Sodium houttuyfonate inhibits biofilm formation and alginate biosynthesis-associated gene expression in a clinical strain of *Pseudomonas aeruginosa in vitro*

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Abstract. The increasing multidrug resistance of *Pseudomonas aeruginosa* has become a serious public-health problem. In the present study, the inhibitory activities of sodium houttuyfonate (SH) against biofilm formation and alginate production in a clinical strain of *P. aeruginosa* (AH16) were investigated *in vitro* using crystal violet dying and standard curve methods, respectively. The cellular morphology of *P. aeruginosa* treated with SH was observed using a scanning electron microscope. Furthermore, reverse transcription-quantitative polymerase chain reaction was used to identify differences in the expression levels of genes associated with alginate biosynthesis as a result of the SH treatment. The results indicated that SH significantly inhibited biofilm formation, and decreased the levels of the primary biofilm constituent, alginate, in *P. aeruginosa* AH16 at various stages of biofilm development. In addition, scanning electron microscopy observations demonstrated that SH markedly altered the cellular morphology and biofilm structure of *P. aeruginosa*. Furthermore, the results from the reverse transcription-quantitative polymerase chain reaction analysis indicated that SH inhibited biofilm formation by mitigating the expression of the *algD* and *algR* genes, which are associated with alginate biosynthesis. Therefore, the present study has provided novel insights into the potent effects and underlying mechanisms of SH-induced inhibition of biofilm formation in a clinical strain of *P. aeruginosa*.

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Introduction

The Gram-negative bacterium, *Pseudomonas aeruginosa*, is a ubiquitous and versatile microorganism that is able to survive in soil, marsh and marine habitats on plant and animal tissue and on non-living surfaces (1). In a clinical context, *P. aeruginosa* functions as an opportunistic pathogen and is a leading cause of nosocomial infections, particularly chronic lung infections in patients with cystic fibrosis (CF) or patients with other immune deficiencies (2). Infections caused by *P. aeruginosa* have become a worldwide problem due to the increasing rates of morbidity and mortality, and the expense associated with hospitalized patients (3).

P. aeruginosa is able to form a biofilm on numerous types of surface, including the lung tissue of patients with CF (4), and on abiotic surfaces, including contact lenses and catheter lines (5,6). As a result of this biofilm-forming ability, *P. aeruginosa* infections are difficult to eliminate, particularly lung infections in CF patients, since the bacteria are highly resistant to a range of antimicrobial agents (7). Certain antimicrobial agents, including azithromycin (AZM), third-generation cephalosporins, carbapenems, monobactams, colistin, tobramycin and quinolones, are effective against the majority of *P. aeruginosa* strains (8). However, *P. aeruginosa* typically develops induced resistance to these agents via a number of mechanisms, including horizontal transfer or over-expression of resistance genes, or gene mutations that target the treatment drug (9). In addition, the ability of *P. aeruginosa* to form a biofilm results in a high level of antibiotic resistance and virulence in patients, since the biofilm is able to protect the bacterium from the inhibitory effects of antibiotics and the host immune system (10). Due to this capability to form a biofilm, *P. aeruginosa* is a model organism in bacterial biofilm research (11). A variety of agents have been investigated with the aim of suppressing the biofilm formation, including products derived from plants (12-14).

Sodium houttuyfonate [SH; chemical composition, $CH_3(CH_2)_8COCH_2CHOHSO_3Na$] is a compound of sodium bisulfite and houttuynin. Houttuynin is the primary constituent of the volatile oil produced by *Houttuynia cordata* Thunb, a wild perennial herb used widely in traditional Chinese medicine (15). SH is easily dissolved in hot water and alkaline

solutions, is slightly soluble in water and ethanol, and is insoluble in chloroform and benzene (16). In China, SH has been clinically used as an antimicrobial agent for numerous years, and has been reported to effectively inhibit Gram-positive bacterial infections, including *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* (17). Ye *et al* reported that Gram-positive bacteria were more sensitive to houttuyfonate homologs compared with Gram-negative bacteria, owing to the interactional differences between SH and the cell membrane (18). In addition, a previous study of transcriptional and functional analysis demonstrated SH-mediated inhibition by autolysis in *S. aureus* (19). However, there are few reports characterizing the effects of SH on the inhibition of Gram-negative bacteria, such as *P. aeruginosa*.

In the present study, the inhibitory activity of SH against biofilm formation and alginate production on a clinical strain of *P. aeruginosa* (AH16) was investigated *in vitro*. Alterations in the cellular morphology of *P. aeruginosa* following treatment with SH were observed using scanning electron microscopy (SEM). Furthermore, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to identify any changes in the expression levels of genes associated with alginate biosynthesis as a result of the SH treatment.

Materials and methods

Bacteria strain and materials. An AH16 *P. aeruginosa* strain was isolated from a patient with chronic pneumonia in the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (Hefei, China). This study was approved by the ethics committee of the First Affiliated hospital of Anhui University of Traditional Chinese Medicine. The strain was found to exhibit higher virulence and produce more biofilm when compared with the wild-type *P. aeruginosa*. SH and AZM were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Luria-Bertani (LB), Mueller-Hilton (MH) and Tryptic Soy Broth (TSB) media were purchased from Beijing Aoboxing Bio-Tech Co., Ltd. (Beijing, China), and crystal violet solution was purchased from bioMérieux, Inc. (Craponne, France). Alginate standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), and Taq polymerase and PCR primers were purchased from Takara Bio, Inc. (Otsu, Japan).

The AH16 strain was inoculated into LB broth, and grown in a constant-temperature shaker (GLY; Fuma, Shanghai, China) at 220 rpm for 6 h at 37˚C. Subsequently, the bacteria were harvested using a GL-20G-II high-speed refrigerated centrifuge (Fuma) at 1,630 x g for 10 min. The supernatant was discarded and the precipitate was resuspended with phosphate-buffered saline (PBS; pH 7.2), after which the samples were centrifuged again at 1,630 rpm for 10 min. The harvested cells were resuspended in PBS (pH 7.2) and adjusted to $2x10^5$ colony-forming units (CFU)/ml using the growth curve method (20).

Evaluation of the effects of SH on biofilm formation. Sauer *et al* (21) previously described five stages of biofilm formation as follows: i) Reversible attachment (0-2 h); ii) irreversible attachment (2 h); iii) maturation stage 1 (day 3); iv) maturation stage 2 (day 6); and v) dispersion (day 9-12). On the basis of these stages, days 1, 3 and 7 were selected as the three time points for observing the growth of the biofilm and to detect any suppression as a result of the drugs used. The minimum inhibitory concentrations (MICs) of SH and AZM were determined to be 512 and 16 μg/ml, respectively, using the microdilution method (22).

Fresh stock solutions of SH and AZM were prepared in MH broth and filtered through a 0.22-μm filter (EMD Millipore, Billerica, MA, USA). Six treatment groups were analyzed, which were treated with SH or AZM at a MIC of 0.5, 1 or 2 (Fig. 1). In a 96-well plate, 28 wells were filled with TSB medium, of which four wells were used for each of the six treatment groups, with four further wells used as the control. Next, 200 μl *P. aeruginosa* suspension was added and the plate was incubated at 37˚C. After 24 h, the culture suspension was discarded, and the sedimented bacteria were washed in 1 ml PBS. Fresh TSB medium with the aforementioned concentrations of antibiotics was added to the relevant wells. The control wells contained TSB medium with no antibiotics. In accordance with the method previously described by O'Toole (23), the medium was exchanged for fresh medium with the appropriate antibiotics every other day. At the end of days 1 (attachment), 3 (maturation stage 1) and 7 (maturation stage 2), cold PBS (4˚C) was used to wash the planktonic bacteria in one well of each group, after which 200 μl crystal violet solution (1%) was added to the wells and left for 20 min. The wells were rinsed with deionized water until no crystal violet was visible, following which the wells were dried and 95% alcohol was added for destaining. Subsequently, the destained solution from each well was transferred to a cuvette and diluted with 3 ml alcohol (95%). The optical density (OD) of the solutions was determined at 570 nm using a UV spectrophotometer (U-2000; Hitachi, Ltd., Tokyo, Japan). The OD value of the negative control was set as 100%, from which the growth of the biofilm was calculated for each group.

Evaluation of the effects of SH on alginate production. Alginate is the predominant constituent of the *P. aeruginosa* biofilm, and functions as a barrier to protect the bacteria from antibiotics and the humoral and cellular host defense system (24,25). Therefore, the effects of SH on alginate production in the *P. aeruginosa* biofilm were evaluated.

The groups analyzed were the same as those described previously (0.5x, 1x and 2x MIC groups each for SH and AZM). A sterile coverglass was used as a carrier for each group. The coverglasses were placed into the wells of a six-well culture plate, and 2 ml TSB medium and 0.2 ml bacterial suspension ($2x10^5$ CFU/ml) were added. The plates were incubated for 1 day at 37˚C, after which the medium was discarded. For each well, the coverglass was removed, and the planktonic bacteria on the coverglass were washed out with sterile PBS. The coverglass was subsequently returned to the well prior to the addition of fresh medium. As aforementioned, the medium added to the antibiotic groups contained the appropriate antibiotic, while the negative control contained medium without antibiotics. All the described steps were repeated every 24 h. At the end of days 1, 3 and 7 of the antibiotic treatment, the coverglasses were removed and the planktonic bacteria were rinsed with PBS. Subsequently, the coverglasses were each placed into a

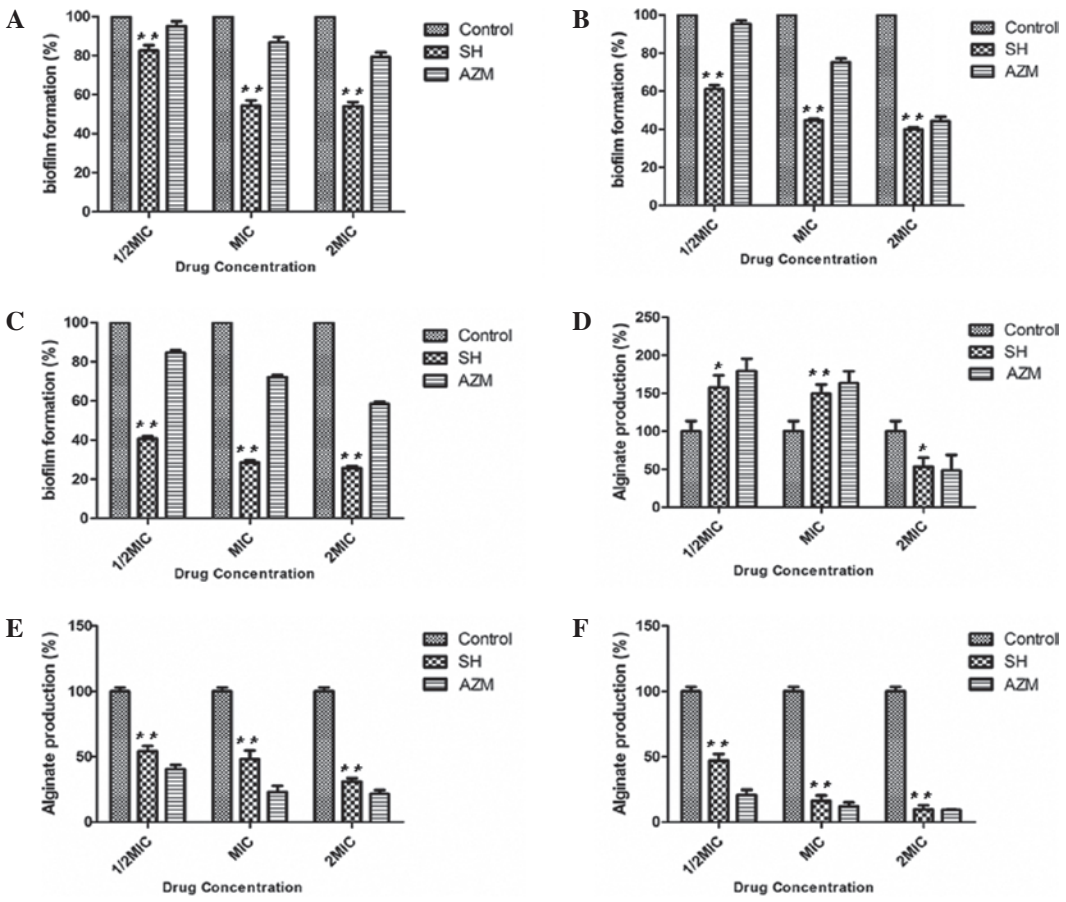

Figure 1. Inhibitory effects of SH on biofilm formation at days (A) 1, (B) 3 and (C) 7 and alginate production at days (D) 1, (E) 3 and (F) 7. *P<0.05 and **P<0.01, vs. control (n=4). Experiments were repeated in quadruplicate, and the results are expressed as the mean of the four replicates. The MICs of SH and AZM were 512 and 16 μg/ml, respectively. Control, negative control; SH, sodium houttuyfonate; AZM, azithromycin; MIC, minimum inhibitory concentration.

test-tube containing 6 ml PBS and 1.2 ml sulfuric acid and sodium borate compound which was prepared by dissolving 2.52 g sulfuric in 1:l sulfuric acid. The tubes were boiled for 5 min and then cooled to 4˚C. Each coverglass received 20 μl hydroxybiphenyl (1%) for colorization, after which the coverglasses were sonicated for 30 min in an ultrasonic cleaning bath (2800; Branson Ultrasonics, Danbury, CT, USA) and the absorbance was measured at 570 nm using the Hitachi UV spectrometer (26). The alginate production of each strain, defined as the quantity of alginate (μg/ml) adhered to the coverglass, was calculated using a standard curve of alginate standards following the subtraction of the blank control.

SEM imaging of the biofilm morphology. Preparation of the carrier coverslips was performed as aforementioned, up to the point at which the planktonic bacteria were rinsed with PBS. Subsequently, the biofilm was subjected to silver staining (27), and cellular morphology was examined under SEM (Sirion 200 field-emission; FEI Company, Hillsboro, OR, USA) at 500 kV and x40,000 magnification.

RT-qPCR of the alginate biosynthesis genes. RT-qPCR was performed to determine the expression levels of genes associated with alginate biosynthesis. The majority of the alginate biosynthesis genes are clustered in the *algD* operon (28). Alginate production is highly regulated, and AlgR is among one of the key regulators (29,30). Therefore, the *algD* and *algR* genes were selected for subjection to RT-qPCR in order to identify whether SH was able to attenuate the expression levels of alginate biosynthesis-associated genes. In addition, the expression levels of the biofilm-associated genes, *pilL* and *rhlI*, were evaluated.

Initially, 1 ml biofilm bacterial suspension culture, treated with 1x or 2x MIC concentrations of SH, was centrifuged at 10,800 x g for 1 min. The supernatant was discarded and the pellet was resuspended in 1 ml TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) at room temperature for 20 min. Each sample received 200 μl chloroform and was vortexed for 15 sec, followed by centrifugation at 4,200 x g at 4˚C for 15 min. The liquid layer of the mixture was transferred to a fresh tube containing 480 μl isopropanol, after which the tube was vortexed for 15 sec and centrifuged at 4,200 x g at 4˚C for 15 min. The total RNA was washed with 70% ethanol, and the tube was centrifuged again at 4,200 x g and 4˚C for 10 min. The liquid was discarded, and the total RNA was dissolved in RNase-free water. Purified RNA (2 μg) was reverse transcribed into cDNA using a one-step method commercial kit (Takara Bio, Inc.). The primers were designed to produce products of 180-240 bp, and the sequences are detailed in Table I. The *rpoD* gene was used as a housekeeping control. PCR assays were conducted using an ABI Prism thermal cycler (Applied Biosystems Life Technologies, Foster City, CA, USA) using the following program: Initial denaturation for 5 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec,

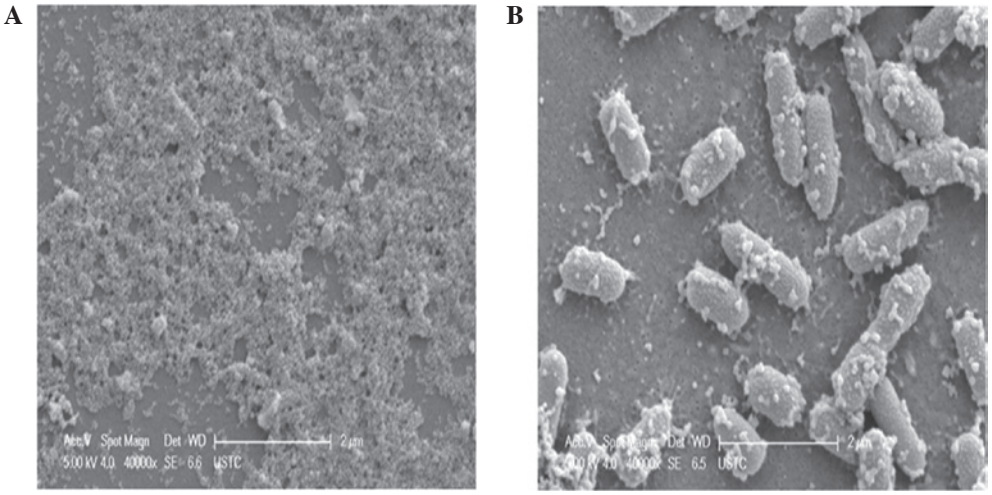

Figure 2. Microscopic morphology of sodium houttuyfonate (SH)-treated *Pseudomonas aeruginosa* biofilm in the (A) control group without drug treatment and the (B) 1X minimum inhibitory concentration SH treatment group (magnification, x40,000).

Table I. Primers used for the reverse transcription-quantitative polymerase chain reaction of the alginate biosynthesis genes.

Gene	Sequence 5'→3'
algR	F: AGACCGGCTACGGCTACA
	R: GCGTCGTGCTTCTTCAGTT
algD	F: AGAAGTCCGAACGCCACA
	R: TCCAGCTCGCGGTAGAT
rpoD	F: AGGCCGTGAGCAGGGAT
	R: GGTGGTGCGACCGATGT

F, forward; R, reverse.

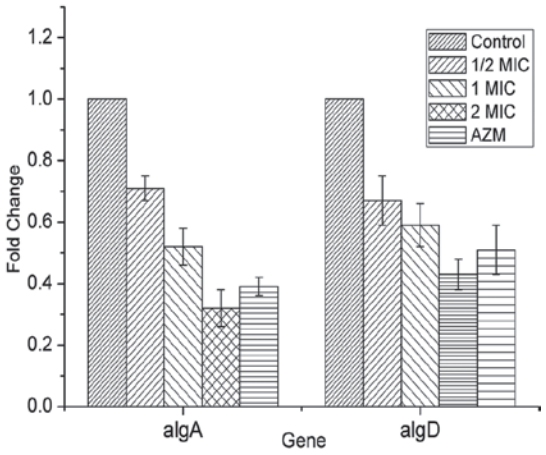

Figure 3. Differences in the expression levels of the alginate biosynthesis genes, *algR* and *algD*, in *Pseudomonas aeruginosa* following sodium houttuyfonate treatment. MIC, minimum inhibitory concentration; AZM, azithromycin.

58˚C for 10 sec and 72˚C for 20 sec. In the experiment, there were three independent biological replicates and two technical replicates. The calculated threshold cycle (Ct) of each gene was normalized against the Ct of the *rpoD* gene amplified from the corresponding sample. Fold change was calculated according to the $2^{-\Delta\Delta Ct}$ method (31).

Statistical analysis. All data were analyzed by SPSS statistical software, version 17.0 (SPSS, Inc., Chicago, IL, USA), and expressed as the mean ± standard deviation. Difference between the various group data were compared using Student's T-test. P-values were calculated by comparison of the data of drug treatment and control groups. Experiments were repeated in quadruplicate, and the results are expressed as the mean of the four replicates.

Results

Effects of SH on biofilm formation. By day 1 of the antibiotic treatment, a significant effect on biofilm growth was observed in the 0.5x, 1x and 2x MIC SH treatment groups when compared with the control group (P<0.01; Fig. 1A), with the 1x and 2x MIC treatments producing a more marked effect compared with the 0.5x MIC treatment. This effect continued throughout day 3 (Fig. 1B) and day 7 (Fig. 1C), with the higher concentrations exhibiting the most notable effects. On day 7 (Fig. 1C), all the biofilms were reduced to <50% of the control (P<0.01). In addition, SH was more effective at repressing biofilm formation compared with AZM. These results indicated that SH significantly inhibited biofilm formation in *P. aeruginosa* (AH16) at various developmental stages.

Effects of SH on alginate production. At day 1 of the antibiotic treatment (Fig. 1D), there was a statistically significant (P<0.05) reduction in the alginate biofilm in the 2x MIC SH group when compared with the control group; however, this effect was not observed in the 0.5x or 1x MIC SH groups, which was similar to the effects of AZM. By day 3 (Fig. 1E), the 0.5x, 1x and 2x MIC SH treatment groups had induced significant reductions in alginate production (P<0.01) in a dose-dependent manner. At day 7 (Fig. 1F), the inhibitory effects of all three SH concentrations had produced reductions in the alginate production of ≥50% compared with the control. Notably, the inhibitory effects of SH were lower compared

with AZM at 0.5x and 1x MIC, but were comparable to AZM at 2x MIC. These results demonstrated that SH exerts an inhibitory effect on alginate production in the *P. aeruginosa* AH16 clinical strain.

Biofilm morphology of P. aeruginosa treated with SH. In the SEM images, the bacterial biofilm morphologies were observed to clearly vary between the two groups. In the control group (Fig. 2A), the bacteria were completely covered by a thick mucous biofilm, and the entire structure exhibited a mushroom shape with interlaced inner pore channels towards the surface. By contrast, in the 1x MIC SH treatment group (Fig. 2B), there was no evident biofilm structure and the rod-shaped bacteria were dispersed over the mucus. Thus, the morphology of the biofilm cells indicated the inhibition of alginate production by SH.

Changes in the expression levels of the alginate biosynthesis genes following SH treatment. Expression levels of the *algD* and *algR* genes were reduced in the SH treatment groups when compared with the control group (Fig. 3). This reduction was dose-dependent, with the expression level being more notably reduced in the group treated with the highest concentration of SH (2x MIC) compared with the medium concentration (1x MIC). By contrast, the biofilm-associated genes, *pilL* and *rhlI*, were not downregulated transcriptionally by SH (data not shown). These results indicated that SH inhibits biofilm formation in *P. aeruginosa* by repressing the expression of genes associated with alginate biosynthesis.

Discussion

The production of a biofilm by a bacterial colony is a key form of growth in environmental and clinical contexts. There are three critical phases of biofilm development, namely adherence, maturation and dispersion. Each of these stages involves reinforcement by, or modulation of, the extracellular matrix (32). The biofilm-forming ability of *P. aeruginosa* is the primary factor enhancing the high level of virulence and antibiotic resistance. The current study investigated the inhibitory capacity of the plant-derived product, SH, which has been reported to possess the ability to inhibit the growth of clinical *P. aeruginosa* strains. SH was observed to significantly repress biofilm formation in the attachment and maturation stages of biofilm development. In addition, the morphology of the biofilm was affected by SH treatment. Thus, the results indicate that SH is able to inhibit the formation of biofilms by a clinical strain of *P. aeruginosa*.

Overproduction of the exopolysaccharide alginate provides *P. aeruginosa* with a selective advantage, and facilitates survival in the lungs of patients with CF (33). The results of the present study revealed that SH significantly inhibits alginate production at the biofilm maturation stage (Fig. 1E-F); however, only the highest concentration of SH was able to repress alginate production at the attachment stage (Fig. 1D), which is comparable to AZM. Furthermore, the alginate biosynthesis genes, *algD* and *algR*, were found to be downregulated as a result of the SH treatment (Fig. 3). Thus, these results indicate that SH inhibits biofilm formation in *P. aeruginosa* by repressing alginate production. Expression of the alginate machinery and biosynthetic enzymes are controlled by the extracytoplasmic sigma factor (33). However, further study is required to elucidate the mechanisms underlying the SH-induced repression of alginate biosynthesis genes in *P. aeruginosa*.

In conclusion, the present study indicated that SH significantly inhibits biofilm formation in a clinical strain of *P. aeruginosa*, and markedly reduced the expression of the primary biofilm constituent, alginate, at various stages of growth. Observations of cellular morphology demonstrated that SH alters the biofilm structure of *P. aeruginosa*, while the results of the RT-qPCR analysis indicated that SH may inhibit biofilm formation by repressing the expression of alginate biosynthesis genes. Thus, the present study provides novel insights into the effects of SH on biofilm formation in the *P. aeruginosa* AH16 strain, and into potential underlying mechanisms. However, further studies are required to confirm the molecular mechanisms underlying the effects of SH against biofilm formation in *P. aeruginosa*.

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