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Punicalagin Inhibits *Salmonella* Virulence Factors and Has Anti-Quorum-Sensing Potential

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Punicalagin, an essential component of pomegranate rind, has been demonstrated to possess antimicrobial activity against several food-borne pathogens, but its activity on the virulence of pathogens and its anti-quorum-sensing (anti-QS) potential have been rarely reported. This study investigated the efficacy of subinhibitory concentrations of punicalagin on *Salmonella* virulence factors and QS systems. A broth microdilution method was used to determine the MICs of punicalagin for 10 *Salmonella* strains. Motility assay and quantitative reverse transcription (RT)-PCR were performed to evaluate the effects of punicalagin on the virulence attributes and QS-related genes of *Salmonella*. The MICs of punicalagin for several *Salmonella* strains ranged from 250 to 1,000 μg/ml. Motility assays showed that punicalagin, at 1/16× MIC and 1/32× MIC, significantly decreased bacterial swimming and swarming motility, which corresponded to downregulation of the motility-related genes (*fliA*, *fliY*, *fljB*, *flhC*, and *fimD*) in RT-PCR assays. RT-PCR also revealed that punicalagin downregulated the expression of most of the selected genes involved in *Salmonella* virulence. Moreover, a QS inhibition assay indicated that punicalagin dose dependently inhibited the production of violacein by *Chromobacterium violaceum* and repressed the expression of QS-related genes (*sdiA* and *srgE*) in *Salmonella*. In addition, punicalagin significantly reduced *Salmonella* invasion of colonic cells ($P < 0.01$) with no impact on adhesion. These findings suggest that punicalagin has the potential to be developed as an alternative or supplemental agent for prevention of *Salmonella* infection.

Salmonella is one of the most important food-borne pathogens worldwide, and it causes infections in both humans and animals with symptoms such as fever, abdominal pain, nausea, diarrhea, and (occasionally) vomiting (1). Pathogenic *Salmonella* strains are distinguished from nonpathgenic *Salmonella* strains by the presence of virulence genes, which are often organized into *Salmonella* pathogenicity islands (SPIs) (2). So far, 15 SPIs have been identified in *Salmonella*. Of these SPIs, SPI-1 and SPI-2 encode two type III secretion systems (T3SSs) that function to deliver into host cells bacterial proteins that can reprogram various aspects of host biology (3, 4).

With the advent of antibiotic resistance of *Salmonella*, especially *Salmonella enterica* serovar Typhimurium definitive type 104, there is an increasing demand for the development of new therapeutics to prevent and treat infections caused by these resistant strains (5). Plant materials have received a great deal of interest for development as an alternative method to control pathogenic microorganisms. Many studies have demonstrated that components derived from plants (such as essential oils) show antimicrobial activity against a broad spectrum of microorganisms (6, 7).

Quorum sensing (QS) is defined as the way that bacteria use autoinducer (AI) molecules for bacterial cell-to-cell communication. AIs include oligopeptides and *N*-acylhomoserine lactones (AHLs) in Gram-positive and -negative bacteria, respectively (8). *Salmonella* is a Gram-negative bacterium and contains at least two types of QS systems, one induced by AHL and the other induced by AI-2. It has demonstrated that pathogens such as salmonellae employ QS to regulate their pathogenicity, such as biofilm formation, virulence factor production, and swarming motility (8). This makes the QS an attractive target for the development of novel anti-infective measures. Because of the role of QS in virulence regulation, many studies have focused on exploring natural QS inhibitors by using various bacterial models such as *Chromobacterium violaceum* CV026 and *C. violaceum* ATCC 12472 (9–11). *C. violaceum* produces a water-insoluble purple pigment called violacein that is regulated by QS via AHL. Therefore, the strain is an AHL biosensor and is considered a good model organism for screening of AHL-mediated QS inhibitors.

Pomegranate (*Punica granatum* L.) is rich in health-promoting compounds, and it has been widely used in traditional medicine for the prevention and treatment of many kinds of diseases, including dysentery, hemorrhage, helminthiasis, diarrhea, and acidosis (12). Punicalagin, the main active compound in pomegranate peel, has been reported to possess many properties, including antioxidant (13), antimicrobial (14), antiproliferative (15), apoptotic (16), antiviral (17), and immunosuppressive (18) activities. Taguri et al. (19) and Glazer et al. (14) have proved that punicalagin has antimicrobial activity against *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and fungi.

Although the antimicrobial activity of pomegranate peel and its extract has been extensively studied (20), little information is available in the literature about the antivirulence capacity and anti-QS potential of punicalagin. Therefore, the aim of this study was to investigate the influence of subinhibitory concentrations of punicalagin on *Salmonella* virulence gene expression and the *in vitro* virulence of *Salmonella*.

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TABLE 1 Differentially expressed virulence-related genes in *S.* Typhimurium SL1344 with or without punicalagin

Gene	Primer sequence (5′–3′)[c]	Relative gene expression at: 1/16× MIC	1/32× MIC	Reference
gyrB	F, GTCGAATTCTTATGACTCCTCC R, CGTCGATAGCGTTATCTACC	1	1	40
fliA	F, CGGAGTATCGTCAGATGTTG R, TTGATGTTCTTCAGTCACCAG	−4.04 ± 0.29[a]	−5.70 ± 0.27[b]	40
fliY	F, GCTTTGCCGATGAGGGTTTG R, GACGCTTTAACGCCCAGATG	−7.06 ± 1.22[b]	−7.83 ± 1.56[b]	40
fljB	F, TGGATGTATCGGGTCTTGATG R, CACCAGTAAAGCCACCAATAG	−13.11 ± 0.67[b]	−12.71 ± 1.42[b]	40
flhC	F, GAAAGTGGGTTGCTTGAATTG R, GCATCTCGGGAAAGTTTACG	−1.89 ± 0.44	−1.83 ± 0.19	40
fimD	F, CGCGGCGAAAGTTATTTCAA R, CCACGGACGCGGTATCC	−2.24 ± 0.44[a]	−2.20 ± 0.80[a]	32
spvB	F, TGGGTGGGCAACAGCAA R, GCAGGATGCCGTTACTGTCA	−0.76 ± 0.15[b]	−0.27 ± 0.15[b]	32
invH	F, CCCTTCCTCCGTGAGCAAA R, TGGCCAGTTGCTCTTTCTGA	−6.70 ± 1.38[b]	−2.54 ± 0.95[a]	32
orf245	F, CAGGGTAATATCGATGTGGACTACA R, GCGGTATGTGGAAAACGAGTTT	−1.94 ± 0.36	−1.23 ± 0.17	32
sipA	F, CAGGGAACGGTGTGGAGGTA R, AGACGTTTTTGGGTGTGATACGT	−6.73 ± 0.83[b]	−3.48 ± 0.83[a]	32
ssaV	F, GCGCGATACGGACATATTCTG R, TGGGCGCCACGTGAA	−0.48 ± 0.10[b]	−0.24 ± 0.01[b]	32
ssrA	F, CGAGTATGGCTGGATCAAAACA R, TGTACGTATTTTTTGCGGGATGT	−0.72 ± 0.17[a]	−0.42 ± 0.10[b]	32
pipB	F, GCTCCTGTTAATGATTTCGCTAAAG R, GCTCAGACTTAACTGACACCAAACTAA	−1.49 ± 0	−0.60 ± 0.26	32
rpoS	F, TTTTTCATCGGCCAGGATGT R, CGCTGGGCGGTGATTC	−3.81 ± 0.66[b]	−4.64 ± 0.18[b]	32
sopB	F, GCGTCAATTTCATGGGCTAAC R, GGCGGCGAACCCTATAAACT	−5.97 ± 0.64[b]	−4.07 ± 0.65[b]	32
hflK	F, AGCGCGGCGTTGTGA R, TCAGACCTGGCTCTACCAGATG	−1.13 ± 0.36	−1.02 ± 0.04	32
lrp	F, TTAATGCCGCCGTGCAA R, GCCGGAAACCAAATGACACT	−2.02 ± 0.31[a]	−2.12 ± 0.19[a]	32
sodC	F, CACATGGATCATGAGCGCTTT R, CTGCGCCGCGTCTGA	−1.61 ± 0.17	−1.42 ± 0.20	32
xthA	F, CGCCCGTCCCCATCA R, CACATCGGGCTGGTGTTTT	−1.60 ± 0.02	−1.80 ± 0.03	32
ssaB	F, ATTCAGG ATATCAGGGCCGAAGGT R, GTGCTGCAAGCAGTAGTGTCACAT	−1.71 ± 0.01	−1.14 ± 0.08	41
hilA	F, CTGTACGGACAGGGCTATCG R, GCAGACTCTCGGATTGAACC	−19.75 ± 1.97[b]	−1.85 ± 0.20[a]	42

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TABLE 1 (Continued)

Gene	Primer sequence (5′–3′)[c]	Relative gene expression at: 1/16× MIC	1/32× MIC	Reference
sdiA	F, TTACATTGGGATGACGTGCT R, AACTGCTACGGGAGAACGAT			This study
srgE	F, GCGCAGGTTGGTATTACTTG R, GGCAGATTGTTCATGATTGC			This study

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] F, forward; R, reverse.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S.* Typhimurium SL1344 and *C. violaceum* ATCC 12472 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Nine other *Salmonella* isolates were taken from our laboratory strain collection and originally isolated from raw chicken in China. All of the *Salmonella* isolates were used in MIC assays, and only SL1344 was used for further experiments because it is commonly used in *Salmonella* virulence studies and it contains phenotypic and genotypic characteristics tested in the following experiments. Punicalagin was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Before each experiment, a fresh overnight culture was prepared by incubation at 37°C for 12 h in Luria-Bertani (LB) broth (Beijing Land Bridge Technology Co., Ltd., Beijing, China) and then the culture was diluted in LB broth to an optical density at 600 nm (OD_{600}) of 0.5 (approximately 10^8 CFU/ml) with a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA).

Determination of MICs. MICs of punicalagin for *Salmonella* strains were determined by a broth microdilution method according to the Clinical and Laboratory Standards Institute, with minor modifications (21). An overnight culture prepared as described above was diluted with LB broth to an OD_{600} of 0.1, and 250 μl of the diluted culture was aliquoted into 96-well plates. Punicalagin was added to each well to obtain final concentrations of 1,000, 500, 250, 125, and 62.5 μg/ml. LB broth with or without a *Salmonella* culture was the control. The plate was incubated at 37°C for 24 h. The MIC was defined as the lowest concentration at which no visible growth was observed.

Growth curves. The method described by Qiu et al. (22) was followed with modifications. Briefly, an overnight culture prepared as described above was diluted with LB broth to an OD_{600} of 0.2, 125 μl of the diluted culture was aliquoted into 96-well plates, and then 125 μl of LB broth containing different concentrations of punicalagin was added. The final concentrations of punicalagin used were 1/64× MIC, 1/32× MIC, 1/16× MIC, 1/8× MIC, 1/4× MIC, 1/2× MIC, and 0 (control). LB broth containing no bacteria was used as a negative control. Bacteria were further cultured at 37°C, and cell growth was determined by measuring OD_{600} at 1-h intervals.

TABLE 2 MICs of punicalagin for different *Salmonella* strains

Strain	Serovar	Source	MIC (μg/ml)
S8XC004c	Shubra	Whole chicken	500
S9xc008b	Enteritidis	Whole chicken	250
S9xc0041	Typhimurium	Whole chicken	1,000
44-1	Indiana	Chicken liver	500
76D	Indiana	Whole chicken	1,000
546D	Shubra	Whole chicken	1,000
1087R	Ball	Whole chicken	1,000
59-1	Infantis	Chicken breast	1,000
60505-10cTT	Thompson	Whole chicken	1,000
SL1344	Typhimurium		500

Motility assay. Swimming motility was evaluated in modified LB broth containing 0.3% (wt/vol) agar as previously described (23). Five microliters of an overnight culture (OD_{600} = 0.5) was stabbed into semi-solid medium containing punicalagin at 1/16× MIC or 1/32× MIC. Medium without punicalagin was the control. The plates were incubated upright at 37°C for 7 h, and the diameter of the bacterial spread halo was recorded.

Swarming medium containing 0.5% (wt/vol) agar, 25 g/liter LB broth, and 5 g/liter glucose was used for swarming assays. Punicalagin (at concentrations of 0, 1/32× MIC, and 1/16× MIC) was added to warm medium, and then the plates were allowed to dry for 1 h at 25°C before use. After inoculation with 5 μl of an overnight culture (OD_{600} = 0.5), the plates were incubated at 37°C for 7 h. Photographs were taken, and the swarm area size was assessed by using AutoCAD to calculate the percentage of inhibition.

Quantitative QS inhibition assay. The effect of punicalagin on the QS-controlled production of violacein was determined with the indicator strain *C. violaceum* ATCC 12472 (24, 25). First, the effect of punicalagin on the growth of *C. violaceum* was studied to determine the concentration used in further experiments. *C. violaceum* was grown to an OD_{600} of 0.1 in LB broth. Two-hundred-microliter culture volumes were placed into the wells of a 96-well microtiter plate. Punicalagin was added to each well to obtain different concentrations. The culture without punicalagin was the control. The plate was incubated at 30°C, and cell growth was determined by measuring OD_{600} at 2-h intervals with a microplate spectrophotometer (model 680; Bio-Rad).

A flask incubation assay was used to quantify the QS-inhibitory activ-

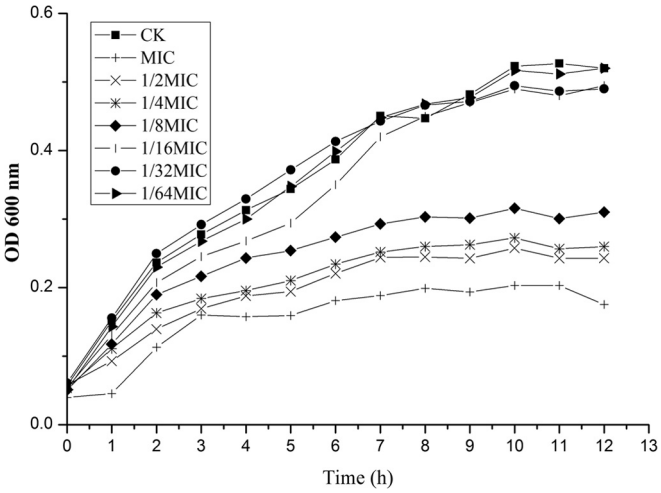

FIG 1 Growth curves of *S.* Typhimurium SL1344 cultured in LB broth with various concentrations of punicalagin. Each value represents the average of three independent experiments. CK, *S.* Typhimurium culture without punicalagin.

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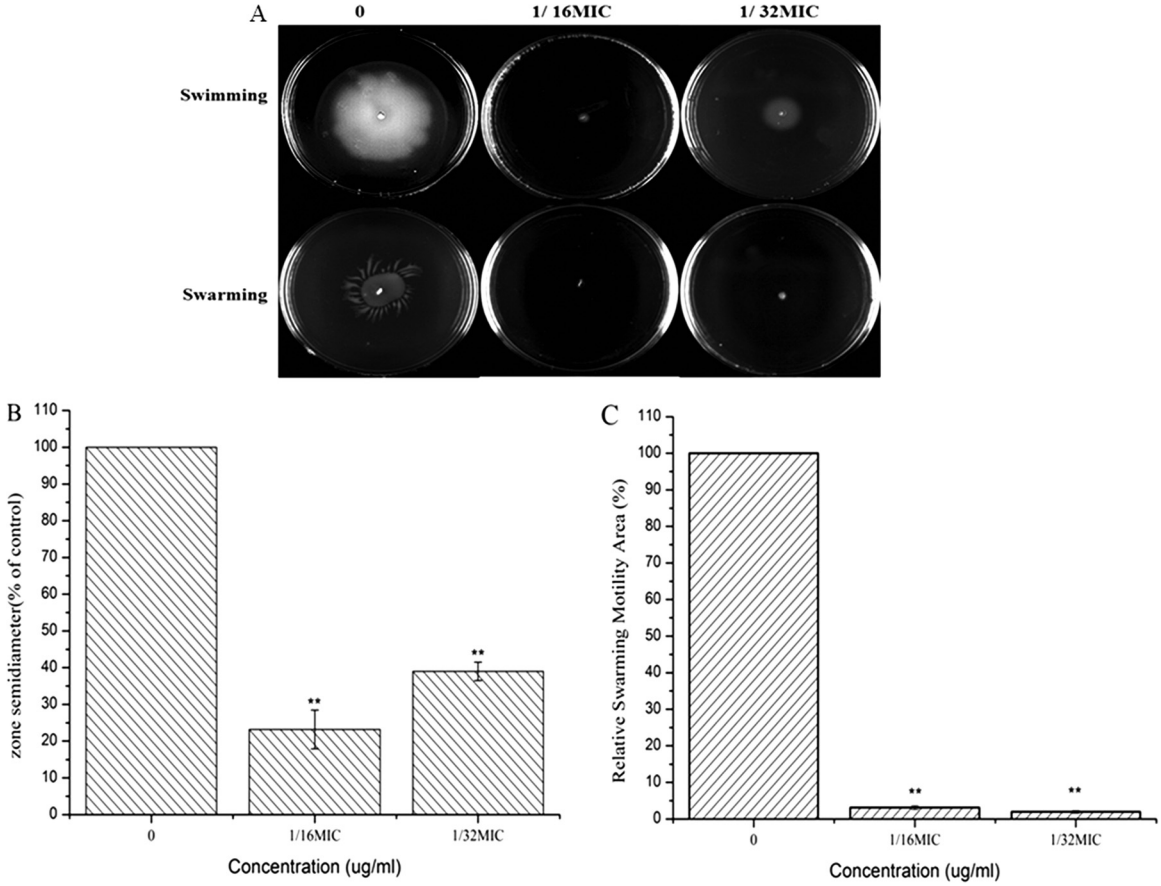

FIG 2 Punicalagin inhibits *S.* Typhimurium SL1344 motility. (A) Swimming and swarming motility of *S.* Typhimurium on soft agar plates containing different concentrations of punicalagin. Cells were inoculated at 37°C and photographed after 7 h of incubation. (B) Measurement of *S.* Typhimurium SL1344 migration in swimming motility assays. The relative zone semidiameter compared to that of the control (0 mM punicalagin, set at 100%) is presented as the mean ± the standard deviation of three independent experiments. (C) Quantification of *S.* Typhimurium SL1344 swarming motility in the presence of punicalagin. The relative swarming motility area of the strain was measured after treatment with punicalagin. Values are normalized to the 100% motility area measured in the absence of punicalagin. Bars showed the mean ± the standard deviation. *, $P < 0.05$; **, $P < 0.01$.

ity of punicalagin. An overnight culture of *C. violaceum* was diluted to an OD_{600} of 0.1. Volumes (3.9 ml) of LB broth that contained different concentrations of punicalagin were placed into flasks. Each flask was inoculated with 100 μl of culture. The flasks were incubated at 30°C for 24 h. Violacein extraction and quantitation were carried out as previously described by Choo et al. (25).

Quantitative RT-PCR. Five microliters of an overnight *Salmonella* culture (diluted to an OD_{600} of 0.5) was cultured in 5 ml of LB broth with or without punicalagin for 7 h (for motility assay) or 13 h (for virulence related genes assay) at 37°C. For QS assays, 5 μl of an overnight culture ($OD_{600} = 0.5$) was diluted in 5 ml of LB broth with or without punicalagin and then the cultures were supplemented with *N*-ketocaproyl-L-homoserine lactone (final concentration, 1 μmol/ml; Sigma-Aldrich, St. Louis, MO). As the control, distilled water was used instead of AHL. After that, the culture was incubated for 13 h at 37°C. RNA was extracted with an RNApure Bacteria kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Briefly, 1 ml of culture was centrifuged at 13,000 × *g* for 5 min (4°C) and then cells were resuspended in Tris-EDTA buffer containing 400 μg/ml lysostaphin (Sigma-Aldrich). The samples were incubated at 37°C for 5 min and then applied to a column to isolate the total RNA. After isolation, traces of contaminating DNA were further eliminated by treating RNA samples with RNase-free DNase I at 37°C for 20 min. The quality, integrity, and concentration of RNA were determined with a nucleic acid and protein minispectrophotometer (Nano-200; Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China). The primer pairs used for reverse transcription (RT)-PCR are listed in Table 1. A 2.5-μl sample of RNA was then reverse transcribed into cDNA with the TaKaRa PrimeScript reagent kit (Perfect Real Time; TaKaRa, Kyoto, Japan) according to the manufacturer's directions. cDNA was stored at −20°C until use. PCRs were performed in a 25-μl system that contained SYBR Premix Ex TaqII (TaKaRa) as recommended by the manufacturer. The reactions were performed with the IQ5 system (Bio-Rad). The cycling conditions included 1 cycle of 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s, and a dissociation step of 95°C for 15 s and 60°C for 30 s. All samples were analyzed in triplicate and normalized to the gyrase subunit B (*gyrB*) gene. Relative quantification based on the expression of a target gene versus the *gyr* gene were determined by the $2^{-\Delta\Delta CT}$ method described previously (26).

Cell culture. The human colonic cell line HT-29 was obtained from the Fourth Military Medical University (Xian, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 1% (vol/vol) nonessential amino acids, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37°C in a humidified 5% (vol/vol) CO_2 atmosphere.

Cell viability assay. The viability of HT29 cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cells were seeded at a density of 1×10^5/ml into the wells of 96-well plates and incubated at 37°C with 5% (vol/vol) CO_2 for 12 h. After incu-

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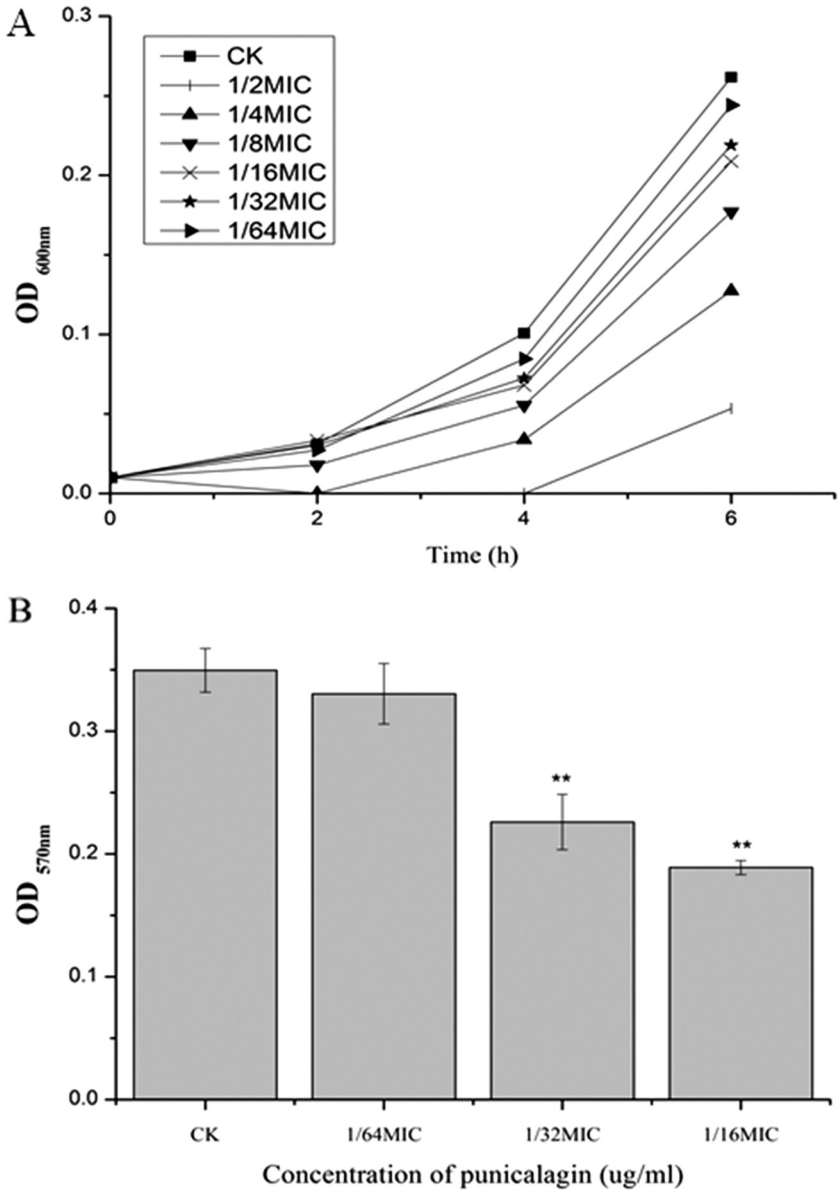

FIG 3 (A) Effect of punicalagin on the growth of *C. violaceum*. (B) Inhibition of violacein production by punicalagin. Violacein production was measured spectrophotometrically as described in Materials and Methods. Data are presented as the mean ± the standard deviation of absorbance at 570 nm. *, $P < 0.05$; **, $P < 0.01$. CK, *C. violaceum* culture without punicalagin.

bation, the medium was removed and 200-μl volumes with different concentrations of punicalagin were added. After incubation for 24 h, cultures were removed, 20 μl of 0.5% (wt/vol) MTT dissolved in phosphate-buffered saline (PBS) was added, and the plates were incubated for 4 h. A 100-μl volume of dimethyl sulfoxide was then added to each well to dissolve the formazan crystals. Absorbance at 570 nm was measured with a microplate reader (Bio-Rad). Cell viability was expressed as a percentage of the control (untreated cells).

Adhesion and invasion of cells. Overnight *Salmonella* cultures were centrifuged and resuspended in DMEM to a density of 1×10^8 CFU/ml. Monolayers of HT29 cells were cultured in 24-well plates as previously described. *Salmonella* culture samples and DMEM containing different concentrations of punicalagin were then added to the wells. Incubation was continued for 1 h at 37°C. The monolayers were washed three times with PBS and lysed in 1% Triton X-100 at room temperature for 20 min. The suspensions were serially diluted, and 100 ml of each dilution was plated on Trypticase soy agar. The plates were incubated for 24 h at 37°C. The bacterial counts were used to calculate the adhesion rate. In the invasion assay, the HT29 monolayers in the wells were washed once with PBS after 1 h of incubation with a bacterial culture containing punicalagin and then incubated for 30 min with 1 ml DMEM containing gentamicin at 100 μg/ml to kill extracellular bacteria. Cells were washed and lysed. The number of intracellular bacteria was determined by colony plating as described above.

Statistical analysis. All experiments were performed in triplicate, and three samples in each replicate were tested for each measurement. Independent Student *t* tests were used for statistical analysis by SPSS19.0 (IBM, New York, NY). A *P* value of <0.05 was considered statistically significant.

RESULTS

MICs. The MICs of punicalagin for 10 *Salmonella* strains are shown in Table 2. Punicalagin showed antimicrobial activity

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against each of the strains tested, and the MICs ranged from 250 to 1,000 μg/ml. The MIC of punicalagin for *S.* Typhimurium SL1344, which was selected for further experiments, was 500 μg/ml.

Growth curves. As shown in Fig. 1, punicalagin, at concentrations ranging from 1/16× MIC to 1/64× MIC, had no significant influence on the growth of *S.* Typhimurium. At concentrations ranging from the MIC to 1/8× MIC, punicalagin could retard the growth of *S.* Typhimurium. The OD_{600}s of cultures treated for 12 h with punicalagin at the MIC, 1/2× MIC, 1/4× MIC, and 1/8× MIC were 33.72, 46.73, 50, and 59.62% of that of a punicalagin-free culture, respectively.

Motility. Figure 2 shows the motility of *S.* Typhimurium on soft agar plates. Punicalagin slightly reduced the swimming motility of *S.* Typhimurium at 1/32× MIC and completely abolished it at 1/16× MIC. The halo diameters were 23.17 and 39.01% of that of the control, respectively, after treatment with punicalagin at 1/16× MIC and 1/32× MIC (Fig. 2A and B). Swarming motility was also greatly impacted by punicalagin. In the presence of subinhibitory concentrations (1/16× MIC and 1/32× MIC) of punicalagin, the strains showed much smaller relative swarm motility areas (about 1.95 and 3.13% of the control swarm motility area) (Fig. 2A and C).

RT-PCR for virulence-related genes. As shown in Table 1, punicalagin significantly ($P < 0.05$) downregulated several genes associated with virulence in *S.* Typhimurium (Table 1). The "early" *flhC* gene of the master flagellar *flhDC* operon was markedly repressed by punicalagin (Table 1). Punicalagin downregulated the expression of the *fliA*, *fliY*, and *fljB* genes to various degrees. The genes also downregulated included *fimD* (critical for regulation of motility), *sopB*, *invH* (adherence and invasion), *sipA*, *pipB*, *orf245* (T3SS), *hflK*, *lrp* (cell membrane and cell wall integrity), *xthA* (exo/endonuclease activity), *rpoS* (involved in metabolism), *sodC* (survival in macrophages), *hilA*, and *ssaB* (both involved in controlling the T3SS of SPI-1 and SPI-2). However, certain genes, such as *spvB* (actin ADP ribosyltransferase 2C toxin), *ssaV* (secretion system apparatus protein), and *ssrA* (sensor kinase), were upregulated by punicalagin.

Anti-QS activity of punicalagin. Figure 3A reveals that the growth of *C. violaceum* was inhibited in the presence of punicalagin at 1/4× MIC to 1/16× MIC and no significant adverse effect on growth was observed at 1/32× MIC and 1/64× MIC. As shown in Fig. 3B, anti-QS activity was shown when punicalagin was used at 1/64× MIC and 1/32× MIC, which was evidenced by less production of violacein (about 94.56 and 64.66% of the control level, respectively). Moreover, we observed that the QS-related genes (*sdiA* and *srgE*) of *S.* Typhimurium were activated by AHL and punicalagin reduced the expression of these two genes to various levels below the control level (Fig. 4).

Adhesion and invasion of cells. To evaluate possible cytotoxicity, HT29 cells were treated with punicalagin for 24 h. Figure 5A shows that there were no detectable cytotoxic effects of punicalagin at concentrations ranging from 125 to 15.125 μg/ml. We found that punicalagin had no significant effect on *S.* Typhimurium adhesion. However, punicalagin remarkably reduced, in a dose-dependent manner, the invasion of HT29 cells by *S.* Typhimurium compared to the control ($P < 0.01$) (Fig. 5B). Punicalagin reduced *S.* Typhimurium invasion of cells by 66 to 79% at concentrations of 15.125 and 62.5 μg/ml, respectively.

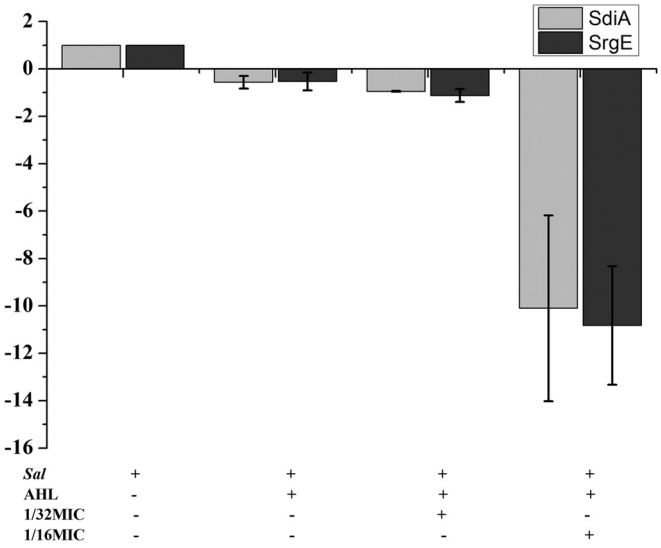

FIG 4 Transcriptional regulation of *sdiA* and *srgE* by punicalagin. Relative quantification based on the expression of a target gene versus that of the *gyr* gene was done by the $2^{-\Delta\Delta CT}$ method, and values are expressed as $-1/2^{-\Delta\Delta CT}$. Values represent the mean ± the standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. *Sal*, *S.* Typhimurium.

DISCUSSION

This study demonstrated that punicalagin decreased the motility of *S.* Typhimurium and downregulated flagellum-associated genes. Motility is correlated with virulence in pathogens, and studies have proved that plant materials could reduce the motility of various pathogens (27, 28) through different mechanisms. Burt et al. reported that carvacrol reduced the motility of *E. coli* because of the absence of the flagellum (29). Inamuco et al. observed that carvacrol inhibited the motility of *S.* Typhimurium not owing to the absence of flagella (28). The loss of motility may be attributed to the loss of functionality of the flagellum. In addition, a previous study showed the relationship between motility and invasion (30). We found that punicalagin reduced the invasion of HT29 cells by *Salmonella*, while adhesion was unaffected. Likewise, Inamuco et al. (28) reported that adhesion of *Salmonella* to intestinal epithelial cells was not affected by carvacrol but invasion was significantly reduced.

The pathogenicity of *S.* Typhimurium is dependent, to a great extent, upon the presence of a large number of defensive, as well as offensive, virulence factors (31). Therefore, an antivirulence strategy is an alternative method of infection control that is gaining increasing interest. Antivirulence agents may impose less pressure on a pathogen than antibiotics do, which could stimulate the emergence of resistance. It has been demonstrated that some plant compounds could influence virulence factor production when used at subinhibitory concentrations (22, 32, 33). Qiu et al. (22) observed that subinhibitory concentrations of thymol decreased the production of α-hemolysin and staphylococcal enterotoxins A and B in *S. aureus*. Upadhyaya et al. (32) showed that carvacrol, thymol, and eugenol downregulated the genes involved in *S. enterica* serovar Enteritidis colonization and macrophage survival. Here we proved that punicalagin downregulates the expression of several virulence genes of *Salmonella*, especially the critical genes required for pathogen colonization. Genes involved in survival in

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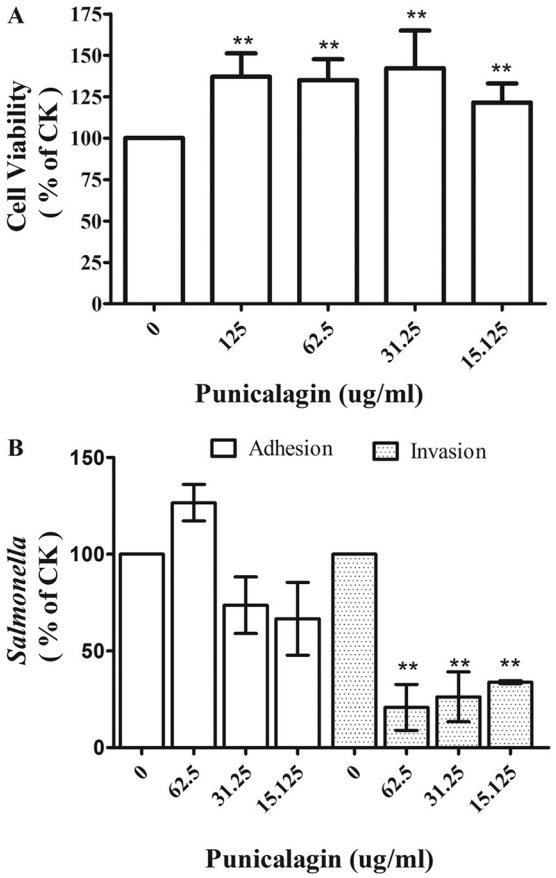

FIG 5 (A) Cytotoxic effects of punicalagin on HT29 cells. (B) Adhesion and invasion of HT29 cells by *S.* Typhimurium. Adhesion and invasion are shown as percentages of the control (0 mM punicalagin) value (set at 100%). Shown are the mean ± the standard deviation of three independent experiments. CK, *C. violaceum* culture without punicalagin. *, $P < 0.05$; **, $P < 0.01$.

macrophages, including *sodC* and *pipB*, were also suppressed by punicalagin. These findings show that punicalagin may attenuate virulence through different mechanisms, the elucidation of which requires genome-wide studies.

Salmonella pathogenesis, especially host invasion and intracellular proliferation, is directly linked to SPI genes. SPI-1 includes invasion genes, while SPI-2 is required for intracellular pathogenesis and has a crucial role in systemic *Salmonella* infections (34). SPI-1 and SPI-2 encode a T3SS, which is a complex of proteins that allows the transfer of virulence factors directly into host cells. The T3SS structural genes include many genes, as well as multiple regulatory and effector genes, and the major T3SS regulatory genes are *hilA* and *ssaB*, which are located on SPI-1 or SPI-2. We demonstrated that punicalagin downregulates the *hilA* and *ssaB* genes. After treatment with punicalagin at 1/16× MIC, the expression levels of *hilA* and *ssaB* were decreased 19.75- and 1.71-fold, respectively. We assumed that downregulation of SPI-1 and SPI-2 virulence genes could be attributed to the decreased *hilA* and *ssaB* expression levels. However, whether punicalagin directly downregulates the *hilA* and *ssaB* genes to repress the expression levels of virulence genes in SPI-1 and SPI-2 needs to be confirmed in further studies.

QS systems, as an attractive target for antimicrobial therapy, have gained more and more attention recently (35). It has been suggested that inactivation of the QS system of a pathogen can result in a significant decrease in its virulence (36). It was demonstrated that extracts from several plants, including *Tremella fuciformis* (37), vanilla (25), and *Terminalia catappa* (24), were able to interfere with bacterial QS. There are at least two QS systems (AI-1 and AI-2) in *Salmonella* (38). For Gram-negative bacteria, the QS system can be interfered with in three ways, inhibition of AHL molecule biosynthesis, degradation of AHL molecules by bacterial lactonases, and the use of small molecules to block AHL receptor protein activation. *S.* Typhimurium contains a transcription factor of the LuxR family, named *sdiA*, that detects and responds to AHLs produced by other species of bacteria. *sdiA* is known to activate two loci (*rck* and *srgE*) containing a total of seven genes (39). We proved that punicalagin inhibited the production of AHL-regulated violacein pigment in *C. violaceum* through disruption of QS signaling systems. RT-PCR assays also confirmed that punicalagin downregulated the expression of the *sdiA* and *srgE* genes in *Salmonella*.

In conclusion, subinhibitory concentrations of punicalagin reduced the virulence factor expression and invasion ability of *S.* Typhimurium. Moreover, punicalagin interfered with the AHL-dependent QS system in *C. violaceum* and inhibited AHL receptor protein expression in *Salmonella*. Therefore, punicalagin could potentially be developed as an alternative or supplemental agent to prevent *Salmonella* infection. Further toxicity analysis and *in vivo* testing are necessary before its real application.

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