Research Article

Inhibition of *Pseudomonas aeruginosa* Biofilm Formation by Traditional Chinese Medicinal Herb *Herba patriniae*

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New antimicrobial agents are urgently needed to treat infections caused by drug-resistant pathogens and by pathogens capable of persisting in biofilms. The aim of this study was to identify traditional Chinese herbs that could inhibit biofilm formation of *Pseudomonas aeruginosa*, an important human pathogen that causes serious and difficult-to-treat infections in humans. A luxCDABE-based reporter system was constructed to monitor the expression of six key biofilm-associated genes in *P. aeruginosa*. The reporters were used to screen a library of 36 herb extracts for inhibitory properties against these genes. The results obtained indicated that the extract of *Herba patriniae* displayed significant inhibitory effect on almost all of these biofilm-associated genes.

Quantitative analysis showed that *H. patriniae* extract was able to significantly reduce the biofilm formation and dramatically altered the structure of the mature biofilms of *P. aeruginosa*. Further studies showed *H. patriniae* extract decreased exopolysaccharide production by *P. aeruginosa* and promoted its swarming motility, two features disparitely associated with biofilm formation. These results provided a potential mechanism for the use of *H. patriniae* to treat bacterial infections by traditional Chinese medicines and revealed a promising candidate for exploration of new drugs against *P. aeruginosa* biofilm-associated infections.

1. Introduction

*Pseudomonas aeruginosa* is a remarkably adaptive bacterial pathogen which can cause persistent infections in burn patients, immune-compromised patients, and individuals with the genetic disease cystic fibrosis. It is one of the most prevalent nosocomial pathogens, and the infections caused by *P. aeruginosa* can be very serious and life-threatening [1].

Biofilm formation is a major characteristic of *P. aeruginosa* chronic infections [2, 3]. *P. aeruginosa* cells in biofilms are surrounded by exopolysaccharides and form a structured aggregates, and these cells exhibit increased resistance to antibiotics and other adversary agents [3–6]. Infections caused by biofilm-forming *P. aeruginosa*, such as those in cystic fibrosis lung, are almost impossible to eradicate [7]. There is an urgent need to find novel antimicrobial agents to control such infections [8, 9].

Traditional Chinese medicines (TCMs) have been widely used to treat infectious diseases for more than a thousand years in China. Many components constituents of TCMs have been found to be very effective in treating bacterial infections such as gastritis, stomatitis, dermatitis, and bacterial pneumonia [10]. However, the mechanisms of these herbs in treatment infectious diseases are mostly unknown.

*Herba patriniae* is a perennial herbal of TCM, which contains various beneficial ingredients such as amino acids, vitamins, minerals, alkaloids, tannins, and saponins. It has been reported to have functions of antioxidant, antibacterial, antiviral, blood-activating and stasis-eliminating, promoting regeneration of liver cells, and anxiety-alleviating. The boiling water extracts of *H. patriniae* had been identified having anticyanobacteria activity against *Microcystis aeruginosa* [11].

In this study, 36 extracts of 18 Chinese herbs that are commonly used for treating infection-like symptoms were
screened for inhibitory effect against *P. aeruginosa* biofilms. We found that the extract of the Chinese herb *H. patriniae* significantly inhibited the expression of the genes associated with biofilm formation in *P. aeruginosa* PAO1. It reduced exopolysaccharide production and biofilm formation and then altered the structure of the mature biofilms.

### 2. Materials and Methods

#### 2.1. Bacterial Strains and Culture Conditions.

Bacterial strains used in this study are listed in Table 1. All the strains were cultured in LB (Luria Bertani) broth at 37°C with orbital shaking at 200 rpm or on LB agar plates supplemented with antibiotic of kanamycin (Kan, 50 𝜇g/mL) or trimethoprim (Tmp, 300 𝜇g/mL) where appropriate.

#### 2.2. Traditional Chinese Medicine Extraction.

Traditional Chinese medicinal herbs were selected according to their efficacy in treatment of infection-like symptoms in Chinese medicine. They were obtained from local pharmacy (Yikang Pharm chain store, China). The pulverized herb was firstly immersed in 75% ethanol or deionized water, respectively, for 2 h and then boiled for 2 h additionally (weight to solvent volume was 1:5). The extracts were filtered by filter paper and then boiled for 2 h and then filtered. The concentrated extracts were then freeze-dried using a lyophilizer and stored at −80°C. The water extracts were dissolved in deionized water and ethanol extracts were redissolved in methanol and then sterilized immediately with 0.22 𝜇m Iwaki filter before use.

#### 2.3. Gene Expression Assay.

Key genes *algU* [12], *pslM* [13,14], *pelA* [15], *algA* [16], *ppyR* [17], and *bdlA* [18] that are known to be involved in biofilm formation in *P. aeruginosa* were selected to construct *luxCDABE*-based promoter-reporter fusions (Table 2). The reporters were constructed as previously described [19, 20] and were subsequently transformed into PAO1. Using *luxCDABE*-based reporters, gene expression in liquid cultures was measured by light luminescence (in counts per second) in a Victor multilabel plate reader.

### Table 1: Bacterial strains used in this study.

| Strains/plasmids | Description | Source |
|------------------|-------------|--------|
| *E. coli* DH10B  | F-mcrA(mrr-hsdRMS-mcrBC)80dlacZ ΔM15ΔlacX74 deoR recA1 endA1 araD139 Δ(ara leu)7697 galU galKΔ-rpsl nupG | Invitrogen |
| *P. aeruginosa* PAO1 | Wild type | This lab |
| pMS402 | Expression reporter plasmid carrying the promoterless *luxCDABE*; Kan', Tmp' | This lab |
| pKD-*pslM* | pMS402 containing *pslM* promoter region; Kan', Tmp' | This study |
| pKD-*pelA* | pMS402 containing *pelA* promoter region; Kan', Tmp' | This study |
| pKD-*algU* | pMS402 containing *algU* promoter region; Kan', Tmp' | This study |
| pKD-*ppyR* | pMS402 containing *ppyR* promoter region; Kan', Tmp' | This study |
| pKD-*algA* | pMS402 containing *algA* promoter region; Kan', Tmp' | This study |
| pKD-*bdlA* | pMS402 containing *bdlA* promoter region; Kan', Tmp' | This study |

### Table 2: Reporter genes and primer sequences used.

| PA number | Gene | Function | Primer | Sequence (5′ → 3′) |
|-----------|------|----------|--------|--------------------|
| PA0762    | *algU* | RNA polymerase sigma factor | pKD-*algU*-S | GCACTCGAGAGGATGCTGAAGACGCTT |
| PA2243    | *pslM* | Succinate dehydrogenase; fumarate reductase flavoprotein | pKD-*pslM*-S | ATCCCTCGAGCCGTCGCAAGAAGACC |
| PA3064    | *pelA* | Glycoside hydrolase; deacetylase | pKD-*pelA*-S | CGTCTCGAGCCGACGCCAGCAAGAC |
| PA3551    | *algA* | Phosphomannose isomerase; guanosine 5′-diphospho-D-mannose pyrophosphorylase | pKD-*algA*-S | TGGGGATCTCGAGCCGACGCCAGCAAGAC |
| PA2663    | *ppyR* | Psl and pyoverdine operon regulator | pKD-*ppyR*-S | TCAGGATCCGAGCTTACACTTACG |
| PA1423    | *bdlA* | Biofilm dispersion locus A | pKD-*bdlA*-S | GCACTCGAGAGGATGCTGAAGACGCTT |

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H. patriniae was inoculated to 1.8 mL TSB supplemented with 3.2 mg grown on cover slip (8 mm diameter) placed at the bottom of wells. Similar to the above described biofilm assay, biofilms were cultured with shaking at 200 rpm for 1 h the plates were kept at stationary state at 37 °C for 3 days and 7 days with a medium replacement at every 24 h interval. Then wells were washed twice with deionized water gently to remove the planktonic cells. The sedentary cells were stained with 1% (v/v) crystal violet solution for 15 min. Unbound dye in the wells were washed off by deionized water before 200 μL of 0.2% L mineral oil was added to the wells to prevent evaporation. The cover slip was then stained with 5% silver nitrate for 15 min, followed by 1% hydroquinone colour-rendering for 2 min and then rinsed with distilled water for 1 min. Fixation was treated with 5% sodium thiosulfate for 1 min, followed by a final water rinse. The cover slip was placed on an inverted optical microscope for biofilm structure observation.

2.7. Swarming Motility Assay. Swarming assay was carried out as previously reported [25]. The medium used for swarming motility assay consists of nutrient broth (0.8%), glucose (0.5%), and agar (0.5%). The plates were dried at room temperature overnight before being used. 2 μL of P. aeruginosa PAO1 culture (OD600 = 0.5) mixed with 106.67 μg of H. patriniae water extract was spotted onto the swarming plate and the one with deionized water was used as blank control. Plates were incubated at 37°C for 24 h before the swarming diameter was measured.

2.8. Measurement of Exopolysaccharide Production. Overnight culture of PAO1 (OD600 = 0.005) was spotted onto Congo red plates (1% Tryptone, 1% agar, 4% Congo red, and 2% Coomassie blue) with or without H. patriniae water extract. The amount of H. patriniae water extract was 64 μg. The colony morphology and staining were observed after 3 days of incubation at 37°C [22].

3. Results and Discussion

3.1. Screening for Herbs with Inhibitory Effect on P. aeruginosa Biofilm-Associated Genes. Eighteen traditional Chinese medicinal herbs were selected because of their common usage for infection-like symptoms. Both boiling water extracts and ethanol extracts were obtained and used to screen for antibiofilm activities. Since P. aeruginosa biofilm formation is directly associated with the activity of several known genes, we constructed luxCDABE-based reporters to examine the effect of herb extracts on these genes. The effects of the crude extracts on the expression of these biofilm-associated genes (algU, pslM, pelA, algA, ppyR, and bdlA) are presented in Table 3. The results indicate that different herbal extracts exhibited various degrees of inhibitory effects on these genes. The water extract of H. patriniae showed the most significant effect on the expression of algU, algA, pslM, and bdlA. Examples of the gene expression profiles in the presence of H. patriniae are shown in Figure 1.

To confirm the results obtained from the lux-based reporter assay, real-time qPCR was carried out using bacterial RNA samples isolated in the presence and absence of H. patriniae. The results are shown in Table 4. In agreement with the results from the reporter assay, the mRNA levels of algU, algA, pslM, and bdlA in PAO1 were all significantly decreased in the presence of H. patriniae extract (at 1.6 mg/mL) compared with those in the absence of H. patriniae extract. It is noted that the inhibition of algU and algA was more pronounced in the qPCR assay.
### Table 3: Effect of water and ethanolic herb extracts on biofilm-associated genes expression.

| The English name of herbs | algU | pslM | pelA | algA | ppyR | bdlA | The English name of herbs | algU | pslM | pelA | algA | ppyR | bdlA |
|---------------------------|------|------|------|------|------|------|---------------------------|------|------|------|------|------|------|
| Atractylodes lancea       | +1.5 | 0    | −2.5 | 0    | 0    | 0    | Atractylodes lancea       | 0    | 0    | 0    | 0    | 0    | 0    |
| Cortex fraxini            | 0    | −3   | −1.5 | −1   | 0    | 0    | Cortex fraxini            | −1.8 | −2.5 | −0.5 | −15  | 0    | 0    |
| Cyrtomium fortunei        | 0    | −1.5 | −2   | −1   | 0    | 0    | Cyrtomium fortunei        | 0    | 0    | 0    | 0    | 0    | 0    |
| Erodium stephanianum Willd.| a    | a    | a    | a    | a    | a    | Erodium stephanianum wild | a    | a    | a    | a    | a    | a    |
| Folium artemisiae Argyi   | −2   | 0    | −2   | 0    | −1.5 | 0    | Folium artemisiae argyi   | 0    | 0    | −2   | 0    | −1.5 | 0    |
| Fruites quisqualis         | a    | a    | a    | a    | a    | a    | Fruites quisqualis         | a    | a    | a    | a    | a    | a    |
| Herba agrimoniae           | a    | a    | a    | a    | a    | a    | Herba agrimoniae           | a    | a    | a    | a    | a    | a    |
| Herba patriniae            | −2   | −2.5 | −0.5 | −2.5 | 0    | −2   | Herba patriniae            | 0    | 0    | 0    | −1.5 | −1   | |
| Herba scutellariae barbatae| 0    | 0    | −1   | 0    | 0    | 0    | Herba scutellariae barbatae| 0    | 0    | 0    | 0    | 0    | −1.5 |
| Pomegranate rind           | −1.5 | −1.2 | −0   | −1   | 0    | 0    | Pomegranate rind           | −1.5 | −1   | −1   | −0.5 | 0    | 0    |
| Portulaceae herba          | +1   | 0    | 0    | +1.5 | 0    | 0    | Portulaceae herba          | 0    | 0    | 0    | 0    | 0    | 0    |
| Radix paemicae             | −0.5 | −0.5 | 0    | 0    | −1   | 0    | Radix paemicae             | 0    | 0    | 0    | 0    | 0    | 0    |
| Radix sanguisorbarae       | −1.5 | −1   | −1.5 | −1   | −0.5 | 0    | Radix sanguisorbarae       | −1.6 | −1.5 | 0    | 0    | −0.5 | 0    |
| Radix lithospermi          | 0    | −1.2 | −2   | −0   | −1   | 0    | Radix lithospermi          | 0    | 0    | 0    | 0    | 0    | 0    |
| Scrophulariae              | −2   | +1.5 | 0    | 0    | 0    | 0    | Scrophulariae              | −0.5 | 0    | −1   | 0    | 0    | 0    |
| Smoked plum                | a    | a    | a    | a    | a    | a    | Smoked plum                | a    | a    | a    | a    | a    | a    |
| Taraxacum mongolicum       | 0    | 0    | 0    | 0    | 0    | 0    | Taraxacum mongolicum       | 0    | +2   | 0    | 0    | 0    | 0    |
| Tripterygium               | −1.5 | 0    | −2.5 | 0    | 0    | 0    | Tripterygium               | 0    | −2   | 0    | −2.5 | 0    | 0    |

Note: herbal extracts were used at 1.6 mg/mL. The values shown represent maximal inhibition (fold changes between experiments with and without herb extract). “+” represents induction of gene expression by herb extracts; “−” represents inhibition of biofilm gene expression by herb extracts; “0” represents no effect of biofilm gene expression by herb extracts; “a” represents inhibition of the growth of bacteria. Numbers represent fold changes at the time points where maximal expression was reached in the absence of herbal extract.
The results indicate that many of these herbs have an inhibitory effect on the genes associated with biofilm formation in *P. aeruginosa*. This is somewhat not surprising because they all have been used for treatment of chronic bacterial infections in traditional Chinese medicine.

The effect from the water extract of *H. patriniae* was remarkable as it inhibited five genes tested. It has been reported that the herb had antibacterial and antiviral activity. However, it was noted that the water extract did not inhibit the growth of *P. aeruginosa* (Figure 1). Even though conventional antibiotic compounds may exist in *H. patriniae* against other bacteria, this result indicates no such component was present against *P. aeruginosa*, at least not at the concentrations used in our experiments.

Lacking of bacterial killing or growth inhibition activity, however, may not be a weakness of such herbs in treating infectious diseases. As discussed in previous reports [26–29], a promising new class of antipathogenic drugs that target virulence factors and/or biofilm formation instead of killing the pathogens has many advantages in clinical use. First, these antipathogenic drugs theoretically are less likely to render drug resistance in the pathogens because they do not assert
a selective pressure on the pathogen’s survival. Second, such therapeutics would unlikely affect other nonpathogenic or beneficial bacteria, that is, the microbiome in the host.

3.2. Extract of H. patriniae Inhibits P. aeruginosa Biofilm Formation. From the gene expression results, the extract of H. patriniae presumably would inhibit the biofilm formation of P. aeruginosa. To verify such an effect, P. aeruginosa biofilm formation was compared in the presence and absence of H. patriniae extract. As shown in Figure 2(a), significantly less biofilm was formed in the presence of the herb extract than that without the extract at the irreversible attachment stage (1 d) and mature stage (3 d and 7 d) after inoculation. The result is in agreement with the gene expression data, suggesting the H. patriniae extract could reduce biofilm formation through inhibiting the genes associated with biofilm formation.

Importantly, the addition of the H. patriniae also dramatically altered the structure of the biofilms (Figure 2(b)). It appears that the herb extract prevented the formation of mature biofilms, only allowing P. aeruginosa to form smaller cell clusters. These results indicate that the water extract of H. patriniae indeed was able to inhibit P. aeruginosa biofilm formation.

3.3. Water Extract of H. patriniae Inhibited P. aeruginosa Exopolysaccharide Production. The exopolysaccharide (EPS) matrix is an important component of biofilm structure [30]. We compared the exopolysaccharide production in the presence of H. patriniae extract to those without H. patriniae extract. As shown in Figure 3, P. aeruginosa PAO1 cells produced more EPS shown in red by Congo red staining than the cells with H. patriniae extract. This result indicates that H. patriniae inhibited P. aeruginosa exopolysaccharide production and hence affected biofilm formation.

3.4. H. patriniae Water Extract Promoted PAO1 Swarming Motility. Upon encountering a surface, the surface-associated behaviors of P. aeruginosa, such as biofilm formation and swarming, are often coregulated [31]. In P. aeruginosa, swarming motility is reversely correlated with biofilm formation. Examination of the swarming motility of PAO1 in the presence and absence of the water extract of H. patriniae showed that H. patriniae promoted P. aeruginosa swarming motility (Figure 4(a)). The diameter of PAO1 grown with 106.67 μg H. patriniae was almost 5.60 cm, while the control was less than 2.0 cm (Figure 4(b)). Considering the reverse relationship between swarming motility and biofilm formation, the enhanced swarming motility by H. patriniae extract could have contributed the inhibition of biofilm formation.

Taken together, the extract of H. patriniae clearly inhibited the biofilm formation of P. aeruginosa. It inhibited several key genes algU, pslM, pelA, algA, and bdlA that are involved in biofilm formation. H. patriniae reduced exopolysaccharide production and promoted swarming motility. Increased motility may reduce adhesion and enable bacteria to actively escape the biofilm matrix to become planktonic bacteria [13, 31, 32]. As depicted in Figure 5, multiple factors/pathways probably have contributed to the reduction of biofilm formation and the altered biofilm structure in the presence of H. patriniae.

In a time of resistance to multiple antimicrobial agents in pathogenic bacteria being spread, there is an urgent need to develop new antibacterial agents [1, 32]. Drugs against infections that involve biofilms are particularly required. Pathogens in biofilm formation are more resistant to conventional antibiotics and other adversary conditions such as nutritional stress. Biofilms also protect bacterial cells from the activity of host immune response [33, 34].

Traditional Chinese medicines are a valuable source for novel antibacterial agents [35–37]. The inhibitory effect of the water extract of H. patriniae against P. aeruginosa biofilms and biofilm-related phenotypes signifies that H. patriniae is a promising candidate for treatment of infections caused by P. aeruginosa biofilms. It could be used in the way of traditional Chinese medicine or it can be explored for active compounds.

4. Conclusions

Our results indicate H. patriniae extract could significantly inhibit the expression of P. aeruginosa genes associated with biofilm formation, alter the structure, and prevent the formation of mature biofilms. It also decreased exopolysaccharide production and promoted swarming motility. These results provided a potential underlying mechanism for the use of H. patriniae to treat bacterial infections in traditional Chinese medicine and revealed a promising candidate for exploration of new drugs against P. aeruginosa biofilm-associated infections.
Figure 2: (a) Inhibition of biofilm production of PAO1 by *H. patriniae* extract. The control did not contain any herb extract. “*” indicates significant difference between the herb group and the control group ($P < 0.05$). “**” indicates very significant difference ($P < 0.01$). (b) Micrographs of biofilms formed with and without *H. patriniae*. Top row, PAO1 silver stained biofilms in the presence of extract of *H. patriniae* after 3 days and 7 days of incubation. Bottom row, PAO1 silver stained biofilms without *H. patriniae*. 
Figure 3: Photograph of exopolysaccharide production on Congo red plates. (a) Without *H. patriniae*. (b) With *H. patriniae*. The colony morphology and staining were observed after 3 days of incubation at 37°C.

Figure 4: Swarming motility of PAO1. (a) PAO1 grown with *H. patriniae* was at right; the one without herb was at left. (b) The swarming diameter of PAO1 with *H. patriniae*, the control was the one without herb. "**" indicates significant difference (P < 0.01).

Figure 5: *P. aeruginosa* biofilm formation and swarming motility in the influence of *H. patriniae*. 
Competing Interests

The authors declare that they have no competing interests regarding the publication of this paper.

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