



FULL PAPER

Bacteriology

Phillyrin is an effective inhibitor of quorum sensing with potential as an anti-*Pseudomonas aeruginosa* infection therapy

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ABSTRACT. In the present study, we evaluated the antibacterial and anti-quorum sensing qualities of phillyrin. The minimum inhibitory concentration (MIC) of phillyrin with regard to *Pseudomonas aeruginosa* is 0.5 mg/ml. The production of virulence factors—such as rhamnolipid (>78.69%), pyocyanin (>85.94%), and elastase (>89.95%)—that affect the pathogenicity of the *P. aeruginosa* strain PAO1 apparently declined in the presence of 0.25 mg/ml phillyrin. Biofilm formation decreased by 84.48%. In a *Caenorhabditis elegans*–*Pseudomonas aeruginosa* infection model, diseased worms lived longer (63.33%) in a phillyrin-containing medium than in a drug-free medium, and the drug did not directly kill the pathogen. Therefore, the present work suggests that phillyrin has potential as an antimicrobial agent for the control of infectious pathogens.

KEY WORDS: *Caenorhabditis elegans*, phillyrin, *Pseudomonas aeruginosa*, quorum sensing inhibitor, virulence factor

Quorum sensing (QS) is the ability of microbes to monitor their population density and control gene expression. QS controls a wide variety of physiological processes including bioluminescence, competence, antibiotic biosynthesis, motility, plasmid conjugal transfer, and biofilm maturation [18]. Numerous pathogens exhibit markedly reduced virulence in infection models when their QS systems are disrupted by mutagenesis [12]. QS systems have been used as effective targets for antibiotics used to treat microbial infection. The enzymes that incapacitate QS signals are often called QQ enzymes, and the chemicals that disrupt QS pathways are known as QS inhibitors (QSIs) [8]. Sources of natural QSIs include diverse species of higher plants from all continents. They comprise many medicinal plants, vegetables, and edible fruits [13, 25]. However, the majority of studies deal with plant extracts from which QSI molecules have rarely been isolated. The present study, we investigated the anti-QS activity of phillyrin, which is one of the main active components found in *Forsythia suspense*.

Forsythia suspense [(Thunb.) Vahl (Oleaceae)] is an ascending plant that is widely dispersed throughout China, Korea, Japan, and many European nations, and is a well-known ingredient of traditional Chinese medicine. Many Chinese medicines—such as Shuanghuanglian oral solutions, Yinqiao Jiedu tablets, and Qinlian tablets—contain *F. suspense*. A number of chemical constituents with diverse structures—including phenylethanoid glycosides, lignans, and flavonoids—have been reported from species of this genus [26, 31]. Phillyrin is an active constituent of *F. suspense*. Researchers have found that phillyrin has anti-obesity activity *in vivo* [6]. However, there have been no studies on the bioactive potential of phillyrin as an anti-QS drug. Therefore, owing to the ethnopharmacological profile of phillyrin, we attempted to assess its anti-QS potential with regard to PAO1, an important pathogenic strain of the bacterium *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Drug preparation

We produced a stock solution of phillyrin (purity >98%; obtained from Sigma-Aldrich, Carlsbad, CA, U.S.A.) by dissolving 20 mg in 1% dimethyl sulfoxide (DMSO), made up to a final volume of 1 ml and a concentration of 20 mg/ml, and stored it at –20°C until required.

Bacterial strains and *Caenorhabditis elegans* cultivation

We used *P. aeruginosa* PAO1, *Escherichia coli* OP50, and *Chromobacterium violaceum* ATCC 12472 standard strains in the present study. Luria–Bertani (LB) broth was used for microbial culture and maintenance. PAO1 and OP50 were pre-cultured

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aerobically in LB broth at 30°C, and were maintained on LB agar plates at room temperature at 25°C for short-term and long-term storage in LB broth containing 20% glycerol at -70°C. The microbial density was monitored by spectrophotometrically measuring the optical density at 600 nm (OD₆₀₀) until it reached 0.4.

We cultured the *C. elegans* nematode worms according to the method described by Puiac *et al.* [22]. Solid plates containing nematode growth medium (NGM; 1.2%) were seeded with *E. coli* OP50 and incubated overnight at 30°C. We maintained the wild-type *C. elegans* N2 strain nematode worms on non-pathogenic *E. coli* strain OP50 microbes, which are uracil auxotrophs, at 20°C. To obtain highly synchronized larvae without bleaching, we isolated the eggs by treating the gravid adults with hypochlorite ("bleaching"). The eggs were seeded on NGM agar and allowed to grow into sterile young adult worms at 25°C. The worms were then used for the infection assay.

Susceptibility testing and qualitative detection of anti-QS activity

We produced serial twofold dilutions of phillyrin in LB broth to assess its bactericidal properties. Each tube was inoculated with 10 μ l of the standardized inoculum. After incubation at 37°C for 24 hr, we calculated the minimum inhibitory concentration (MIC), i.e., the lowest concentration of phillyrin that completely inhibited visible growth.

C. violaceum ATCC 12472 is used as a biosensor strain to detect anti-QS activity [2]. We inoculated 10 ml of molten LB agar (0.5% w/v) with 100 μ l of *C. violaceum* 12472 grown overnight in LB broth. The agar-culture solution was immediately poured over the surface of the LB agar plates. Subsequently, 2-mm wells were punched through the agar and filled with 50 μ l of $1/2 \times$ MIC phillyrin. We incubated the plates for 24 hr at 30°C and examined them for the production of violacein pigment. Violacein inhibition was assessed by measuring the diameter of the yellowish opaque halo surrounding each well (indicating bacterial growth) in the absence of the purple violacein pigmentation of the bacterial lawn (indicating QS inhibition). We used 50 μ l of 1% DMSO as a negative control.

Quantitative assessment of *P. aeruginosa* virulence factors

We added phillyrin ($1/2 \times$ MIC, $1/4 \times$ MIC, or $1/8 \times$ MIC) to test-tubes containing LB broth. *P. aeruginosa* that had been cultured overnight was inoculated into the drug-supplemented LB broth. We centrifuged 1 ml of each culture at 12,000 rpm for 5 min, and tested the resulting supernatants *in vitro* for inhibition of *P. aeruginosa* virulence factors (pyocyanin, rhamnolipid, and elastase).

Pyocyanin Inhibition: A rapid and accurate chemical technique was used to determine the pyocyanin inhibition ratio. We extracted the pyocyanin from 2 ml of each cell-free supernatant with 1.5 ml of chloroform, then mixed the chloroform layer with 2 ml of 2 M HCl and measured the absorbance at 520 nm (OD₅₂₀) in acidic solution using a UV spectrophotometer [24].

Rhamnolipid assays: We implemented the method described by Chandrasekan *et al.* with some modifications, to study the efficiency of rhamnolipid inhibition by phillyrin *in vitro* [4]. Briefly, 1 ml of each supernatant was extracted with 1 ml of diethyl ether and vortexed immediately. The diethyl ether layer was pooled and evaporated to dryness using a vacuum centrifuge; 100 μ l of sterile H₂O was then added to each extract. We then added 800 μ l of 0.16% orcinol (in 70% H₂SO₄) to each 100- μ l sample. We maintained the reaction at 80°C for 30 min and then measured the OD of each reaction sample at 495 nm.

Elastase assays: We added 100 μ l of each supernatant to 900 μ l of ECR buffer (100 mM Tris and 1 mM CaCl₂ (pH 7.5) containing 20 mg of ECR (Sigma)), and incubated the mixture at 37°C for 3 hr while rotating slowly. The assay tubes were centrifuged at low speed to remove insoluble material. We then determined the concentration of elastase by measuring the OD at 495 nm using a spectrophotometer [21].

Biofilm formation assays

We carried out biofilm formation assays by labelling with crystal violet (CV; 0.1% (w/v) in water) as previously described with modifications [33]. Briefly, cultures of PAO1 were treated overnight with sub-MIC concentrations of phillyrin, or were left untreated. We then washed the samples with sterile water to remove the cells and added 0.1% CV solution. The excess dye was removed by washing with deionized water and the absorbed dye was dissolved in 95% ethanol. We quantified biofilm formation by measuring the absorbance at 650 nm of the ethanol solutions obtained.

Swimming and twitching motility assays

Swimming motility was determined on 0.3% LB agar (Merck, Shanghai, China) plates. We injected 0.5 μ l of the standardized culture below the surface of the agar, and incubated the plates overnight at 30°C. Twitch assays were conducted on 1% LB agar plates. We inoculated the bacteria by picking a colony from each LB plate that had been cultured overnight, using a sterile tip. The tip was used to inoculate the bacteria at the bottom of each plate, which was then incubated at 37°C for 24 hr. We measured the zone of motility on each plate [3] and recorded it photographically using a Canon EOS 5D Mark IV camera.

Caenorhabditis elegans–*Pseudomonas aeruginosa* infection model

We assessed the mortality of *C. elegans* nematode worms living on a lawn of *P. aeruginosa* PAO1 in the presence of 0.25 mg/ml of phillyrin. The ability of phillyrin to ensure the survival of infected nematodes was assessed by comparing the survival rate in a phillyrin-treated nematode population to that in a non-treated population [28]. A non-treated population was used as a control. We counted the nematodes at 5-hr intervals over 24 hr using a microscope [30]. To assess the nematodes, we compared their swallowing rates in the different experimental groups for 1 min [16]. To obtain scores for total progeny (brood size) and male self-

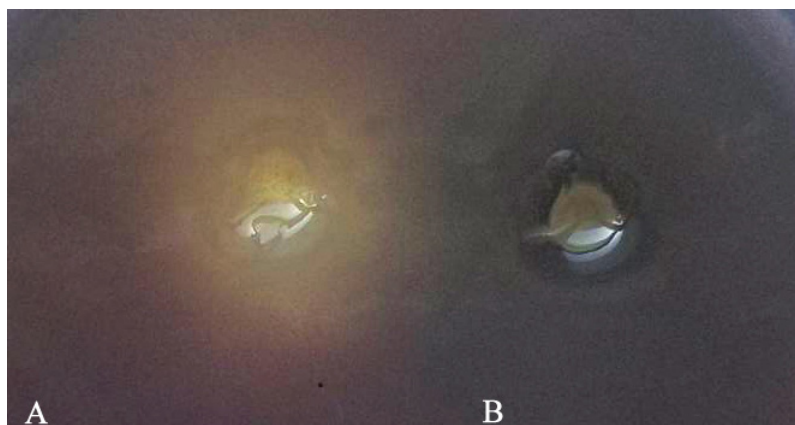


Fig. 1. Assessment of the anti-quorum sensing (anti-QS) properties of phillyrin by subjecting the reporter strain *Chromobacterium violaceum* ATCC 12472 to a diffusion assay. (A) With 0.125 mg/ml phillyrin; (B) with dimethyl sulfoxide (DMSO).

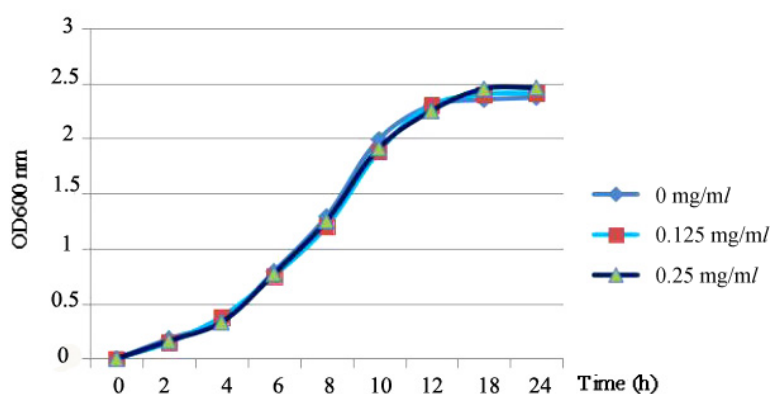


Fig. 2. Growth curve analysis of the influence of phillyrin on the growth of *Pseudomonas aeruginosa*. *P. aeruginosa* strain PAO1 was grown in the presence of 0, 0.125, and 0.25 mg/ml phillyrin.

progeny, we transferred L4 adult nematodes to individual NGM plates seeded with or without PAO1, and allowed them to lay eggs for 5 days, transferring them to new plates every day. We counted the eggs laid on each plate after removing the parent [29].

Statistical analysis

The data were statistically analyzed using the GraphPad (Prism 5) program. The data are presented as the mean \pm standard deviation of three replicate assays. We carried out analysis of variance (ANOVA) ($P \leq 0.05$) to determine significant differences between treatments. The letters indicate significant differences between means ($P < 0.05$).

RESULTS

Anti-bacterial and anti-quorum sensing activity of phillyrin

A 0.5 mg/ml DMSO solution of phillyrin had no visible effect on *P. aeruginosa*, but a 0.25 mg/ml solution did. Therefore, the MIC of phillyrin with regard to *P. aeruginosa* is 0.5 mg/ml. The MIC of phillyrin with regard to *C. violaceum* ATCC 12472 was also 0.5 mg/ml. We used $1/2 \times \text{MIC}$ for anti-QS detection. The colorless and opaque halos without purple pigmentation around the bacterial colonies clearly indicated QS inhibition (Fig. 1). Phillyrin had an effect on the planktonic cell growth of *P. aeruginosa* PAO1 at sub-inhibitory concentrations. The growth of PAO1 was unaffected by phillyrin at sub-MIC concentrations (Fig. 2).

Pyocyanin inhibition

Virulence factors play a vital role in the pathogenesis of *P. aeruginosa* by assisting its successful infection of its host. We defined 100% pyocyanin production as that taking place in an untreated *P. aeruginosa* culture. Phillyrin concentrations of 0.25, 0.125, and 0.0625 mg/ml reduced pyocyanin secretion by up to 85.94, 65.16, and 50.17%, respectively (Fig. 3A). The reduction in pyocyanin levels resulted in an attenuation of microbial virulence.

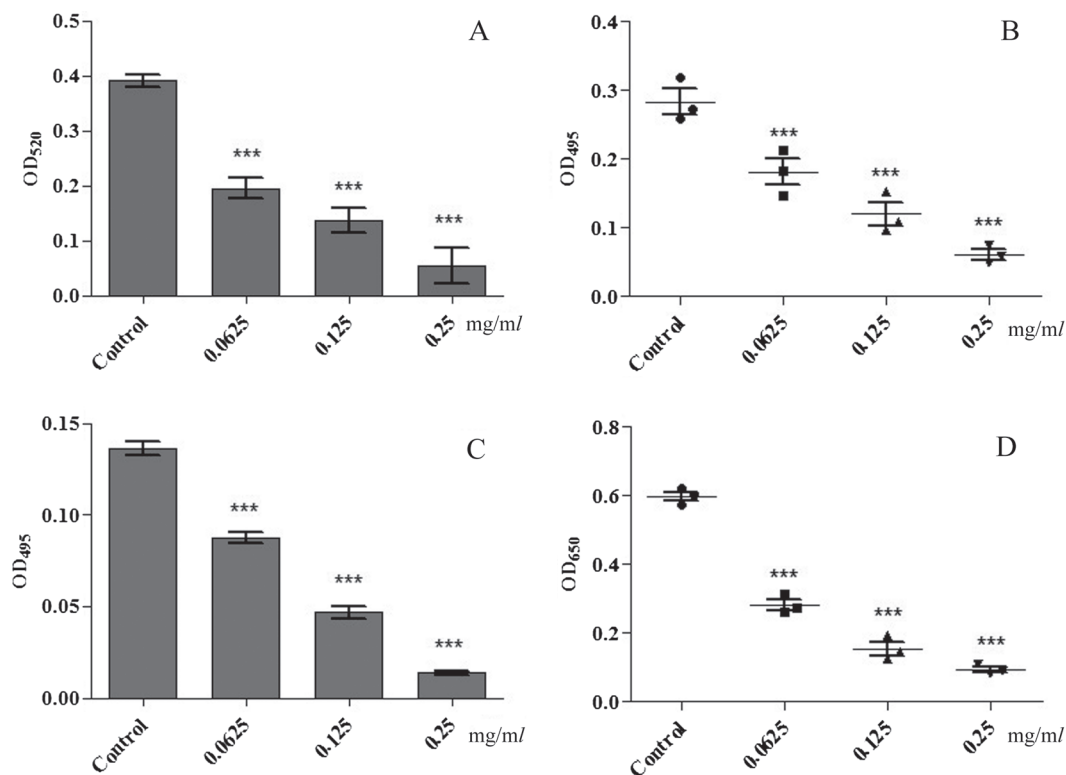


Fig. 3. Yields of virulence factors pyocyanin (A), rhamnolipid (B), and elastase (C), and significantly reduced biofilm formation (D) in phillyrin-treated *Pseudomonas aeruginosa* compared to the untreated control ($P < 0.01$). Error bars represent the standard error of three independent experiments ($n = 3$).

Rhamnolipid assays

Both the *rhl* and *rhlAB* (encoding a rhamnosyltransferase) QS systems are required for rhamnolipid production in PAO1 [21]. Consistent with the suppression of QS activity, 0.25 mg/ml phillyrin controlled the production of rhamnolipid, as evidenced by the 78.69% reduction ($P < 0.01$) (Fig. 3B).

Elastase assays

As shown in Fig. 3C, there were significant reductions ($P < 0.01$) in the levels of elastase (35.53, 65.68, and 89.95%) when the concentration of phillyrin was in the range 0.0625–0.25 mg/ml.

Biofilm formation

There were remarkable reductions in biofilm formation (52.81, 74.31, and 84.47%) when *P. aeruginosa* cells were treated with increasing concentrations of phillyrin (0.0625–0.25 mg/ml), as shown in Fig. 3D.

Swimming and twitching motility assays

We investigated the ability of phillyrin to reduce the swimming and twitching motility of the opportunistic pathogen *P. aeruginosa*. The average swimming zone diameters were 67 ± 4.3 and 13 ± 3.8 mm in the untreated control and the plate treated with 0.25 mg/ml phillyrin, respectively (Fig. 4A). The average twitching zone diameters were 54 ± 2.8 and 11 ± 2.1 mm in the untreated control and the plate treated with 0.25 mg/ml phillyrin, respectively (Fig. 4B). Collectively, these data confirm that the inhibition of motility by phillyrin is not due to growth.

C. elegans nematode model

The *C. elegans*–*P. aeruginosa* host–pathogen model provided a powerful platform with which to examine the activity of phillyrin *in vivo*. Phillyrin protected the nematodes from *P. aeruginosa* and improved their survival rate (48.33%) when co-administered with the pathogen (Fig. 5A). To assess the ability of phillyrin to protect nematodes from *P. aeruginosa* and improve their survival rate, we determined their reproductive capacity by counting the number of eggs produced following inoculation with PAO1 (Fig. 5B). Phillyrin improved egg productivity by 79.93% when used at a concentration of 0.25 mg/ml. Phillyrin also increased the rate of food ingestion (87.97%) without exerting any bactericidal effect (Fig. 5C).

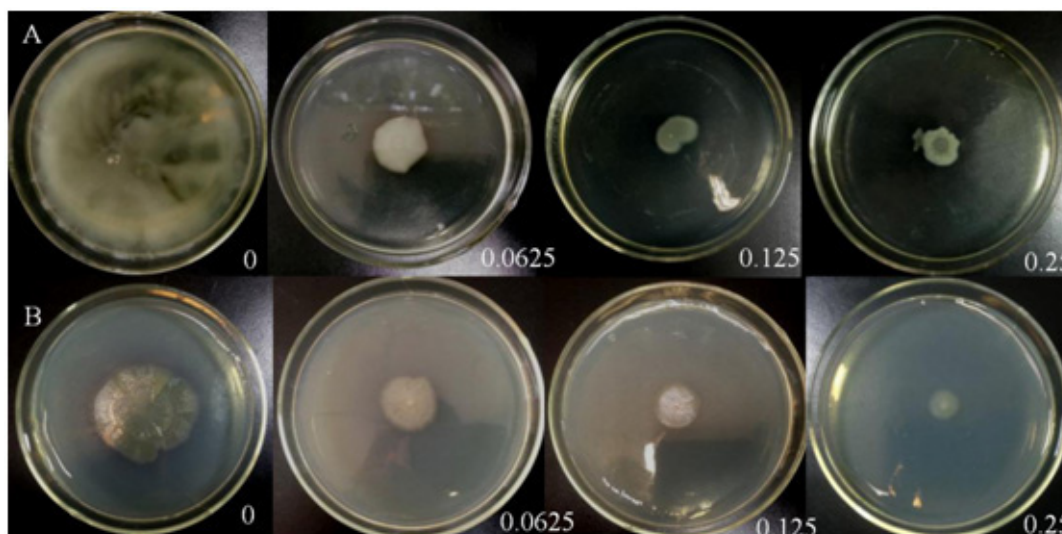


Fig. 4. Motility of *Pseudomonas aeruginosa* at various concentrations of phillyrin. The flagellum-mediated movement generated a turbid zone. (A) Swimming zones at 0.0625–0.25 mg/ml phillyrin; (B) twitching zones at 0.0625–0.25 mg/ml phillyrin.

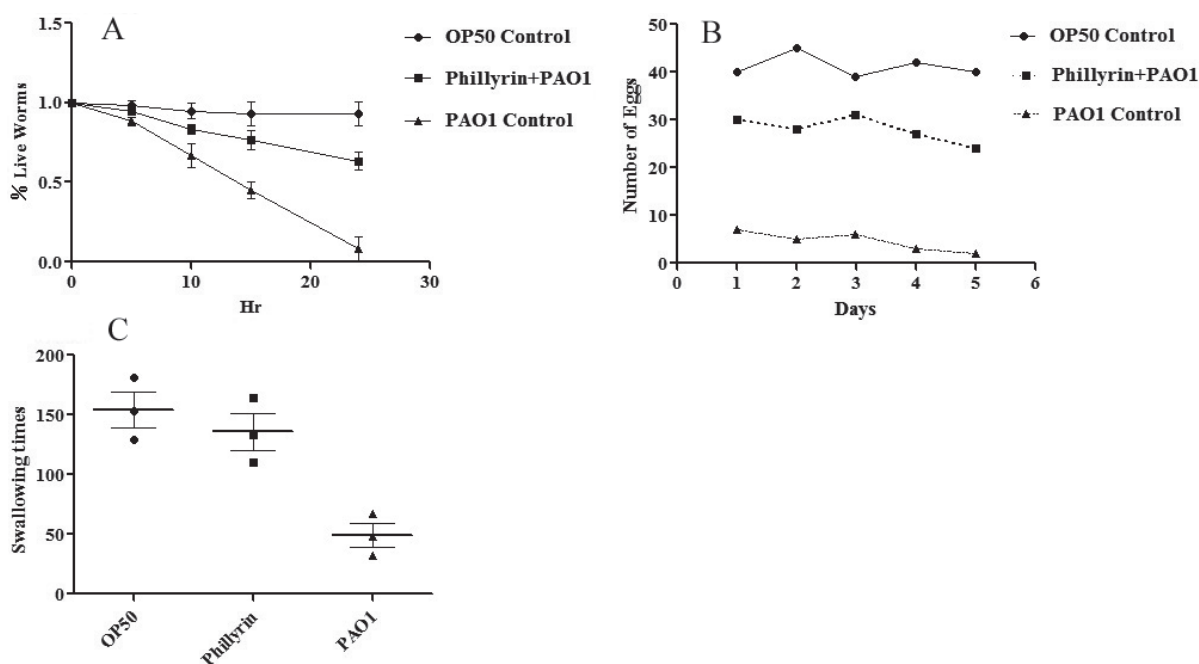


Fig. 5. (A) Phillyrin prevents *Pseudomonas aeruginosa* strain PAO1 from killing *Caenorhabditis elegans*. (B) Assessment of the ability of the nematode worms to reproduce. (C) Quantification of the swallowing rates of the nematode worms. All values are plotted relative to an untreated positive control. Error bars represent the SD of two replicates.

DISCUSSION

Over the past few decades, it has become increasingly clear that herbal medicines are an abundant source of antibacterial and anti-QS compounds. Examples include Yunnan Baiyao, *Camellia sinensis* (green tea), *Terminalia chebula*, *Nymphaea tetragona* (waterlily), eugenyl acetate, and *Callistemon viminalis*, which reportedly inhibit quorum sensing in *P. aeruginosa* [1, 11, 19, 20, 27, 35]. There is increasing interest in selecting efficacious QSIs from traditional Chinese medicines instead of using conventional antibiotics to fight pathogens. Remarkably—considering the huge quantity of literature describing the identification of QSI activity in traditional Chinese medicinal herbs—only a few QSIs have been finely characterized at the molecular level. In a previous study, we demonstrated that a water extract of *Forsythia suspense* exhibited anti-QS activity [34]. However, it is unclear which active

ingredients of *F. suspense* are responsible for the anti-QS activity. Fortunately, we have discovered that phillyrin—a major active component of *F. suspense*—has promising QSI activity, and warrants further study.

In the present study, we investigated for the first time the anti-QS activity of phillyrin to verify it as an alternative antibiotic. *In vitro* assays have demonstrated the potential of phillyrin to inhibit the production of QS-regulated virulence factors such as pyocyanin, elastase, and rhamnolipid in *P. aeruginosa*. QS regulates virulence, swimming and twitching motility, and the development of biofilms. Rhamnolipids are biodegradable surfactants that are predominantly produced by PAO1 and play a major role in the pathogenesis of *P. aeruginosa* extracellular factors. They comprise a hydrophilic head of one or two rhamnose molecules and a hydrophobic tail portion of one or two fatty acids. Rhamnolipids are involved in protecting cells from oxidative stress and play central roles in immune cells and erythrocyte destruction [17]. Bacterial biofilms are surface-associated, multicellular groups of microorganisms, and are a prerequisite of *P. aeruginosa* invasion. For example, they are sometimes found in medical devices or in the lungs of immune-compromised patients [9]. Swimming motility is achieved by long and hyperflagellated cells formed on the swimming plate. In *P. aeruginosa*, the *rhlAB* operon, which encodes a rhamnolipid biosurfactant that promotes swarming motility, is positively regulated by the *rhlI/R* C4–HSL QS system [7]. In *P. aeruginosa*, swimming is mediated by a QS system, whereby *rhlI/rhlR* mutants reduce and delay swimming, and *lasI/lasR* mutants completely diminish the ability to swim. Swimming is regulated by genes that are controlled by QS [10].

Generally, the drug-enhanced survival of nematodes confronted by pathogens depends on two mechanisms: the triggering of innate immune signaling and/or the suppression of pathogen virulence factors [14]. Therefore, we conclude that in survival and reproductive assays, phillyrin protects *C. elegans* from *P. aeruginosa*, possibly by suppressing pathogen virulence factors. Lewenza *et al.* have proposed that the feeding behavior of *C. elegans* can be used as a sensitive indicator of the virulence for *P. aeruginosa* PAO1 [16]. Numerous pharmacological experiments have been carried out on phillyrin. It has a positive effect on cigarette smoke-induced lung injury, lipopolysaccharide D-galactosamine-induced liver injury, transient cerebral ischemia in gerbils, and learning and memory deficits in senescence-accelerated mouse prone 8 (SAMP8) mice [5, 15, 23, 32].

In conclusion, our results support the potential use of phillyrin as an alternative antibiotic for combating bacterial infections. We hope that the present study will encourage greater collaboration between experimental and theoretical researchers.

CONFLICTS OF INTEREST. The authors declare that there are no conflicts of interest regarding the publication of this paper.

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