An In Vitro Coumarin-Antibiotic Combination Treatment of *Pseudomonas aeruginosa* Biofilms

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**Abstract**

The drug resistance of *Pseudomonas aeruginosa* is a worldwide problem due to its great threat to human health. A crude extract of *Angelica dahurica* has been proved to have antibacterial properties, which suggested that it may be able to inhibit the biofilm formation of *P. aeruginosa*; initial exploration had shown that the crude extract could inhibit the growth of *P. aeruginosa* effectively. After the adaptive dose of coumarin was confirmed to be a potential treatment for the bacteria’s drug resistance, “coumarin-antibiotic combination treatments” (3 coumarins—simple coumarin, imperatorin, and isoimperatorin—combined with 2 antibiotics—ampicillin and ceftazidime) were examined to determine their capability to inhibit *P. aeruginosa*. The final results showed that (1) coumarin with either ampicillin or ceftazidime significantly inhibited the biofilm formation of *P. aeruginosa*; (2) coumarin could directly destroy mature biofilms; and (3) the combination treatment can synergistically enhance the inhibition of biofilm formation, which could significantly reduce the usage of antibiotics and bacterial resistance. To sum up, a coumarin-antibiotic combination treatment may be a potential way to inhibit the biofilm growth of *P. aeruginosa* and provides a reference for antibiotic resistance treatment.

**Keywords**

*Pseudomonas aeruginosa*, drug resistance, biofilms, quorum sensing, coumarin, antibiotic, *Angelica dahurica*

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for novel specific intervention strategies. Thus, developing QS inhibitors (QSIs) as anti-infective drugs provide a new strategy for coping with bacterial resistance.

Radix Angelicae Dahuricae (AD) is a natural medication promulgated by the National Health Commission of the People’s Republic of China to treat headaches and toothache. At the same time, it is also an important spice used in food preservation. AD has displayed many bioactivities, such as antitumor, antibiotic, antitumor, and anticholinesterase. Coumarins are one of AD’s prominent bioactive constituents. Coumarins and their derivatives have antibacterial, antiviral, antifungal, anti-inflammatory, anticancer, anticoagulant, and antihypertensive activities. Some types of coumarin derivative also have in vitro antibacterial activity against P. aeruginosa.

Based on our previous studies, we found that different AD extracts could stifle biofilm formation of S. aureus and P. aeruginosa effectively, most likely resulting from the presence of coumarin. Combination therapies have become hot due to their considerable antibacterial effect both in animal and clinical trials. In this article, the effect is reported of a coumarin-antibiotic (C-A) combination treatment toward biofilm formation of P. aeruginosa. Furthermore, this study could provide pointers to clinical trials with C-A combination treatment, in order to solve the currently urgent drug-resistance problem of P. aeruginosa and develop potential QSIs.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

*Pseudomonas aeruginosa* CICC 10419 used in this study was provided by the China Industrial Microbial Culture Preservation Center (CICC). *Pseudomonas aeruginosa* CICC 10419 was cultured in Luria-Bertani (LB) medium containing 5% glucose at 37 °C. The coumarins and antibiotics used were purchased from Chengdu Pusi Biotechnology Co., Ltd. All other chemical reagents were guaranteed and purchased from Chengdu Haoboyou Technology Co., Ltd.

**Extraction of AD**

AD root was dried, powdered, sieved, and then soaked in 75% ethanol solution for 3 hours. The mixture was heated under reflux for 3 times, each time for 3 hours. The liquids were then combined, the insoluble material removed by filtration, and the filtrate evaporated to dryness in a rotary evaporator under reduced pressure. The dry extract was dissolved in pure water and extracted 3 times with ethyl acetate. The solvent extracts were combined and concentrated to dryness under reduced pressure to give the dry extract, which was dissolved in water, filtered, and sterilized by passage through a 0.22-µm sterile filter membrane, prepared into certain concentrations of an aqueous solution, and then stored at low temperature for the next experiment.

**Observation of Biofilm Formation**

*Pseudomonas aeruginosa* CICC 10419 was inoculated into LB liquid medium, cultured at 37 °C at 120 r/min overnight, and diluted to 10^9 CFU/mL. Approximately 200 µL of the suspension was added to 96-well plates and then cultured for 1, 2, 3, 4, 5, 6, and 7 days. The supernatant was removed, and crystal violet staining was performed. Finally, absorbance was measured at 570 nm, and the average of three measurements was taken.

After biofilm formation had been observed, approximately 20 mL of a 10^6 CFU/mL suspension was added to a clean glass slide that had been autoclaved and sterilized under ultraviolet
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(UV) light for 24 hours. After incubation for 1, 3, 5, and 7 days at 37 °C, crystal violet stain was applied, and the slides were observed under an optical microscope.

Effects of Extracts on Biofilm Formation

Pseudomonas aeruginosa CICC 10419 strain suspension was diluted to 10^6 CFU/mL in LB medium. Approximately 100 µL of the bacterial suspension and 100 µL of the different extract concentrations were added to 96-well plates. The extract was prepared at final concentrations of 1, 10, 20, 30, 40, and 50 mg/mL. Anhydrous ethanol served as the control group. The culture was allowed to stand for 24 hours. Approximately 20 µL of triphenyltetrazolium chloride (TTC) was added to each well, incubated at 120 r/min, and protected from light. After 4 hours of culture, each well was observed for color change. The experiment was repeated 3 times, and the results were averaged. Approximately 10 µL of the suspensions containing different drug concentrations were obtained from the 96-well plates in LB solid medium containing 5% glucose, uniformly coated, and cultured at 37 °C for 24 hours to observe bacterial growth.

The overnight culture suspension was diluted to 10^6 CFU/mL, and 100 µL was inoculated into a 96-well plate. Different extract concentrations were added to obtain final concentrations of 1/8, 1/4, 1/2, 1.5, and 2 minimum inhibitory concentrations (MICs). The mixture was subjected to static culture at 37 °C for 24 hours. The supernatant was removed, and then crystal violet staining was performed. Finally, absorbance was measured at 570 nm on a microplate reader, and the experiment was repeated 3 times to obtain an average value.

### Table 1. Effects of Angelicae Dahuricae Extract at MIC and MBC on Bacterial Biofilms.

| Strain          | Concentration (mg/mL) | 1   | 10  | 20  | 30  | 40  | 50  |
|-----------------|-----------------------|-----|-----|-----|-----|-----|-----|
| Pseudomonas     | MIC                   | +   | +   | +   | +   | +   | −   |
| aeruginosa CICC | MBC                   | +   | +   | +   | +   | +   | −   |

Abbreviations: MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

**Note.** “+” means bacterial growth; “−” means sterile growth.

Destruction of Mature Biofilms

Given that the concentration of coumarins in the extract is low, accurately determining their effect on mature biofilms is difficult. Therefore, standard coumarins were used in this experiment (identification reports are shown in the Supplemental Material).

The mature biofilm cultured for 3 days was washed 3 times with phosphate-buffered saline (PBS). Approximately 100 µL of medium and 100 µL of coumarin of different concentrations were added to the mature biofilm to obtain final concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL, with anhydrous ethanol serving as the control group. The mixtures were statically cultured for 24 hours, and 20 µL of TTC was added to each well and cultured for 4 hours (20 r/min) in the dark. The color change of each well was observed. This experiment was repeated 3 times, and the results were averaged. Approximately

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![Figure 3. Effect of Angelicae Dahuricae extract on biofilm formation, as determined by crystal violet staining. **P < 0.01.](image-url)
10 µL of the bacterial suspensions with different drug concentrations were aspirated from the above 96-well plates in LB solid medium, uniformly coated, and cultured at 37 °C for 24 hours to determine the minimum bactericidal concentration (MBC) of coumarins on the mature biofilm.

**C-A Combination Treatment**

The antibiotics selected for *P. aeruginosa* CICC 10419 were ampicillin and ceftazidime. Simple coumarin, imperatorin, and isoimperatorin standards were used for the drug combination experiment.

The mature biofilm cultured for 3 days was washed 3 times with PBS. To each well were added antibiotics (final concentrations of 3, 4, 5, 6, 7, and 8 µg/mL) and coumarin (final concentrations of 0, 10, 20, 30, 40, and 50 µg/mL). After incubation at 37 °C for 24 hours, the supernatant was removed, and crystal violet staining was performed. Finally, absorbance was measured at 570 nm, and the average value was obtained from 3 measurements.

**Statistical Analysis**

Three independent biological replicates were performed for all experiments. Statistical analysis was performed using a 2-tailed paired Student’s *t*-test. Differences were considered significant when the *P* value was ≤0.05. Differences were considered extremely significant when the *P* value was ≤0.01.

**Results**

**Determination of Biofilm Formation**

With prolonged culture time, the amount of biofilm produced by the strain gradually increased (Figure 1). When the strains were cultured to the fifth day, the amount of biofilm tended to be constant, indicating that the mature biofilm had been formed. The results of optical microscopy are shown in Figure 2. With prolonged time, the structure of the biofilms gradually changed from loose to dense. When their culture reached the third day, mature biofilms began to form.

**Inhibition of Bacterial Biofilm by Extracts**

The inhibitory effect of the extract on the biofilms was gradually enhanced after various concentrations of the extract were applied to the strain for 24 hours (Table 1). *Pseudomonas aeruginosa* CICC 10419 biofilm formation could be completely inhibited, with a MIC and MBC of 50 mg/mL.

**The Effect of Extracts on Biofilm Formation**

Figure 3 showed that the biofilm yield of the strain gradually decreased with increasing coumarin concentration. When the drug concentration was either greater than or equal to the MIC, the strain biofilm formation could be completely inhibited.
Determination of MIC and MBC of the Extracts on Bacterial Mature Biofilm

The results recorded in Table 2 show that the extract minimally affected mature biofilms due to its low coumarin concentration. When used alone, coumarin reacted positively on the mature biofilms but could not completely destroy them.

C-A Combination Treatment of P. aeruginosa Mature Biofilm

Simple coumarin and antibiotics. Figure 4 showed that coumarin alone, in all groups, inhibited the formation of P. aeruginosa CICC 10419 biofilms. The simple coumarin-ceftazidime combination showed a more considerable impact than the simple coumarin-ampicillin group. The disintegration rate of the ceftazidime group dramatically decreased when the ceftazidime concentration was over 5 µg/mL. However, the disintegration rate of ampicillin was modest.

Imperatorin and antibiotics. Figure 5 showed that the inhibitory effect on P. aeruginosa CICC 10419 biofilms was enhanced when imperatorin was combined with antibiotics. The imperatorin-ampicillin combination seemed to be the best. For the imperatorin-ceftazidime combination, when the antibiotic concentration was 4 µg/mL, the disintegration rate of all groups was significantly reduced.

Isoimperatorin and antibiotics. As showed in Figure 6, when isoimperatorin was combined with either ampicillin or ceftazidime, the inhibitory effect on P. aeruginosa CICC 10419 biofilm was obvious. When 3 µg/mL ceftazidime and 50 µg/mL isoimperatorin was applied, the inhibition effect was much more significant than that of 3 µg/mL ceftazidime alone. Moreover, when the concentration of antibiotics was more than 6 µg/mL, all groups showed a similar inhibitory effect on P. aeruginosa CICC 10419 biofilm growth.

Discussion

Antibiotics resistance has become a threat to peoples’ health, and, therefore, some novel treatments should be explored to treat it, including some traditional medicines and herbs.\textsuperscript{133-35}
has been used as a medical food for thousands of years in southwest Asia and shows valuable bioactivities.\textsuperscript{35,38} In this study, AD extracts showed promising anti-QS activity on a \textit{P. aeruginosa} strain. Moreover, coumarin and its monomers have stronger antibiotic activity than certain abused antibiotic drugs. However, Figures 4–6 show that once biofilms formed, even with high concentrations of antibiotics, the change in the bacteriostatic effect was extremely limited, but combined with coumarin, the biofilm started to collapse. Research has shown that coumarin can significantly negatively regulate genes which are responsible for QS synthesis in \textit{P. aeruginosa}.\textsuperscript{39} Dense and thick biofilms of \textit{P. aeruginosa} are one of the clinical challenges in terms of infection because QS gets involved in biofilm formation.\textsuperscript{39,41} However, biofilms dramatically decreased after the coumarin was added. Therefore, the C-A combination treatment was more likely to act directly on the bacterial biofilms, killing the colonies to a certain extent and to be a new QSI.\textsuperscript{42,43}

From the present outcomes, we could not figure out which combination is best to prevent the biofilm formation. It is true that the reliability of in vitro experiment is lower than that of in vivo experiment due to the influence of drug absorption, metabolism in vivo, strains, species, and so on. Nevertheless, according to this study, there is no doubt that C-A combination treatment could be a potential QSI to tackle \textit{P. aeruginosa} drug resistance. Future exploration will go on to explore further the mechanism of C-A combination treatment of \textit{P. aeruginosa} CICC 10419 biofilm formation, which could help us to decide whether this treatment could have the potential for use in the clinic.

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\textbf{Supplemental Material}

Supplemental material for this article is available online.

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