

Hyperoside inhibits biofilm formation of *Pseudomonas aeruginosa*

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Abstract. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common pathogen in hospital-acquired infection and is readily able to form biofilms. Due to its high antibiotic resistance, traditional antibacterial treatments exert a limited effect on *P. aeruginosa* biofilm infections. It has been indicated that hyperoside inhibits *P. aeruginosa* PAO1 (PAO1) biofilm formation without affecting growth. Therefore, the current study examined the biofilm formation and quorum sensing (QS) system of PAO1 in the presence of hyperoside. Confocal laser scanning microscopy analysis demonstrated that hyperoside significantly inhibited biofilm formation. It was also observed that hyperoside inhibited twitching motility in addition to adhesion. Data from reverse transcription-quantitative polymerase chain reaction indicated that hyperoside inhibited the expression of *lasR*, *lasI*, *rhlR* and *rhlI* genes. These results suggest that the QS-inhibiting effect of hyperoside may lead to a reduction in biofilm formation. However, the precise mechanism of hyperoside on *P. aeruginosa* pathogenicity remains unclear and requires elucidation in additional studies.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a common pathogen in hospital-acquired infections (1). Due to increasing multidrug resistance, *P. aeruginosa* infection is an increasingly common cause of mortality and morbidity (2). The mechanisms of antibiotic resistance in *P. aeruginosa* include the expression of multiple antibiotic modifying enzymes, antibiotic efflux pumps and acquisition of chromosomally or plasmid encoded antibiotic resistance genes. Additionally, chromosomal mutations and lower membrane permeability for the antibiotics also contribute to antibiotic resistance (3).

P. aeruginosa is a biofilm-forming pathogen and is difficult to eradicate due to its high antibiotic resistance and the ability of the biofilm to evade the immune system (4-7). Quorum sensing (QS) is a system of stimuli and response correlated to population density. *P. aeruginosa* uses the QS system to coordinate gene expression according to the density of its local population. Thus, it can coordinate certain behaviors such as biofilm formation, virulence and antibiotic resistance. QS inhibitors (QSIs) are the most well reported alternative therapeutics that can be used to overcome the problem of increasing antibiotic resistance in *P. aeruginosa*. QSIs target the virulence of the organism and therefore are also termed antipathogenic drugs. The virulence of *P. aeruginosa* depends on its cell-to-cell communication system, or QS system that uses diffusible signaling molecules that accumulate with increasing cell density and allows *P. aeruginosa* to trigger coordinated responses and achieve outcomes that would otherwise remain impossible to achieve by individual bacterium (8). Previous studies have demonstrated that traditional treatments for bacteria exert some effect on biofilm infections (9,10). Therefore, the effects of constituents from marine organisms, traditional Chinese herbs and plants (11-13) on biofilm infections are being assessed.

Flavonoids are plant polyphenols present in vegetables, fruits and beverages of plant origin and are well known for their antipyretic, analgesic and anti-inflammatory physiological properties (14-18). Hyperoside is a type of modified flavonoid. It has been demonstrated that hyperoside has weak antibacterial activity against gram-positive bacteria and no antibacterial activity against gram-negative bacteria (19,20). Furthermore, it has been identified that hyperoside exhibits an inhibitory effect on *P. aeruginosa* PAO1 (PAO1) biofilms (21). While the incidence of infections caused by antibiotic resistant strains has increased, the discovery of novel classes of antibiotics has slowed down which made it imperative to search for alternative treatment strategies. Therefore, the current study examined the biofilm formation, adhesion and motility of PAO1 in the presence of hyperoside. And the hyperoside may become a more effective method to treat the infection of *P. aeruginosa*.

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Materials and methods

Bacterial strains and culture conditions. *P. aeruginosa* PAO1 was acquired from Bioplus Biotech Co., Ltd. (Shanghai, China) and cultured in Lubria-Bertani (LB) medium (BD

Diagnostics, Sparks Glencoe, MD, USA) at 37°C for 24 h in all experiments. The hyperoside was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China).

Dose effect of hyperoside on biofilm formation. PAO1 was activated in LB medium overnight at 37°C prior to 1:1,000 (v/v) dilution in a tissue culture microtiter plate. The final concentrations of hyperoside in the tissue culture microtiter plate were 8, 16, 32, 64, 128 and 256 µg/ml. Following 24 h incubation at 37°C, the medium was removed and wells were washed three times with ddH₂O. The microtiter plate was dried prior to the addition of 1% crystal violet (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) for 15 min at room temperature. Following staining, the dye was removed and the wells were washed three times with tap water. The microtiter plate was dried prior to the addition of 30% glacial acetic acid to solubilize the dye bound to the biofilm. The absorbance was measured by xMark Microplate Spectrophotometer (Bio-Rad, Laboratories, Inc., Hercules, CA, USA) at 590 nm. The optimal concentration for biofilm inhibition was used in the later experiments.

Growth assays. Cells were grown in LB medium in the presence or absence of 16 µg/ml hyperoside. Bacterial culture turbidity was measured by xMark Microplate Spectrophotometer (Bio-Rad, Laboratories, Inc) at 600 nm at intervals of 0 h up to 24 h.

Microscopy analysis. Confocal laser scanning microscopy (CLSM) was performed to analyze the effect of hyperoside on the PAO1 biofilm at 24 h. Prior to the CLSM experiments, cell cultures were divided into control and hyperoside (16 µg/ml)-treated groups. Biofilms on the culture dish were fixed with 2.5% glutaraldehyde at room temperature for 3 h. Following washing with phosphate-buffered saline (PBS), 5 µg/ml propidium iodide (Sigma-Aldrich) was added and the biofilms were incubated for 15 min at 4°C. The plate was then washed, 50 µg/ml fluorescein isothiocyanate-concanavalin A (Sigma-Aldrich) was added and the plate was incubated for 30 min at 4°C. The stained biofilm was then observed using CLSM.

Adhesion assays. Adhesion assays were performed as previously described with minor modifications (22). Following PAO1 activation, the control and hyperoside groups were cultured in a 96-well microtiter plate and incubated for 4 h at 37°C. Following incubation, the attached cells were stained with filtered 1% crystal violet (Sigma-Aldrich) at room temperature for 15 min. The dye was dissolved in 30% glacial acetic acid (Sigma-Aldrich), and the absorbance was measured by xMark Microplate Spectrophotometer at 590 nm.

Motility assays. Twitching motilities were assayed on agar plates (freshly prepared LB agar plates with 1% Bacto agar were used for the twitching assay) in the presence or absence of 16 µg/ml hyperoside (23). An overnight culture was stabbed with a toothpick to transfer PAO1 through the agar layer (point-incubation) to the bottom of the Petri dish and plates were then incubated at 37°C for 48 h. The agar was removed and attached cells were stained with 1% crystal

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer direction	Sequence (5'-3')
lasR	Forward	CTGTGGATGCTCAAGGACTAC
	Reverse	ACCGAACTTCCGCCGAAT
lasI	Forward	CGTGCTCAAGTGTTCAAGGA
	Reverse	GCGTCTGGATGTCGTTCTG
rhIR	Forward	CCGATGCTGATGTCCAACC
	Reverse	GCTACATCGTCGCCATGAG
rhII	Forward	GCTACATCGTCGCCATGAG
	Reverse	TCTCGCCCTTGACCTTCTG
16S	Forward	ATCTTCGGACCTCACGCTATC
	Reverse	CCAAGTTGCTGAACCACTAC

violet (Sigma-Aldrich) at room temperature for 15 min. The plates were washed gently with PBS to remove unattached cells prior to staining. The diameter of the stained zone was measured by graduated scale to assess the twitching motility.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to detect the transcription levels of lasI, lasR, rhII and rhIR genes in *P. aeruginosa* with or without 16 µg/ml hyperoside. The primers used to amplify these genes are listed in Table I. Total RNA was isolated from PAO1 using a FastRNA Pro Blue Kit (MP Biomedicals, Santa Ana, CA, USA). Cells were grown overnight at 37°C in the presence or absence of hyperoside and harvested by centrifugation at 16,100 x g for 10 min, and the deposit was resuspended in TRIzol (Tiangen Biotech Co., Ltd., Beijing, China). Preparation of total RNA was performed according to the manufacturer's protocol. The RNA sample was treated with 50 units of DNase I (Roche Applied Science, Penzberg, Germany) for 2 h at 37°C to remove contaminating DNA. DNaseI was eliminated by phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated H₂O. Reverse transcription was performed using the First Strand cDNA Synthesis kit (Toyobo Co., Ltd., Osaka, Japan) and qPCR using SYBR® Green Real-time PCR Master mix (Toyobo Co., Ltd.) according to the manufacturer's protocol. The reaction procedure involved two-step PCR programme: 94°C for 5 min, (94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec) X40 cycles. The 16S rRNA gene was selected as the internal control to normalize the data. Relative expression of gene (RQ) was calculated by $2^{-\Delta\Delta C_q}$ and percent reduction was calculated as (1-RQ) X 100 (24). Experiments were repeated independently three times.

Statistical analysis. The results are expressed as the mean ± standard deviation and the results from at least three independent experiments were statistically analyzed by one-way analysis of variance, using SPSS 17.0 software (SPSS,

Inc., Chicago, IL, USA). $P < 0.05$ was considered to represent a statistically significant difference.

Results

Effect of hyperoside on *P. aeruginosa* biofilm formation. Hyperoside is a flavonol glycoside with variety of biological activities, including antioxidant, anticancer, antihyperglycemic and anti-inflammatory functions (25-27). However, prior to the current study, hyperoside has demonstrated little antibacterial activity (20,28). The dose-effect experiment of the present study demonstrated that 256 $\mu\text{g/ml}$ hyperoside exhibited no inhibitory effects, and 16 $\mu\text{g/ml}$ hyperoside had the strongest inhibitory effect on *P. aeruginosa* biofilm formation. All doses of hyperoside between 16-64 $\mu\text{g/ml}$ demonstrated biofilm inhibition to an extent (Fig. 1). As hyperoside concentration decreased, (from 64 to 16 $\mu\text{g/ml}$) the inhibition rate of biofilm formation increased. However, the biofilm inhibition activity was not concentration dependent. A similar mode of action has been observed for another traditional medicine component, catechins (29). Although hyperoside does not exhibit antibacterial activity at experimental concentrations, higher hyperoside concentrations may have other pharmacological activities that weaken the effect of biofilm formation.

Effect of hyperoside on growth. A growth curve using 16 $\mu\text{g/ml}$ hyperoside is presented (Fig. 2) and the negligible difference in coincident bacterial growth suggests that the biofilm inhibition effect was completely unrelated to antibacterial activity.

CLSM observation. The inhibitory effect of 16 $\mu\text{g/ml}$ hyperoside was confirmed by CLSM micrographs of the *P. aeruginosa* biofilm. Figure 3 shows that the biofilm of the hyperoside group was sparse (Fig. 3B) compared with the control group (Fig. 3A) and the amount of bacteria and polysaccharide was clearly decreased.

Effect of hyperoside on adhesion. Hyperoside had a significant inhibitory effect on PAO1 cell adhesion, a process involved in initial biofilm formation ($P < 0.05$; Fig. 4). The inhibition of initial adherence by hyperoside suggests that it may decrease and delay new biofilm formation.

Effect of hyperoside on motility. Twitching motility is mediated by type 4 pili (30) and this was inhibited by hyperoside (Fig. 5). Accordingly, twitching motility may be decreased by inhibiting the activity of type 4 pili. The movement of pili is the first stage of biofilm formation. As an essential step for irreversible adhesion, it can affect the morphology and structure of the biofilm. Therefore, hyperoside may inhibit the biofilm formation of *P. aeruginosa* through inhibiting its type 4 pili.

Effect of hyperoside on gene expression. *P. aeruginosa* has two well-studied quorum sensing (QS) systems, las and rhl. The las system is comprised of the transcriptional activator LasR and the autoinducer (AI) synthase LasI. The rhl system is comprised of the transcriptional regulatory protein RhlR and the RhlI AI synthase (31). The las and rhl systems are important for *P. aeruginosa* biofilm development (32,33).

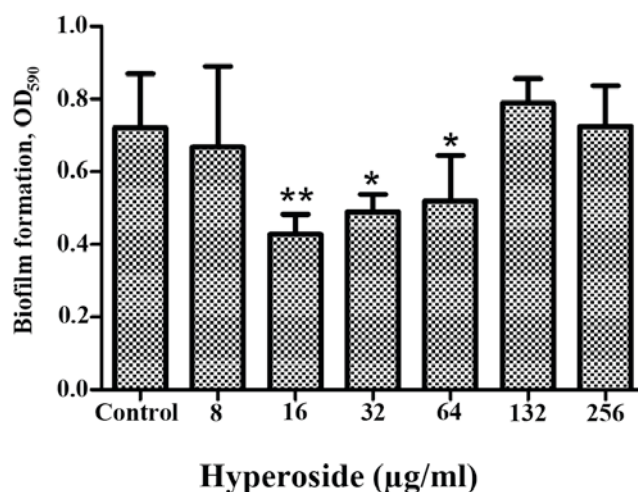


Figure 1. Effect of hyperoside on biofilm formation at different concentrations. Results are presented as the mean \pm standard deviation obtained from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. the control group. OD₅₉₀, optical density at 590 nm.

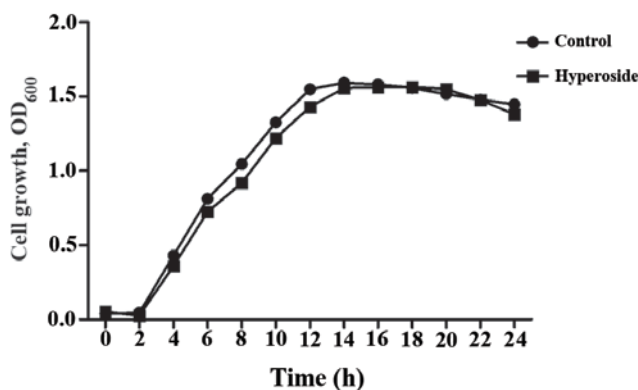


Figure 2. Growth curve of *P. aeruginosa* in the presence or absence of 16 $\mu\text{g/ml}$ hyperoside over 24 h. OD₆₀₀, optical density at 600 nm.

Hyperoside inhibits a multitude of factors involved in biofilm formation; therefore, it may inhibit the QS systems. RT-qPCR was used to detect lasR, lasI, rhlR and rhlI gene expression. The results from RT-qPCR indicated that hyperoside inhibited the expression of the lasR, lasI, rhlR and rhlI genes as the downregulation of transcription in these genes was significant ($P < 0.05$; Fig. 6). Similar effects have been observed regarding other flavonol glycosides in previous studies (34).

Discussion

Bacteria living in biofilms cause 80% of bacterial infections (35). Due to the high antibiotic resistance of biofilms, novel drugs are required to treat biofilm infections. Previous studies have shown that the formation of biofilm can be inhibited with sub-minimum inhibitory concentrations of certain antibiotics, such as imipenem and erythromycin (36,37). However, the long-term use of antibiotics will give rise to drug-resistant bacteria. An increasing number of studies have found that natural products, including baicalin, allicin and garlicin, exhibit an inhibitory effect on biofilms (38,39).

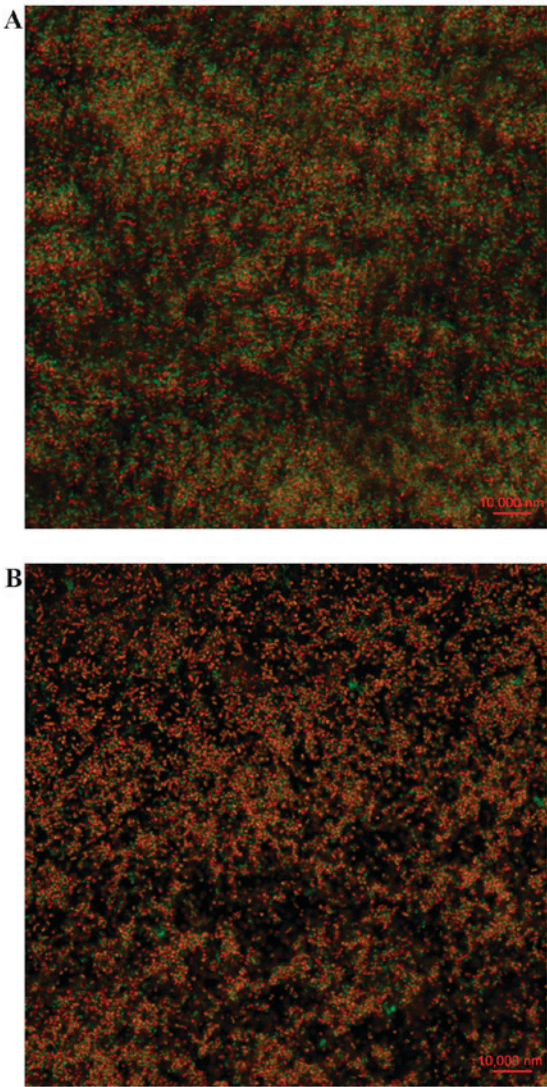


Figure 3. Confocal laser scanning microscopy showing the effect of hyperoside on *P. aeruginosa* biofilm formation. Concanavalin A (green) and PI (red) staining were used to generate the images. (A) CLSM image of untreated PAO1 biofilm. (B) CLSM image of PAO1 biofilm treated with 16 µg/ml hyperoside. CLSM, confocal laser scanning microscopy; PAO1, *Pseudomonas aeruginosa* PAO1.

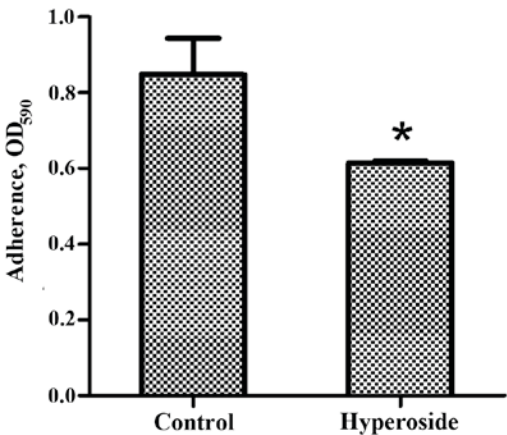


Figure 4. Adhesion ability of *P. aeruginosa* in the presence or absence of 16 µg/ml hyperoside. Results are presented as the mean ± standard deviation as obtained from three independent experiments. *P<0.05 vs. the control group. OD₅₉₀, optical density at 590 nm.

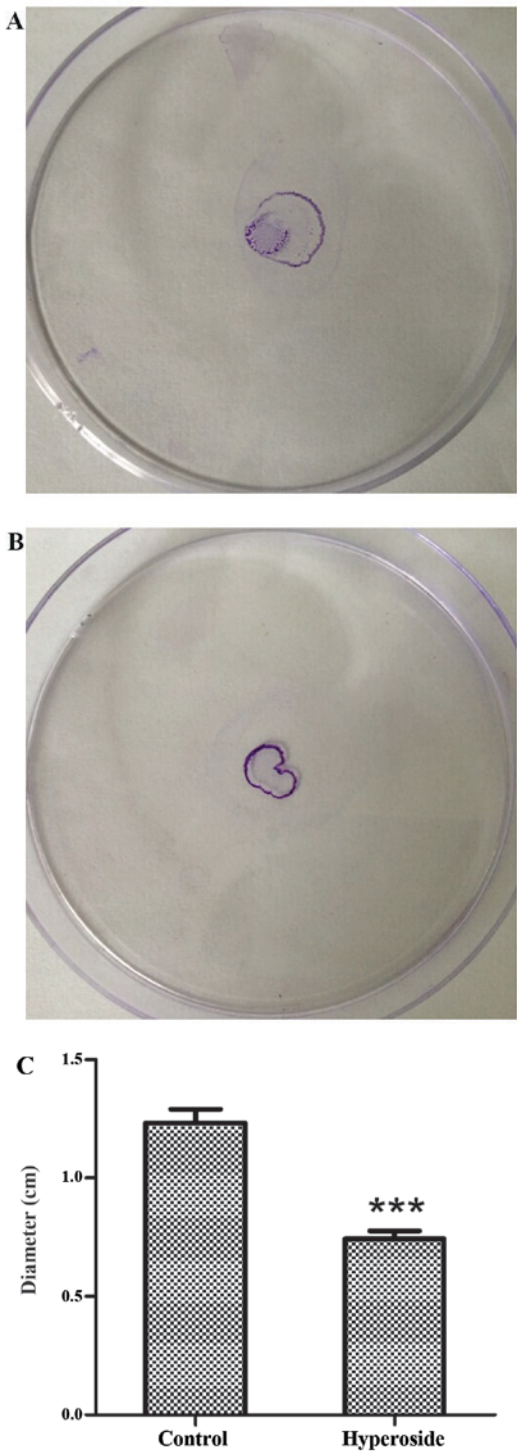


Figure 5. Twitching motility of *P. aeruginosa* standard strain PAO1. (A) Absence of hyperoside and (B) presence of 16 µg/ml hyperoside. (C) Graph indicating the diameter of the stained zone. Results are presented as the mean ± standard deviation obtained from three independent experiments. ***P<0.001 vs. the control group. PAO1, *Pseudomonas aeruginosa* PAO1.

The use of them for biofilm inhibition has been successful and has the potential to identify novel medicines. Currently, there is no natural drug permitted for use in the clinical treatment of biofilm infection. In this study, we demonstrated that hyperoside treatment of PAO1 attenuated biofilm formation, which is related to the QS system. Twitching motility is a

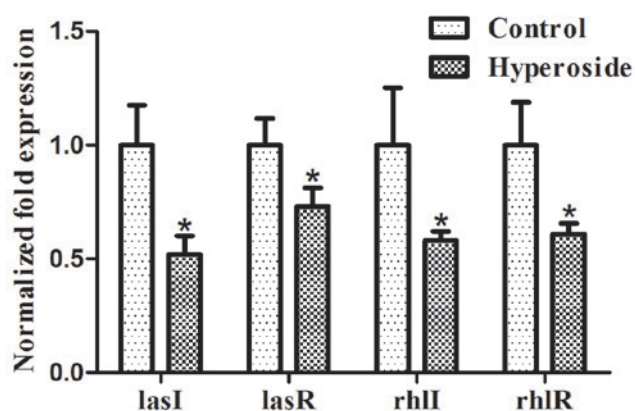


Figure 6. Comparison the transcription levels of *lasI*, *lasR*, *rhlI* and *rhlR* genes related to QS system in *P. aeruginosa* with or without 16 μ g/ml hyperoside. Expression levels were determined by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation. The experiment was repeated three times. * $P < 0.05$ vs. control group.

type IV pili-driven movement by which bacteria can adhere and spread biofilm over a surface (40,41). Type IV pili is also controlled by QS in *P. aeruginosa* (42). In the current study, it was found that hyperoside-treated PAO1 exhibited reduced adherence, as well as twitching motility, suggesting that hyperoside impairs the QS system to inhibit PAO1 adherence, motility and biofilm formation. Additionally, quantitative analysis of gene expression showed that hyperoside inhibited the expression of *lasR*, *lasI*, *rhlR* and *rhlI* genes involved in the QS system, which indicates that hyperoside affected *P. aeruginosa* biofilm formation by repressing the activity of *las* and *rhl* systems.

In view of its structure and function, biofilm is difficult to be removed completely by a single drug treatment. Therefore, drug combinations are often used to overcome biofilm formation, especially antibiotic-antibiotic combinations (43-45). Despite a favorable anti-biofilm effect, the combination of antibiotics can result in the generation of drug resistant bacteria, and enhance the toxicity to humans. Several studies have showed that phytochemicals can potentiate the activity of eradicating biofilm of antibiotics in combination (46-49). The combinations of antibiotic sulfamethoxazole with protocatechuic acid/ellagic acid/gallic acid and tetracycline with gallic acid, cefoperazone with allicin showed synergistic mode of interaction and were highly effective in inhibition *P. aeruginosa* biofilm under *in vitro* conditions (50). Vitexin has been found to potentiate the anti-biofilm activity of azithromycin and gentamicin on *P. aeruginosa* (51). As a type of nature product, whether hyperoside also has synergistic effects with antibiotics that inhibit *P. aeruginosa* biofilm formation requires further study. Although hyperoside exhibits antibacterial properties against *P. aeruginosa in vitro*, its anti-bacterial effects against *P. aeruginosa in vivo* are largely unknown. Defined clinical trials should be conducted to confirm its efficacy. Furthermore, little is known regarding the mechanism of hyperoside, and thus warrants further investigation. Overall, the effect and mechanisms of hyperoside require further assessment, including analysis using *in vivo* techniques.

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