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Flavone Reduces the Production of Virulence Factors, Staphyloxanthin and α-Hemolysin, in *Staphylococcus aureus*

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Abstract *Staphylococcus aureus* is a leading cause of nosocomial infections due to its resistance to diverse antibiotics. This bacterium produces a large number of extracellular virulence factors that are closely associated with specific diseases. In this study, diverse plant flavonoids were investigated to identify a novel anti-virulence compound against two *S. aureus* strains. Flavone, a backbone compound of flavonoids, at subinhibitory concentration (50 μg/mL), markedly reduced the production of staphyloxanthin and α-hemolysin. This staphyloxanthin reduction rendered the *S. aureus* cells 100 times more vulnerable to hydrogen peroxide in the presence of flavone. In addition, flavone significantly decreased the hemolysis of human red blood by *S. aureus*, and the transcriptional level of α-hemolysin gene *hla* and a global regulator gene *sae* in *S. aureus* cells. This finding supported the usefulness of flavone as a potential antivirulence agent against antibiotic-resistant *S. aureus*.

Introduction

Staphylococcus aureus is an important human pathogen that often exhibits antibiotic resistance and is responsible for worldwide outbreaks of nosocomial infections [14]. This pathogen can secrete several exotoxins, such as hemolysin, enterotoxins, coagulase, TSST-1, and protein A, which are associated with specific diseases [16]. *S. aureus* strains are also capable of producing the golden carotenoid pigment, staphyloxanthin that acts as a virulence factor, primarily being a bacterial antioxidant which protects the pathogen from the host's immune system in the form of reactive oxygen species [3, 13].

Over the past several decades, numerous antibiotics have been developed and used for bacterial infections. However, there has been a significant decrease in the rate of discovery of new antibiotics [13]. Furthermore, current usage of bactericidal compounds is often unsuccessful because of the emergence of methicillin-resistant *S. aureus* [2, 12]. Hence, unlike antibiotics that mostly aim to inhibit cell growth, alternative approaches such as antivirulence compounds have attracted strong research interest. The antivirulence approach aims to reduce the production of virulence factors without affecting bacterial growth to impede the possible emergence of drug resistance [2, 7].

Major discoveries in the antivirulence approach against *S. aureus* include the inhibition of (i) the virulence factor staphyloxanthin [13], (ii) enterotoxins and hemolysins [24] (iii) antibiotic resistant biofilm formation [1, 8, 9], and (iv) bacterial quorum sensing [18]. Recently, several plant compounds have been reported to decrease the virulence of *S. aureus* without affecting its growth. For example, thymol found in thyme [20] reduced enterotoxins and α-hemolysin production; luteolin [19] and chrysin [23] reduced α-hemolysin production; and fisetin [5] and olelic acid [22] inhibited the biofilm formation in *S. aureus*.

The overall aim of this study was to identify novel and potent antivirulence compounds from the screening of plant flavonoids against *S. aureus*. We investigated the effects of 12 flavonoids on the production of virulence factors, such as staphyloxanthin and α-hemolysin in *S. aureus*. Among the tested flavonoids, a subinhibitory concentration of flavone was identified as the most potent

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antivirulence compound without antimicrobial activity. This is the first article reporting on the use of flavone to reduce the production of both staphyloxanthin and α-hemolysin of *S. aureus*.

Materials and Methods

Bacterial Strains and Chemicals

All experiments were conducted at 37 °C, and trypticase soy broth (TSB) was used for the cultures of *S. aureus* (ATCC 25923) and *S. aureus* (ATCC 6538), which were obtained from the Korean Agricultural Culture Collection. Two *S. aureus* strains were used to reinforce our findings. Chemicals including 12 flavonoids (flavone (99 %), 6-aminoflavone (97 %), 6-hydroxyflavone (98 %), apigenin (97 %), chrysin (97 %), curcumin (94 %), daidzein (98 %), fisetin (98 %), genistein (98 %), luteolin (98 %), phloretin (99 %), and quercetin (98 %)) were purchased from Sigma-Aldrich Co. (Missouri, USA). The structures of the flavonoids are shown (Fig. 1). All the 12 flavonoids were dissolved in dimethyl sulfoxide (DMSO).

Bacterial Culture

Staphylococcus aureus strains were initially streaked from −80 °C glycerol stock on LB plates, and a fresh single colony was inoculated in TSB (2 mL) in 14-mL tubes and cultured at 37 °C and 250 rpm for all experiments except cell growth measurement. Overnight cultures were re-inoculated at 1:100 dilution in the medium. For cell growth measurements, a fresh single colony was inoculated in TSB (25 mL) contained in 250-mL flasks, and cultured at 37 °C and 250 rpm, and optical densities were measured at 600 nm using a spectrophotometer (UV-160, Shimadzu, Japan). Each experiment was performed with at least two independent cultures.

Staphyloxanthin Assay

The bright golden coloration of this virulence factor facilitates the anti-virulence screening by the simple observation of color change [6]. Also, a quantitative carotenoid assay method was adapted from the previous method [15]. In brief, cells were re-inoculated at 1:100 dilution in TSB medium and incubated for 16 h at 37 °C with or without flavonoids. Cells (1 mL) were then collected by centrifugation at 16,600×*g* for 1 min and washed with 1 ml of phosphate-buffered saline (PBS). At this point, cell pellets were photographed to compare the staphyloxanthin production. For the extraction of carotenoid pigments, the cell pellets were resuspended in 0.2 mL of methanol by vortexing, and this mixture was heated at 55 °C for 3 min. Pigment extraction was separated from cell debris by centrifugation at 16,600×*g* for 10 min. This pigment extraction step was repeated 3 times, and the optical densities of collected extractions were measured at 465 nm using a spectrophotometer (UV-160, Shimadzu, Japan). Each data point was averaged from at least three independent cultures.

Hydrogen Peroxide Resistance Assays

The resistance assay (survival test) with hydrogen peroxide was adapted from previous study [13]. Overnight cultures grown for 16 h in TSB were re-grown to mid-log phase in TSB (turbidity at 600 nm of 1). Then, 0.1 mL of each culture was incubated with H_2O_2 at a final concentration of

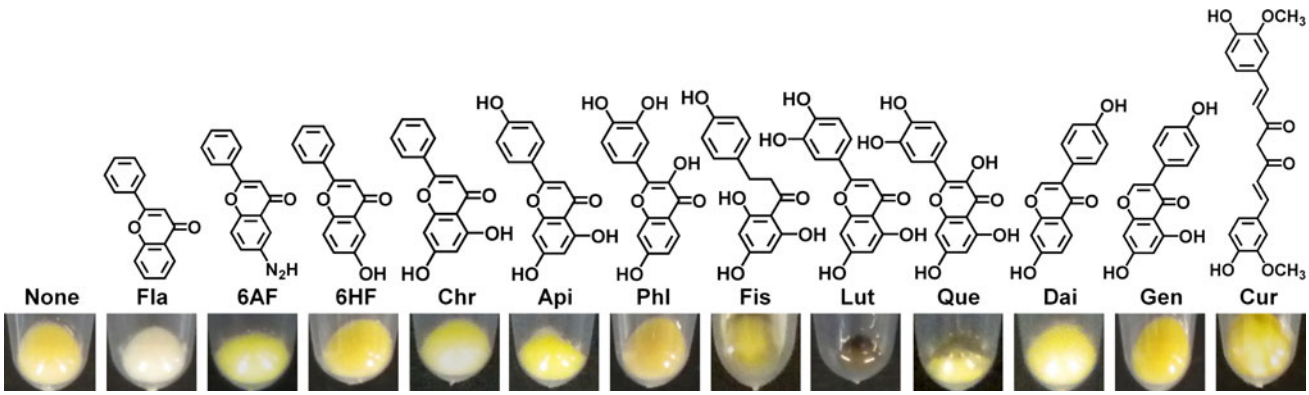

Fig. 1 Effect of flavonoids on the production of staphyloxanthin in *S. aureus*. *S. aureus* (ATCC 25923) was used. Cell pellets of 16-h grown *S. aureus* with or without flavonoids were observed for the production of staphyloxanthin. *Fla* flavone, *6AF* 6-aminoflavone, *6HF*: 6-hydroxyflavone, *Chr* chrysin, *Api* apigenin, *Phl* phloretin, *Fis* fisetin, *Lut* luteolin, *Que* quercetin, *Dai* daidzein, *Gen* genistein, and *Cur* curcumin. All flavonoids were used at 50 μg/mL, except luteolin, which was used at 25 μg/mL because of its antimicrobial activity. All the compounds were dissolved in DMSO. DMSO was used as a control. The structures of the flavonoids are shown. The experiment was done in triplicate, and representative images are shown

Table 1 Primer sequences for quantitative RT-PCR

Gene	Name	Primer
hla	Alpha-hemolysin	Forward 5′-CGG CAC ATT TGC ACC AAT AAG GC-3′
		Reverse 5′-GGT TTA GCC TGG CCT TCA GC-3′
sae	Histidine protein kinase	Forward 5′-CGT ACA TTC AGA GTA GAA AAC TCT CGT AAT AC-3′
		Reverse 5′-GTT GCG CGA GTT CAT TAG CTA TAT AT-3′
agr	Quorum-sensing regulator	Forward 5′-GTG AAA TTC GTA AGC ATG ACC CAG TTG-3′
		Reverse 5′-TGT AAG CGT GTA TGT GCA GTT TCT AAA C-3′
sigB	RNA polymerase sigma factor	Forward 5′-TCA CTG ATA GAA GGT GAA CGC TCT-3′
		Reverse 5′-AGT GAG CGA TGA ACT AAC CGC-3′
sar	Biofilm regulator	Forward 5′-GAG TTG TTA TCA ATG GTC-3′
		Reverse 5′-GTT TGC TTC AGT GAT TCG-3′
seo	Enterotoxin O	Forward 5′-AGT CAA GTG TAG ACC CTA TT-3′
		Reverse 5′-AGA TAT TCC ATC TAA CCA AT-3′
16 s rRNA	A component of ribosomes	Forward 5′- TGT CGT GAG ATG TTG GG-3′
		Reverse 5′-CGA TTC CAG CTT CAT GT-3′

1.5 % (v/v) for 60 min with shaking at 250 rpm. The percentage of cells surviving the stresses was calculated as the number of colony-forming units (CFU)/mL remaining after each stress divided by the initial CFU/mL. Three independent experiments were conducted.

Hemolysis Assay

Hemolysis analysis was modified from the previous method [10]. The lysis efficacy of human red blood cells was measured with whole cultures of *S. aureus* grown in the presence of flavonoids. In brief, *S. aureus* cells were diluted at 1:100 with an overnight culture in TSB and cultured with or without all flavonoids at 50 μg/mL except luteolin at 25 μg/mL at 37 °C for 16 h with shaking at 250 rpm. The cell cultures (50 μL including cells and culture supernatant) were added into diluted human red blood cells that had previously been separated by centrifugation at 900×*g* for 5 min, washed with PBS buffer three times and diluted at 3 % of red blood cells in PBS buffer. For hemolytic activity, the mixture was incubated at 37 °C for 1 h with 250 rpm shaking. The supernatant was collected by centrifugation at 16,600×*g* for 10 min, and the optical density was measured at 543 nm.

RNA Isolation and Real-time qRT-PCR

Staphylococcus aureus (ATCC 25923) was cultivated in TSB with or without flavone for 16 h at 250 rpm. Before taking samples, RNase inhibitor (RNAlater, Ambion, TX, USA) was added, and cells were immediately chilled for 30 s with dry ice and 95 % ethanol (to prevent RNA degradation) before centrifugation at 13,000×*g* for 2 min. The cell pellets were immediately frozen with dry ice and stored at −80 °C. Total RNA was isolated using a Qiagen RNeasy mini Kit (Valencia, CA, USA). To remove all DNA, the purified RNA was treated for 15 min with 30 Units of DNase I. To investigate the transcriptions of *hla* (α-hemolysin gene), *sae* (a global regulator), *agrA* (quorum-sensing gene), *sar* (accessory regulator A), *sigB* (RNA polymerase sigma factor), and *seo* (enterotoxin), they were quantified using qRT-PCR. The primer pairs for qRT-PCR are presented in Table 1. The 16S rRNA housekeeping gene was used. Real-time qRT-PCR was performed using the StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA) and SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA).

Results

Investigation of Flavonoids for Staphyloxanthin Reduction in *S. aureus*

To investigate the antivirulence activity against *S. aureus*, (ATCC 25923), 12 flavonoids at subinhibitory concentrations (10, 25, and 50 μg/mL) were screened for the reduction of staphyloxanthin production. The golden pigment staphyloxanthin could be visually identified in the cell pellets of *S. aureus*. Among the tested flavonoids, flavone, which is the backbone compound of flavonoids, most significantly reduced the staphyloxanthin production in *S. aureus* (Fig. 1). Quantitative analysis also clearly indicated that flavone reduced the staphyloxanthin production by tenfold compared with non-treatment control (data not shown). In the case of luteolin, its antimicrobial activity at 25 μg/mL [19] reduced the cell growth and

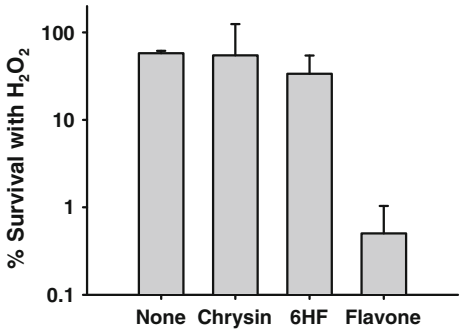

Fig. 2 Effect of flavonoids on hydrogen peroxide resistance. The survival of *S. aureus* (ATCC 25923) with or without flavonoids (chrysin, 6-hydroxyflavone (6HF), and flavone) was measured after H_2O_2 (1.5 %, v/v) treatment for 60 min. Flavonoids were used at 50 μg/mL. The percentage of survive cells was calculated as the number of colony forming units (CFU) per ml remaining after the H_2O_2 stress divided by the initial CFU per ml. The experiment was performed in triplicate

staphyloxanthin production. The result of staphyloxanthin inhibition by flavone was similar to those in another *S. aureus* strain (ATCC 6538) (data not shown).

Flavone Reduced Hydrogen Peroxide Resistance

Staphyloxanthin acts as an antioxidant by enabling the detoxification of host-immune system-generated ROS such as oxygen radical (O^{2-}) and hydrogen peroxide (H_2O_2) [13]. Hence, we examined the effect of flavone on the survival rate of *S. aureus* in the presence of H_2O_2. As expected, flavone reduced H_2O_2 susceptibility by 100-fold, while structurally similar chrysin and 6-hydroxyflavone had no or much less effect on the survival rate (Fig. 2). This result was attributed to the effect of flavone in reducing staphyloxanthin production in *S. aureus.*

Flavone Reduced Hemolysis by *S. aureus* without Affecting the Growth of Planktonic Cells

As *S. aureus* can produce α-hemolysin, which is a pore-forming cytotoxin and causes hemolysis, we investigated the effect of flavonoids on blood hemolysis by *S. aureus*. Among the 12 tested flavonoids, eight showed a significant antihemolytic activity (Fig. 3). This result is consistent with that of the previous study [19] in that luteolin at subinhibitory concentration abolished the hemolysis activity of *S. aureus*. Moreover, seven more flavonoids, flavone, 6-aminoflavone, 6-hydroxyflavone, apigenin, phloretin, fisetin, and genistein, also markedly reduced hemolysis activity at their subinhibitory concentrations. Particularly, flavone clearly and dose-dependently inhibited the hemolytic activity of two *S. aureus* strains after 16-h culture (Fig. 4a, b). The reduction of hemolytic activity by flavone was similar at the different culture-time points, such as 12 and 24 h (data not shown).

A potential antivirulence compound without antimicrobial activity is preferred as this avoids the possible development of bacterial drug resistance. Thus, the toxicity of flavone was investigated by measuring the growth of planktonic *S. aureus* cells. Although flavone at 50 μg/mL slightly delayed the cell growth of two *S. aureus* strains, the growth was recovered at 14 h (Fig. 4c, d). In addition, the specific growth rates have been measured. In the absence of flavone, the specific growth rates of *S. aureus* ATCC 25923 and ATCC 6538 were 1.35 ± 0.18/h and 1.23 ± 0.21/h, whereas the growth rates were 1.05 ± 0.12/h and 1.22 ± 0.21/h with flavone at 50 μg/

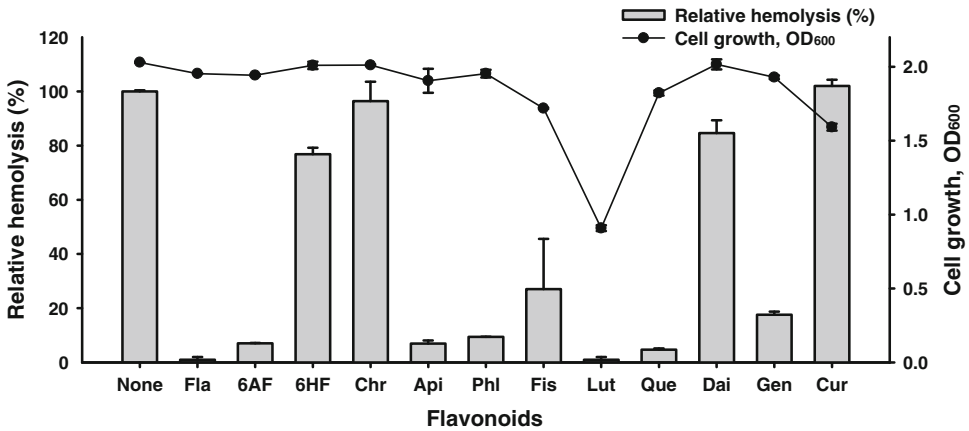

Fig. 3 Effect of flavonoids on hemolysis. The hemolysis screening was performed using human red blood cells upon adding *S. aureus* (ATCC 25923) cultures (50 μL) grown with flavonoids for 16 h. *Fla* flavone, *6AF* 6-aminoflavone, *6HF* 6-hydroxyflavone, *Chr* chrysin, *Api* apigenin, *Phl* phloretin, *Fis* fisetin, *Lut* luteolin, *Que* quercetin, *Dai* daidzein, *Gen* genistein, and *Cur* curcumin. All flavonoids were used at 50 μg/mL, except luteolin, which was used at 25 μg/mL because of its antimicrobial activity

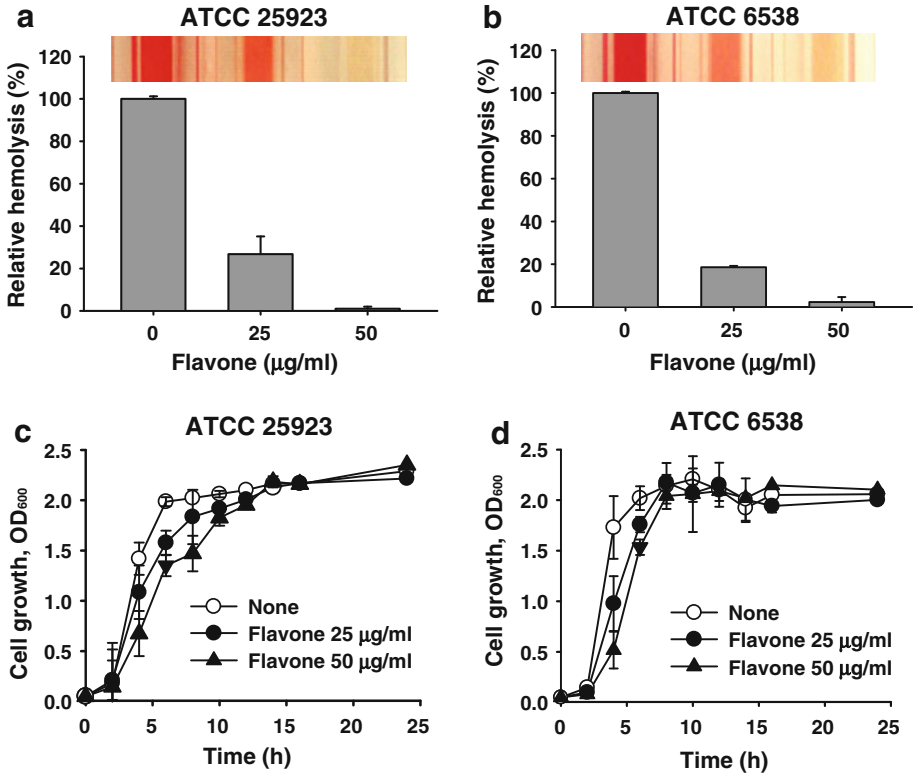

Fig. 4 Effect of flavone on hemolysis and cell growth in two *S. aureus* strains. The hemolysis assay was performed using human red blood cells upon adding two *S. aureus* (ATCC 25923 and ATCC 6538) cultures (50 μL) grown with flavone (0, 25, and 50 μg/mL) for 16 h. *Pictures* of the spectrophotometer cuvettes are shown for the hemolysis activity. Planktonic cell growth of *S. aureus* was measured at 600 nm in 250 mL-flasks with 250 rpm

mL, respectively. Furthermore, the numbers of viable cells were not significantly affected by flavone at 50 μg/mL (data not shown). The overall growth data indicated that the reduction of staphyloxanthin and antihemolytic activity of flavone was due to its antivirulence activity rather than antimicrobial activity.

Flavone Repressed the Transcription of α-Hemolysin

To investigate the mechanism of flavone's antivirulence activity, real-time qRT-PCR was used to determine a differential expression of virulence factor-related genes, such as *hla* (α-hemolysin gene), *sae* (a global regulator inducing *hla* [17], and *agrA* (quorum-sensing gene), in *S. aureus* cells with and without flavone. Flavone clearly repressed the transcription of *hla* by 11-fold and *sae* by fourfold (Fig. 5), which supports the reduction of hemolysis in *S. aureus* cells by flavone (Fig. 4). However, flavone elevated *agrA* transcription by fourfold and did not change the transcription of other virulence factor genes such as *sar*, *sigB* and *seo* (Fig. 5). The results support the previous finding that the *agr* and *sae* might be inhibiting each other [17].

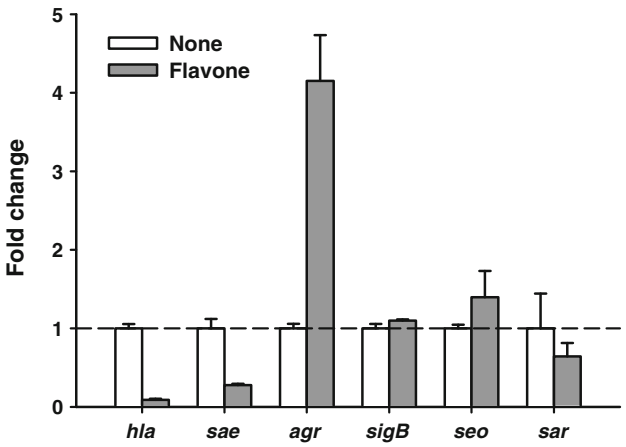

Fig. 5 Transcriptional profiles of *S. aureus* cells in the presence of flavone. Flavone was used at 50 μg/mL. Transcriptional profiles were measured by qRT-PCR. *Fold change* represents the change (*n*-fold) in transcription compared to the data in the absence of flavones (*white bars*, value of 1.0). The experiment was performed in duplicate

Discussion

In this study, we utilized dual screening to inhibit various virulence factors, such as staphyloxanthin and α-hemolysin, in *S. aureus*. Among 12 plant flavonoids, flavone reduced the production of staphyloxanthin, H_2O_2 resistance, and blood hemolysis without inhibiting the planktonic growth of *S. aureus*. This article is noteworthy as it is the first one to report on the use of flavone to reduce both the hemolytic ability and staphyloxanthin production of *S. aureus* (Figs. 1, 3, and 4).

Flavonoids are ubiquitous in plants and are commonly found in fruit, vegetables, nuts, seeds, stems, and flowers.

They are biologically active in combating diseases in humans because of their diverse biological functions, such as antioxidative, antifungal, antiviral, antibacterial, and anticarcinogenic activities [4]. As the daily dietary intake of mixed flavonoids is estimated to be in the range of 500–1,000 mg [21], they are likely to have minimal toxicity to humans [4], but further study is warranted to confirm this. Recently, the flavonoids luteolin [19] and chrysin [23] at subinhibitory concentrations showed an ability to inhibit the hemolysis of *S. aureus*, and fisetin reduced the antibiotic-resistant biofilm formation in *S. aureus* [5], which demonstrated the potential antivirulence activity of these flavonoids. Compared with luteolin, chrysin, and fisetin, flavone specifically reduced the virulence factor of staphyloxanthin and the H_2O_2 resistance. Therefore, the present results have expanded the scope of previous studies and demonstrated that the functional groups of flavonoids differentially control several virulent phenotypes of *S. aureus*. Flavone is the simplest form among flavonoids used in this study. Although it is speculative, only this simple flavone can be easily transported into *S. aureus* cells and bound to regulatory proteins, while other larger flavonoids may have a transport problem into cells and have a less binding affinity to some regulatory proteins. Further investigation is required to understand how flavone rather than other larger analogs specifically works in *S. aureus* cells.

The expansion in bacterial resistance to antibiotics has created an urgent need for effective antimicrobial agents as well as antivirulence compounds against pathogenic bacteria. In this study, the dual screening of 12 flavonoids for two virulence factors was performed against *S. aureus*, and flavone demonstrated potential as a new potent antivirulence compound. Although the exact action mechanisms of flavone's antivirulence activity remains to be determined, the results suggest that the screening of a larger library of flavonoids will generate more potent therapeutics for the human pathogen *S. aureus*, and possibly for other pathogens as well. Recently, the flavonoid phloretin, which is abundant in apples, reduced the attachment of *Escherichia coli* O157:H7 to human colonic epithelial cells and also diminished colon inflammation in a rat model [11]. Therefore, natural flavonoids are important sources for antivirulence compounds and flavone can be used as a basic structure in the design of potent antivirulence drugs.

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