with a transparent bottom. Fresh cultures (5ìl) were inoculated into the wells containing a total of 95ìl medium supplemented with extract or equal volume solvent as control. 50ìL of filter-sterilized mineral oil (Sigma) was added to prevent evaporation. Promoter activities were measured every 30 min for 24 h. The growth of bacterial was monitored at the same time by measuring the OD_{600} in the Victor[3] multilabel plate reader.

Measurement of pyocyanin production

Pyocyanin was measured by a previously method with minor modification[8]. The *P. aeruginosa* PAO1 was grown in LB liquid medium supplemented with crude extract or equal volume solvent as control overnight. After centrifugation, 3 ml of chloroform was added to 5 ml of supernatant of PAO1 overnight cultures. The chloroform phase was transferred to a fresh tube and mixed with 1 ml

Table 3. Diameter of zones of clearance of MRSA

The English names of herbs	Diameter of zones of clearance (mm)			
	LB		BHI	
	aqueous extract	ethanolic extract	aqueous extract	ethanolic extract
Radix Paeoniae Rubra	14.27	15.82	10.92	15.08
Herba Scutellariae Barbatae	/	15.47	/	8.85
Radix Lithospermi	/	9.03	/	8.40
Cortex Moutan	/	14.06	/	15.05
Radix Sanguisorbae	/	/	/	17.97
Fructus Cnidii	/	/	/	8.9
Tet (10mg/ml)	25.35	40.27		

Note: Tet (10mg/ml), Positive control; "/"represents there was no zone of clearance, extract had no inhibition on the growth of MRSA compared with the negative control (equal volume solvent, distilled water or 50% methanol)

Table 4. Diameter of zones of clearance of *E.coil*

The English names of herbs	Diameter of zones of clearance (mm)			
	LB		BHI	
	aqueous extract	ethanolic extract	aqueous extract	ethanolic extract
Herba Scutellariae Barbatae	9.94	14.25	/	/
Radix Paeoniae Rubra	9.89	11.62	9.41	10.30
Cortex Moutan	9.97	12.51	8.36	10.68
Radix Lithospermi	/	10.05	/	/
Fructus Bruceae	/	8.37	/	/
Radix Sanguisorbae	14.27	13.85	/	/
Rhizoma Belamcandae	/	9.87	/	/
Van (10mg/ml)	21.44	18.85		

Note: Tet (10mg/ml), Positive control; "/"represents there was no zone of clearance, extract had no inhibition on the growth of MRSA compared with the negative control (equal volume solvent, distilled water or 50% methanol)

Table 5. Diameter of zones of clearance of PAO1

The English names of herbs	Diameter of zones of clearance (mm)			
	LB		BHI	
	aqueous extract	ethanolic extract	aqueous extract	ethanolic extract
Radix Paeoniae Rubra	12.31	14.25	12.75	12.5
Cortex Moutan	12.3	14.88	10.43	12.79
Tc (10mg/ml)	18.38	23.99		

Note: Tet (10mg/ml), Positive control.

of 0.2 M HCl after extraction. After centrifugation, the top layer (0.2 M HCl) was removed and its absorption was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per ml of culture supernatant, were calculated using an extinction coefficient at 520 nm of 17.072.

Motility Assay

The bacterial motility assay was carried out as reported previously[9]. Swimming motility medium consisted of 10 g/L tryptone, 5g/L NaCl, and 0.3% w/v agar and swarming motility medium used consisted of 8 g/L Nutrient broth, 5 g/L glucose and 0.5% w/v agar. 2 µL of overnight cultures of PAO1 were carefully spotted on the swimming plates supplemented with extract or equal volume solvent as control, and were incubated at room temperature for 16 h; Swarming plates were typically allowed to dry at room temperature overnight before being used. Overnight cultures of PAO1 were spotted on the swarming plates[9] supplemented with extract or equal volume solvent as control and incubated at 37°C for 24 h.

RESULTS

Anti-bacterial activity of 20 Chinese medicinal herbs

Twenty Chinese medicinal herbs were selected for investigation of anti-bacterial activity. They were chosen because they are known for the functions of Qing Re Jie Du (i.e. treating symptoms resembling infections).The antimicrobial activities in the water and ethanol extracts of these 20 herbs were tested against using several pathogens including *P. aeruginosa*, *Staphylococcus aureusÿ*methecilin resistant strain (MRSA), and *Escherichae coli*. Two different media, the LB and BHI broth, were used and herbal medicine extracts exhibited different degrees of antimicrobial activity using different broths. The antimicrobial activity of the ethanol extract of herbs was higher than the aqueous extract in the MRSA (Table 3) and *E. coli* (Table 4). Among all of the extracts, Paeonia suffruticosa Andr and Paeonia lactiflora Pall exhibited anti-bacterial in all of three strains. In addition, we noticed that only two extracts of herbs was found anti-bacterial activity in *P. aeruginosa* (Table 5), confirming the high resistance existed in *P. aeruginosa*.

The effect of herbal medicines on virulence factors in *P. aeruginosa* PAO1

Using these *lux*-based promoter-reporter fusions, virulence gene expression was measured as light production and the effect on growth was reflected by clear halo. Serial 2-fold dilutions of 20 herbal extracts (listed in Table 2) were tested at a final concentration of 172.50mg/ml086.25 mg/ml043.13 mg/ml021.56 mg/ml. The effects of crude extracts (Table 2) on the expression of virulence factors (*lasI0rhlI0lasR0rhlR0phzA10exoS* and *fliC*) were listed in the Table 6. Some herbal medicines exhibited various degree of inhibition on the virulence factors. However, we noticed that the aqueous extract of Folium artemisiae argyi inhibited more virulence factors and exhibited more inhibition on the virulence factors than other herbs. Crude extract of Folium artemisiae argyi represses the expression of QS associated virulence genes in PAO1 without affecting growth

Considering the result of extracts effect on the expression of virulence factors, we observed that the crude aqueous extract of Folium artemisiae argyi inhibited a number of virulence genes expression related with QS. Serial two-fold dilutions of aqueous extracts were tested at a final

Table 6. Effect of theaqueous and ethanolic extract of 20 herbs on the QS associated genes expression with discs diffusion assay

The English name of herbs		*phzA1*	*lasI*	*lasR*	*rhlI*	*rhlR*	*exoS*	*filC*
Folium Artemisiae Argyi	aqueous	- - + +	0 + + +	- - - -	- - - -	- - - -	- - - -	- - - -
	ethanolic	- - 0	0 0 0 0	- - - -	- - - -	d dd	d d 0 0	- - 0
Herba Lobeliae Chinensis	aqueous	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	ethanolic	+ 0 +	+ + +	0 0 0	- - -	0 0 0	- - -	- 0 0
Herba Scutellariae Barbatae	aqueous	d + +	- - 0 0	- - 0 0	- - - 0	- 0 0	- 0 0 0	- - -
	ethanolic	- 0 0	- + + 0	- - - 0	- - - -	- 0 0	- 0 0 0	- 0 0
Caulis Sargentodoxae	aqueous	0 0 0 0	0 0 0	0 0 0	d d 0	0 0 0 0	0 0 0	0 0 0 0
	ethanolic	d d +	0 0 0 0	- - - 0	- - 0 0	- - -	+ + + +	- - 0
Flos Lonicerae Japonicae	aqueous	- - d d	- - - -	- - - 0	- - - -	- - - 0	- - 0 0	- - - 0
	ethanolic	+ + +	+ + +	- - -	0 0 0	- - -	- - -	- - 0
Cortex Moutan	aqueous	+ + + +	0 0 0 0	+ 0 0 0	+ + + +	+ + 0 0	+ + + +	+ + + +
	ethanolic	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Rhizoma Paridis Chinensis	aqueous	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
	ethanolic	0 0 0	+ + 0	0 0 0	0 0 0	0 0 0	- - -	0 0 0
Radix Lithospermi	aqueous	d + 0	- 0 0 0	- - - 0	- 0 0 0	- 0 0	- 0 0 0	- - 0
	ethanolic	- - 0	0 0 0 0	- - - 0	- - 0 0	- - -	+ + + 0	- - 0
Semen Cassiae	aqueous	0 0 0	d d +	d + +	d d +	- - 0	- 0 0	- - -
	ethanolic	- - - 0	- - 0 0	- 0 0 0	- - 0 0	- - 0 0	- - - 0	- - 0 0
Herba Agrimoniae	aqueous	+ + +	- - -	+ + +	- - +	+ + +	d - -	- - -
	ethanolic	- - 0	- 0 0 0	d dd -	- - - -	- - -	DD + +	+ + +
Radix Isatidis	aqueous	0 0 0	0 0 0	0 0 0	+ 0 +	0 0 0	0 0 0	0 0 0
	ethanolic	+ 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Radix Sanguisorbae	aqueous	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	ethanolic	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Fructus Cnidii	aqueous	- - 0	- - 0	- - 0	- - d	- - -	- - -	- - -
	ethanolic	+ + +	+ + +	+ 0 0	+ + +	0 0 0	- - -	- - 0
Radix Paeoniae Rubra	aqueous	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
	ethanolic	d dd	d dd	d dd	- - -	d dd	d dd	d dd
Fructus Bruceae	aqueous	d d +	d dd	- - -	d - -	d dd	d - -	d dd
	ethanolic	d dd	d dd	d dd	d dd	d dd	d dd	d dd
Rhizoma Belamcandae	aqueous	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	- - 0	0 0 0
	ethanolic	0 0 0	0 0 0	0 0 0	- 0 0	0 0 0	0 0 0	0 0 0
Fructus Quisqualis	aqueous	d + +	d - -	d dd	d dd	- - -	- - -	- - -
	ethanolic	d d +	+ d -	- - -	- - -	0 0 0	- - -	0 0 0
Radix Stellariae	aqueous	+ + +	0 + +	0 0 0	0 0 0	0 0 0	- - 0	0 0 0
	ethanolic	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	- - 0	0 0 0
Herba Portulacae Herba	aqueous	- - - 0	0 0 0 0	0 0 0 0	- 0 0 0	D D 0 0	0 0 0 0	D + 0 0
	ethanolic	- 0 0	+ + + +	+ 0 0 0	+ + + 0	0 0 0	+ 0 0 0	+ + +
Herba Solani Nigri	aqueous	0 0 0	0 0 0	0 0 0	+ + +	- 0 0	0 0 0	0 0 0
	ethanolic	d 0 0	+ + +	- - 0	- - 0	0 0 0	- 0 0	0 0 0

"+"represents induction of the virulence gene expression by extractÿ"-" represents inhibition of the virulence gene expression by extract. "0" represents no effect on the virulence gene expression by extractÿ"d" represents different compounds in extract exhibited inhibition and induction on the expression of virulence factors. For example, "- - - 0" represents the effects of serial 2-fold dilutions of herbal extracts 101/201/4 and 1/8 on the expression of virulence factorÿ"+ + +"represents the effects of serial 2-fold dilutions of the herbal extracts 101/201/4 on the expression of virulence factor; 101/201/4 and 1/8 represent the aqueous extracts of Folium artemisiae argyi at the concentration of 172.5mg/ml086.25 mg/ml043.13 mg/ml021.56 mg/ml respectively.

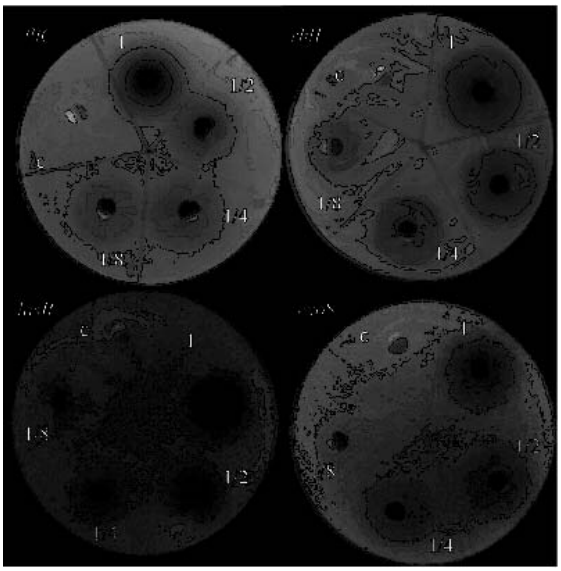

Fig. 1. Inhibition on *fliC*, *rhlI*, *lasI* and *exoS* by the aqueous extract of Folium artemisiae argyi. 101/201/4 and 1/8 represent the aqueous extracts of Folium artemisiae argyi at the concentration of 172.5mg/ml086.25 mg/ml043.13 mg/ml021.56 mg/ml respectively

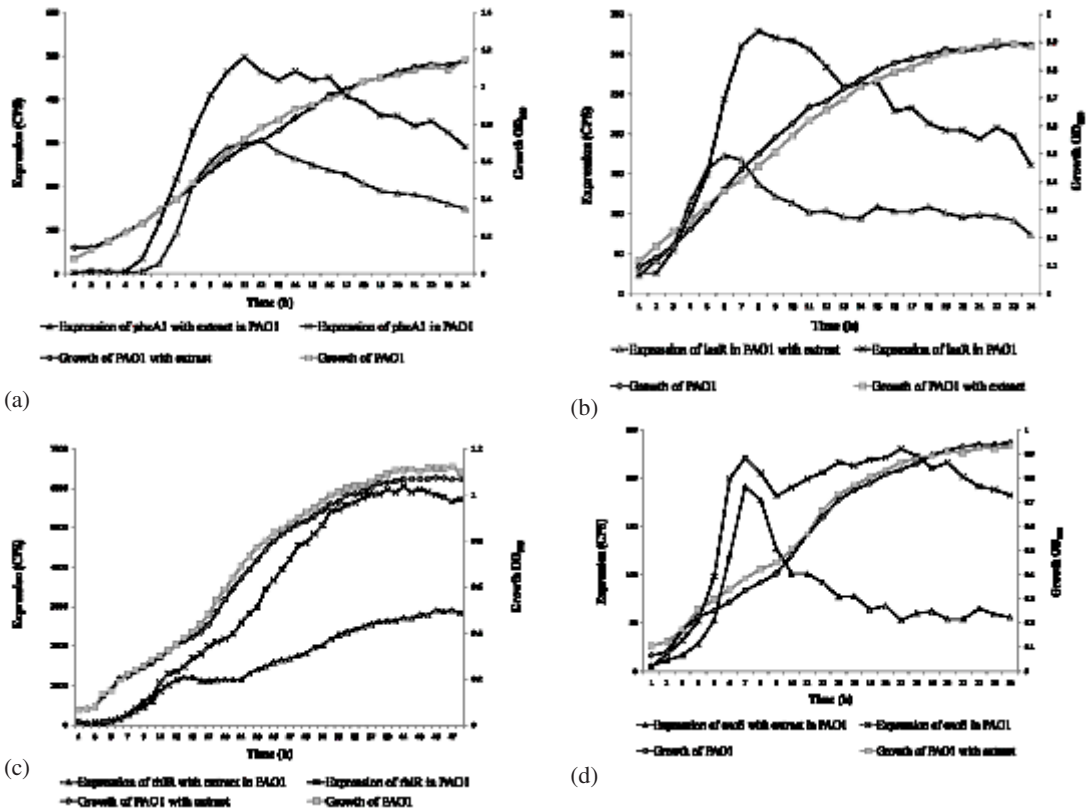

Fig. 2. Regulation of *phzA1* (Fig. 2A), *lasR* (Fig. 2B), *rhlR* (Fig. 2C) and *exoS* (Fig. 2D) by the aqueous extract of Folium artemisiae argyi. The aqueous extract of Folium artemisiae argyi was added at 0.86mg/ml

phzA2 (precursor of pyocyanin synthesis clusters) were inhibited by the aqueous extract of Folium artemisiae argyi (Fig. 4), we tested the effect of the aqueous extract of Folium artemisiae argyi on the production of pyocyanin. In agreement with the decreased expression of pyocyanin synthesis genes, aqueous extract of Folium artemisiae argyi reduced the pyocyanin production of *P. aeruginosa* PAO1.

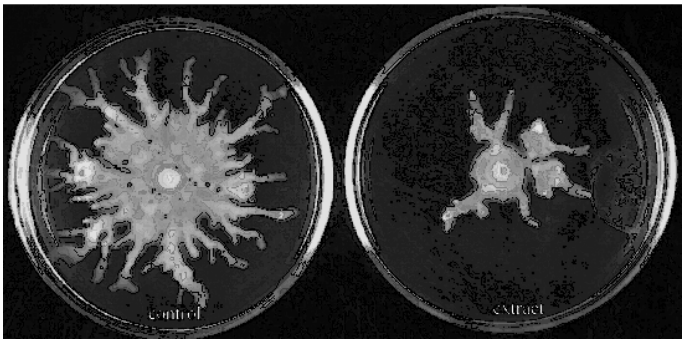

Fig. 3. Repression on swarming motility by the aqueous extract of Folium artemisiae argyi. The aqueous extract of Folium artemisiae argyi was added at 0.86mg/ml

DISCUSSION

With the ever increasing mergence of multidrug-resistant bacteria, new antibiotics and novel strategies to combat bacterial infections are needed. Traditional Chinese Medicine has been used to treat diseases for thousands of years while the mechanism remains unclear. A growing number of researches focused on the active compounds and the mechanism of action of TCMs which provide a potentially rich resource for new antibiotics. Many components of TCMs have been identified as being effective in treating various diseases such as gastritis, stomatitis, dermatitis, and pneumonia[4], Quorum sensing regulating bacterial virulence has afforded a novel target to control bacterial infections without interfering with growth. The first natural QS inhibitor (QSI), a furanone compound was isolated from the marine macro alga Delisea pulchra[19]. Other potential QSIs, the halogenated furanones C-302 and C-56 have been developed through chemical modifications[20]. Extensive studies has revealed several QSIs existed in fungi[21], sponges[22] and herbs[23-26] as well as commercially available drugs[27,28].

Folium artemisiae argyi has been widely used in China for many years, which is known for the functions of Qing Re Jie Du (i.e. treating symptoms resembling infections) for treatment of human diseases such as urinary tract infection,

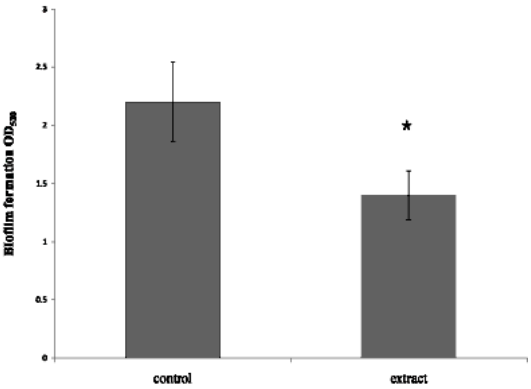

Fig. 4. Inhibition of the aqueous extract of Folium artemisiae argyi on pyocyanin production in *P. aeruginosa* PAO1. The aqueous extract of Folium artemisiae argyi was added at 0.86mg/ml.ÿ compared with PAO1 $p<0.05$

skin infection and carbuncle. However, the mechanism of treatment of infections with remains unclear. Our data in our study indicate that the crude extract of Folium artemisiae argyi could inhibit the expression of virulence factors in *P. aeruginosa,* such as pyocyanin production and swarming motility, while had no influence on cell viability. The result indicates that one or some active compound (s) exhibiting QSI activity in the extract of Folium artemisiae argyi, which may, at least partially, account for the efficacy of this herb for treating bacterial infections. Because it does

concentration of 3.45mg/ml01.73mg/ml00.86mg/ml00.43mg/ml. Then we took further investigation into the quorum sensing inhibition activity of Folium artemisiae argyi. Monitoring expression assay was performed in liquid to test the effect of the aqueous extract of Folium artemisiae argyi with the *lux*-based promoter-reporter fusions. As shown in the Table 7, the aqueous extract of Folium artemisiae argyi repressed a number of virulence factors such as *phzA1* (Fig. 2A), *lasR* (Fig. 2B), *rhlR* (Fig. 2C) and *exoS* (Fig. 2D) in liquid medium without affecting the growth of bacterial, which were consistent with the observation in the double agar diffusion assay.

Inhibition of PAO1 swarming motility by Folium artemisiae argyi

P.aeruginosa swarming motility is important for *in vivo* virulence[10] and regulated by quorum sensing. Previous studies have suggested that rhamnolipid production and the flagellum all contribute to swarming[11, 12]. Rhamnolipid production is controlled by *rhl* system positively. Mutants with defects in rhamnolipid synthesis genes showed an abnormal swarming pattern or defect in swarming motility[13]. The results obtained in gene expression assay showed that the aqueous extract of Folium artemisiae argyi inhibited the expression of *rhlI, rhlR* and *fliC* (flagella protein biosynthesis gene). We investigated the effect of the aqueous extract on the swarming motility of PAO1. Compared with the control, the aqueous extract of Folium artemisiae argyi inhibited the swarming motilitiy in *P.aeruginosa* PAO1 apparently (Fig.3). In addition, twitching motility is caused by type IV pili-mediated bacterial translocation on a solid surface and swimming motility requires flagellar. However, the aqueous extract of Folium artemisiae argyi had no apparent effect on twitching motility and swimming motility of *P. aeruginosa* PAO1 (data not shown).

Extract of Folium artemisiae argyi inhibits pyocyanin production

Pyocyanin (PCN) is one of the predominant phenazines produced by *P. aeruginosa* and functions as an important virulence factor[14,15]. In the lungs of individuals with cystic fibrosis, pyocyanin inhibits the ciliary function of respiratory epithelial cells in vitro[16] and alters the host immune and inflammatory response[17,18]. Since the expression of *phzA1* and not seem to affect *P. aeruginosa* growth, it is unlikely to be bactericidal or bacteriostatic. Hence, the active compound(s) in Folium artemisiae argyi probably won't assert a selective pressure on the pathogens and therefore less likely render resistance. Folium artemisiae argyi seems be a promising source for the development of anti-pathogenic drugs. Further investigations are required to identify the active compound(s) in Folium artemisiae argyi.

In conclusion, the Chinese medicinal herbs traditionally used in Chinese medicine for treating infectious diseases seem to function through inhibiting both bacterial viability and virulence, representing a promising source for new anti-infective development.

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