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Subinhibitory concentrations of punicalagin reduces expression of virulence-related exoproteins by *Staphylococcus aureus*

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One sentence summary: Punicalagin reduces expression of virulence-related exoproteins by *Staphylococcus aureus*.
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ABSTRACT

Staphylococcus aureus produces a number of virulence factors. The major virulence factors exhibited by *S. aureus* include various antigens, enzymes, cytotoxins and exotoxins (e.g. hemolysins, enterotoxins and toxic shock syndrome toxin). In this report, we show the influence of punicalagin on the secretion of exoprotein from *S. aureus* by western blotting, tumor necrosis factor (TNF) release assay and quantitative RT-PCR. When added to *S. aureus* cultures at an OD600 of 0.9, graded subinhibitory concentrations of punicalagin reduced the production of α-toxin, SEA and SEB in methicillin-resistant *Staphylococcus aureus* in a dose-dependent manner. Consistently, punicalagin reduced TNF-inducing activity by *S. aureus* culture supernatants. Here, the transcriptional level of *agr* (accessory gene regulator) in *S. aureus* was inhibited by punicalagin, suggesting that the reduced transcription may affect the secretion of exotoxins. These findings suggest that the expression of α-toxin and enterotoxins in *S. aureus* is sensitive to the action of punicalagin, which may be an advantageous candidate in the treatment of toxigenic staphylococcal disease.

Keywords: methicillin-resistant *Staphylococcus aureus*, punicalagin; α-toxin; enterotoxin; tumor necrosis factor

INTRODUCTION

Staphylococcus aureus is among the prevalent members of the normal flora of the skin, oral cavity and intestine. *Staphylococcus aureus*, the most significant infectious agents of humans, causes miscellaneous severe systemic diseases such as endocarditis, osteomyelitis, pneumonia and toxemia. The species invades the skin through wounds, follicles or skin glands and produces a number of cell-associated and secreted virulence factors. The secreted factors include enzymes (coagulase, hyaluronidase, staphylokinase and lipase) and toxins (hemolysins, leukocidin, enterotoxins, exfoliative toxins and toxic shock syndrome toxin) (Pinchuk, Beswick and Reyes 2010; Qiu *et al*. 2010). Most of the exotoxins of *S. aureus* are classified as blood cell toxins (hemolysins and leukocidins), intestinal toxins and epithelial toxins. α-Toxin (α-hemolysin) possesses the far-reaching biological function, lyses red blood cells by disrupting membranes and damages leukocyte, skeletal and heart muscle, and renal tissue (Leng *et al*. 2011; Melo et al. 2016). Staphylococcal enterotoxin (SE) that act upon the gastrointestinal tract of

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humans induces vomiting and diarrhea with pyrogenic human diseases that include gastroenteritis and food intoxication. This toxin has the immunomodulatory role of superantigens that stimulate T-cell activation and release T-cell-derived cytokines (Yoh *et al.* 2000). The result of SE-induced T-cell activation is a cytokine bolus leading to an acute toxic shock (Pinchuk, Beswick and Reyes 2010). According to Varshney *et al.* (2009), most *S. aureus* isolates obtained from three separate hospitals had more than one enterotoxin gene. More than 20 distinct SEs have been identified, of which SEA and SEB are the best characterized. SEA is most common toxin associated with staphylococcal food poisoning and SEB, while it is associated with food poisoning, has been studied for potential use as a biological weapon (Omoe *et al.* 2002; Ler, Lee and Gopalakrishnakone 2006). Most of the antibiotic-associated diarrhea isolates of *S. aureus* are resistant to methicillin. How the production of the toxin affects the immunopathogenesis of MRSA-associated diarrhea is not yet clear.

Punicalagin (2,3-hexahydroxydiphenoylgallagyl-D-glucose) is an abundant ellagitannin isolated from pomegranate polyphenols, and possesses a variety of properties including antimicrobial (Taguri, Tanaka and Kouno 2004), antioxidant (Xu *et al.* 2015), anti-inflammatory (Xu *et al.* 2014), antiviral (Yang *et al.* 2012) and antitumor (Aqil *et al.* 2012) bioactivities. However, there have been no studies on the exposure of staphylococcal virulence factor to punicalagin.

This study was aimed at investigating the influence of subinhibitory concentrations of punicalagin on the production of α-toxin and staphylococcal enterotoxins (SEA and SEB).

MATERIALS AND METHODS

Bacterial strains and medium

MRSA strain ATCC 33591 was obtained from the American Type Culture Collection (Manassas, VA, USA). Bacteria were incubated on Mueller–Hinton agar (MHA) in a 37°C incubator and were suspended in Muller-Hinton broth (MHB). Punicalagin (≥98%, HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blotting

The MRSA strain (ATCC 33591) was grown to an OD_{600} value of 0.9 in MHB with graded subinhibitory concentrations of punicalagin for western blotting. Cells were harvested after 30 min and 4 h of treatment. The harvested cells were centrifuged at 13 000 rpm for 10 min and suspended in SMART bacterial protein extraction solution (iNtRON Biotechnology, Inc., Korea) according to the manufacturer's procedures. Cell lysates were centrifuged at 13 000 rpm for 10 min to separate the soluble and insoluble fractions. The soluble protein was present in the supernatant. Equal protein amounts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoresed gels were transferred to Amersham Hybond-P-membranes (GE Healthcare, Piscataway, NJ, USA) for the western blot analyses. Membranes were blocked in TBS-T (tween-20) containing 5% skim milk, and then probed with rabbit polyclonal anti-*Staphylococcus* alpha hemolysin antibody, rabbit polyclonal anti-*Staphylococcus* enterotoxin A antibody and rabbit polyclonal anti-*Staphylococcus* enterotoxin B antibody (diluted 1:500; Abcam, UK) for 5 h. After washing three times, membranes were washed with TBST and were reprobed with goat anti-rabbit IgG secondary antibody (diluted 1:1,000; Thermo Scientific, USA) for 90 min. Loading differences were normalized with monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (diluted 1:500; Santa Cruz Biotechnology, USA). The membrane was then supplemented with ECL Prime western blotting detection reagent (GE Healthcare Life Sciences, Korea). Immunoreactive bands of membrane were detected using the Imagequant LAS-4000 mini chemical luminescent imager (GE Healthcare Life, Korea).

Tumor necrosis factor release assay

Preparation of bacterial supernatants

The tumor necrosis factor (TNF) release assay was performed as described by Qiu *et al.* (2010) and Bernardo *et al.* (2004) with some modification. Overnight culture (OD_{600} value of 0.9) of MRSA ATCC 33591 in RPMI 1640 (Welgene, Korea) was diluted 30-fold in RPMI 1640. Graded concentrations of punicalagin (1/8, 1/4 and 1/2 MIC) were added to the diluted bacterial suspensions. *Staphylococcus aureus* supernatant without punicalagin treatment was used as a control. After 4 h incubation, the supernatants (700 μL) collected (protein secretion) were filtered through a 0.2 μm filter and immediately analyzed as described below.

Preparation of RAW 264.7 cells

RAW 264.7 cells were prepared in RPMI 1640 (supplemented with 10% FBS, 100 IU/mL^{-1} penicillin and streptomycin). Cells were seeded at a density of 10^6/mL in RPMI, dispensed (100 μL) into 96-well tissue culture plates and then incubated in 5% CO_2 at 37°C in an incubator for 18 h to allow adherence. Cell culture media were washed and RPMI 1640 medium (150 μL) was added. *Staphylococcus aureus* supernatants (50 μL) were added to the tissue culture plate. After incubation for 16 h, supernatants were collected, centrifuged (1000 g for 5 min) and measured by enzyme-linked immunosorbent assay (ELISA).

ELISA

The TNF level in the supernatants was analyzed using the mouse TNF-α ELISA MAX Set Standard (Biolegend Inc., San Diefo, CA, USA) according to the manufacturer's instructions.

Reverse transcription and quantitative RT-PCR

Strain ATCC 33591 was grown to an OD_{600} value of 0.9 in MHB and subjected to subinhibitory concentrations (1/8 MIC, 1/4 MIC and 1/2 MIC) of punicalagin for 4 h. A control without punicalagin was included. Total RNA was prepared using the Easy-RED BYF total RNA extraction kit according to the manufacturer's procedure (iNtRON Biotechnology). The RNA concentration was determined by measuring A_{260} on a Nanodrop spectrophotometer (BioTek, Korea). RNA was reverse transcribed into cDNA using a cDNA synthesis kit (iNtRON Biotechnology) for first-strand cDNA synthesis in accordance with the manufacturer's instructions, in order to synthesize the RNA template for quantitative reverse

Table 1. Primers used in quantitative RT-PCR.

Primer	Sequence
sea	Forward; 5′-ATGGTGCTTATTATGGTTATC-3′ Reverse; 5′-CGTTTCCAAAGGTACTGTATT-3
seb	F; 5′-TGTTCGGGTATTTGAAGATGG-3′ R; 5′-CGTTTCATAAGGCGAGTTGTT-3′
agrA	F; 5′-TGATAATCCTTATGAGGTGCTT-3′ R; 5′-CACTGTGACTCGTAACGAAAA-3′
16S RNA	F; 5′-GCTGCCCTTTGTATTGTC-3′ R; 5′-AGATGTTGGGTTAAGTCCC-3′

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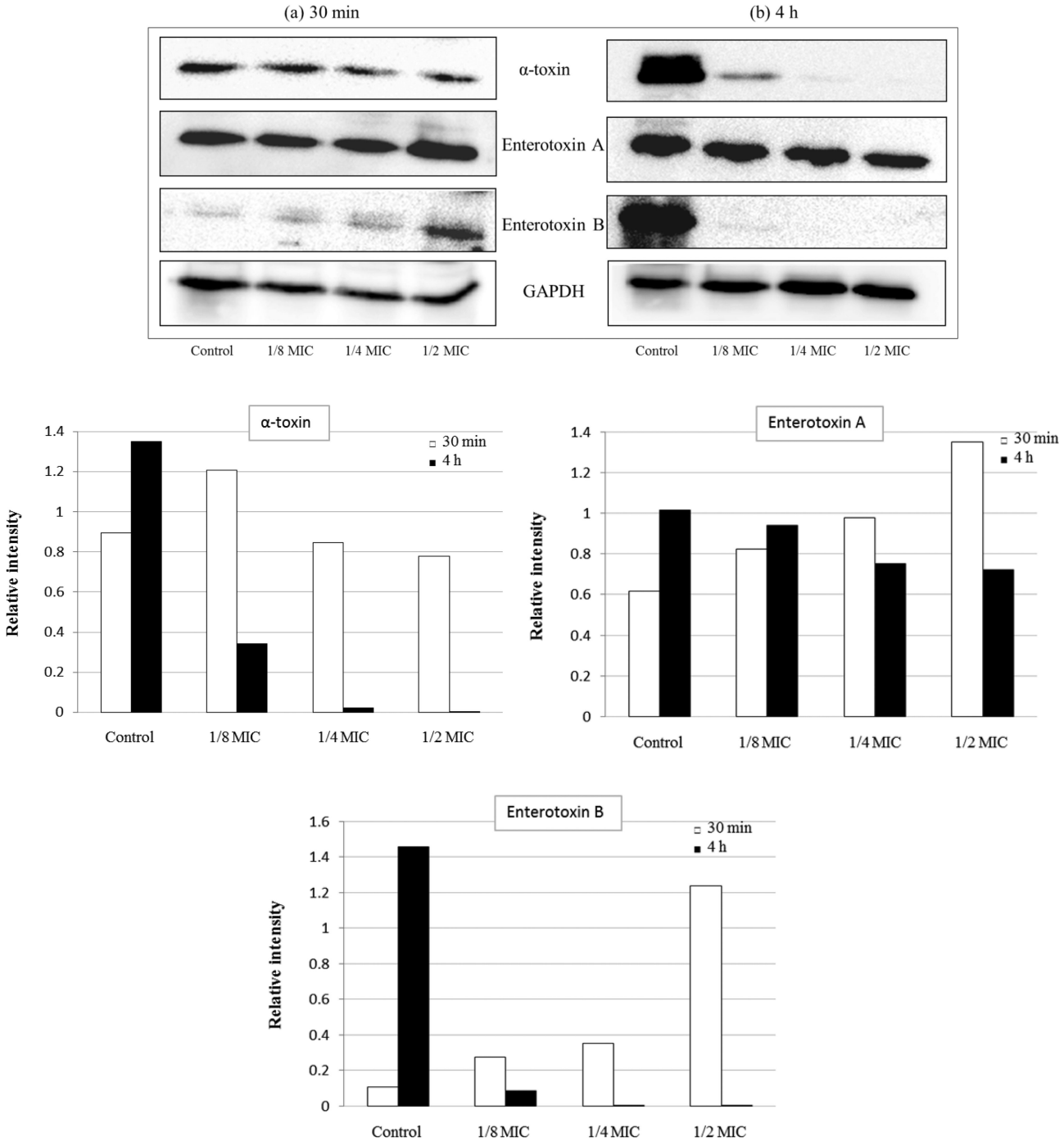

Figure 1. Western blot analysis of α-toxin, SEA and SEB production by strain ATCC 33591 after exposure to (a) 30 min and (b) 4 h with graded subinhibitory concentrations of punicalagin. Proteins were stained with the indicated antibodies against α-toxin, SEA and SEB. The control was not treated with drugs (lane 1). After exposure to 4 h of treatment, the graded subinhibitory concentrations of punicalagin suppressed production of α-toxin and enterotoxins from *S. aureus* in a dose-dependent manner (b) (lanes 2–4). GAPDH was used as the loading control.

transcriptase PCR (qRT-PCR). The primer pairs used for the qRT-PCR are presented in Table 1. The PCR was set up was as follows: 10 μL of 2 $\times$SYBR premix (Life technologies), 1 μL sample cDNA and 1 μL of each primer (10 μM), and deionized water to a total volume of 20 μL. The PCR was run using the StepOnePlus real-time PCR system (Applied Biosystems, France).

Statistical analysis

Statistical differences were examined using Dunnett's *t*-test for multiple comparisons. The data are presented as the mean $\pm$ standard deviations (SD). A *p*-value less than 0.05 was considered to be statistically significant.

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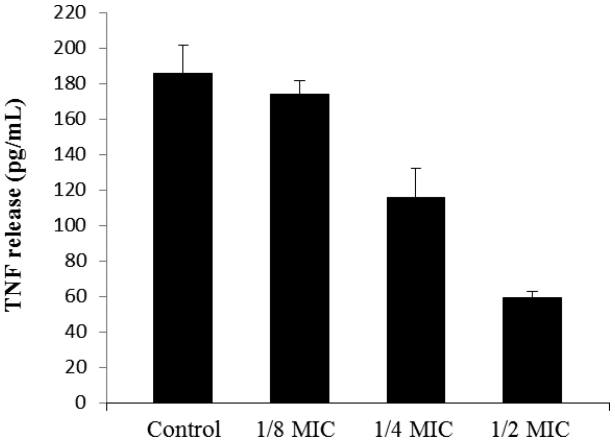

Figure 2. TNF release from RAW 264.7 cells stimulated with the supernatants of *S. aureus* grown in the presence of graded subinhibitory concentrations of punicalagin. After stimulation for 16 h with RAW 264.7 cells, TNF levels were measured by ELISA. Values represent the means ± SD for two independent experiments.

RESULTS

Punicalagin reduces the level of α-toxin and enterotoxin expression by *Staphylococcus aureus*

The secretion of staphylococcal α-toxin and two major enterotoxins (SEA and SEB) when exposed to subinhibitory concentrations of punicalagin was analyzed by western blotting. As shown in Fig. 1(a), SEA and SEB were induced at the increasing concentrations of punicalagin after exposure to punicalagin for 30 min. After exposure to 4 h of treatment, a dose-dependent inhibition of all exotoxins was observed in Fig. 1(b). Growth of MRSA in the presence of 1/8 MIC punicalagin led to a measurable reduction of α-toxin and SEB secretion, and none or little protein could be detected in the MRSA strain while at 1/4 MIC (15.6 μg/mL). We suppose that the organisms are under tremendous stress against drugs immediately after exposure to punicalagin (Fig. 1a). These exotoxin expression levels in MRSA after adding graded subinhibitory concentrations of punicalagin corresponded to low expression of the relative genes after primer extension (Fig. 3). GAPDH was used as the loading control.

Punicalagin reduces TNF-inducing activity of *Staphylococcus aureus*

Among the virulence factors secreted by *S. aureus*, α-toxin is primarily responsible for the hemolytic activity of *S. aureus*, and enterotoxins are the most important exotoxins of *S. aureus* that could act as superantigens, stimulating T cells to release proinflammatory cytokines (Qiu et al. 2011a). Therefore, to clarify the biological relevance of reduction in Staphylococcal exotoxin secretion caused by punicalagin, we performed a TNF release assay. The culture supernatants of *S. aureus* grown in the subinhibitory concentrations of punicalagin reduced TNF-inducing activity in a dose-dependent manner (Fig. 2).

Punicalagin represses the transcription of *hla*, *agrA*, *sea* and *seb* in *Staphylococcus aureus*

As punicalagin remarkably reduced the production of α-toxin, SEA and SEB from *S. aureus*, we therefore hypothesized that punicalagin could affect the transcription of *hla*, *agrA*, *sea* and

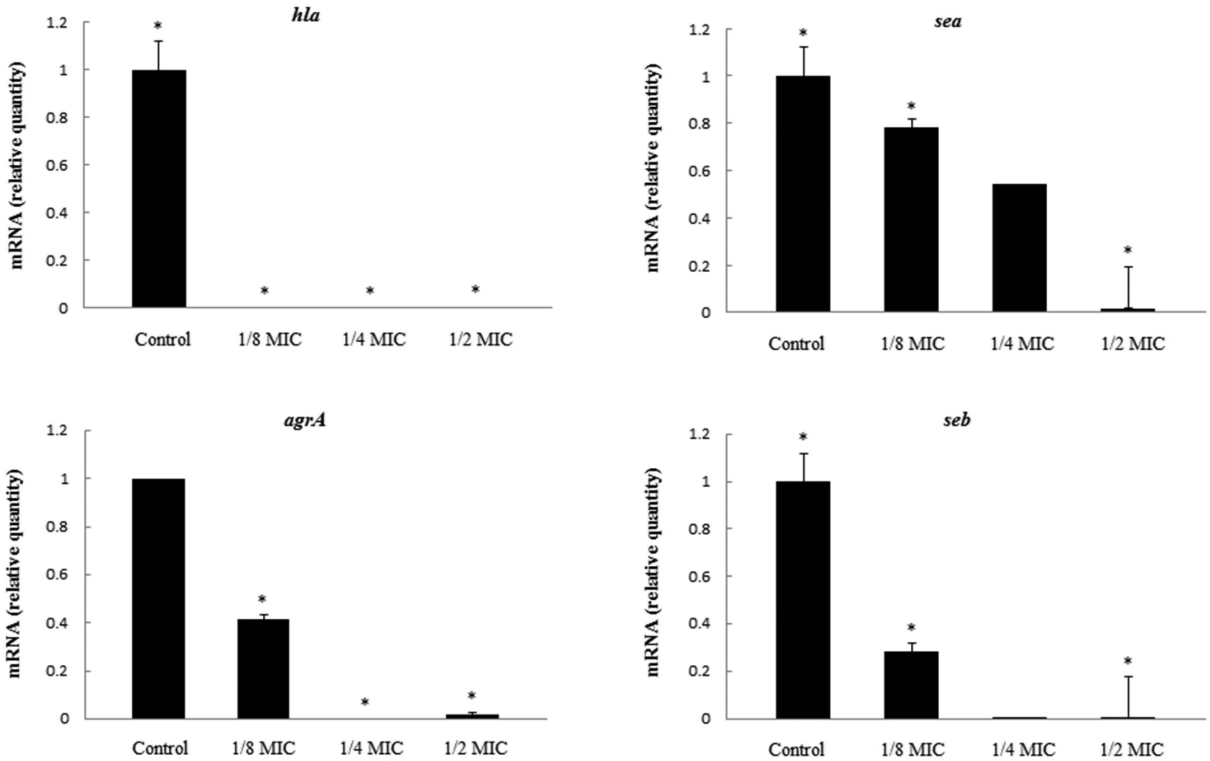

Figure 3. Relative gene expression of *hla*, *agrA*, *sea* and *seb* in *S. aureus* after growth with subinhibitory concentrations of punicalagin. The *hla* gene could not be detected, and *agrA* and *seb* expression were almost couldn't find in the presence of 1/4 MIC punicalagin. The sea gene was also reduced in a dose-dependent manner. Values represent the mean and standard error of three independent experiments. * represents $P < 0.05$.

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seb. As expected, the transcriptional level of these genes in *S. aureus* was affected by treatment with graded subinhibitory concentrations of punicalagin. When exposed to 1/8 MIC of punicalagin, the transcriptional levels of *hla* could not be detected, and *agr*A, an accesory gene regulator, was reduced by 2.4-fold. The transcriptional levels of *sea* and *seb* were reduced by 1.28- and 3.57-fold, respectively (Fig. 3). The expression of *hla*, *agr*A, *sea* and *seb* was significantly inhibited in MRSA in a dose-dependent manner when it was treated with sub-MIC (7.8–31.25 μg/mL) concentrations of punicalagin.

DISCUSSION

The pathogenicity of *Staphylococcus aureus* is related to the secretion of numerous staphylococcal virulence factors that allow it to adhere to host tissues, invade the immune system and cause harmful toxic effects to the host (Gordon and Lowy 2008; Bien, Sokolova and Bozko 2011). The number of virulence factors produced by *S. aureus* plays an important role in the pathogenesis of a broad range of *S. aureus* infection (Dinges, Orwin and Schlievert 2000; Pinchuk, Beswick and Reyes 2010). *Staphylococcus aureus* exoproteins such as exotoxins and enzymes convert host tissue into nutrients for bacterial growth, and possess properties including pyrogenicity and superantigenicity. Therefore, the antibiotic used to treat *S. aureus* infection should not only have antimicrobial activity, but must have an ability to suppress the emergence of virulence factors expressed by killed or stressed bacteria.

We provide here that punicalagin is able to repress production of α-toxin and enterotoxins from *S. aureus* isolates with subinhibitory concentrations ranging from 7.8 to 31.25 μg/mL. α-Toxin plays significantly contributes to *S. aureus*-induced cell death, and as a trigger of caspase activation via the intrinsic death pathway independently of death receptor signaling (Bantel *et al*. 2001). SEs, as superantigens, can result in staphylococcal gastroenteritis, one of the causes of food poisoning in humans (Leng *et al*. 2011). For the management of toxic *S. aureus* infections, protein synthesis inhibitors such as clindamycin and linezolid inhibit the production of virulence factors produced by *S. aureus* (Bernardo *et al*. 2004). In contrast, β-lactam and glycopeptide antibiotics induce α-toxin, enterotoxins and toxic shock syndrome toxin-1 production through the stimulatory effect of exoprotein synthesis, indicating that β-lactam antibiotics may fail in *S. aureus* infections, especially MRSA infections, caused by toxin-producing organisms (Stevens *et al*. 2007; Xiang *et al*. 2010; Qiu *et al*. 2011b). Punicalagin was observed to inhibit α-toxin, SEA and SEB expression at concentrations lower than 62.5 μg/ml (MIC). In particular, at 1/8 or 1/4 MIC of punicalagin, no immunoreactive protein (α-toxin and SEB) could be found (Fig. 1). The sub-MIC (7.8–31.25 μg/mL) of punicalagin significantly suppressed production of exoprotein genes *hla*, *agr*A, *sea* and *seb* in MRSA. A virulence factor gene, *hla*, is controlled by several regulatory systems such as Agr and Sar. *agr*A encodes accessory gene regulator A, which positively regulates exotoxin encoding genes (Qiu *et al*. 2011a; You, Choi and Kim 2013). The accessory gene regulator *agr*A is one of the characterized global regulatory systems involved in the regulation of α-toxin (Cheung *et al*. 2004; Oogai *et al*. 2011). Previous studies have shown that inhibition of regulatory genes such as *agr*A and *sar*A alter the transcription of exotoxin-encoding genes (Leng *et al*. 2011; You, Choi and Kim 2013). The sub-MIC of punicalagin reduced production of α-toxin by *S. aureus*, possibly in part due to inhibition of the *agr* gene. Similarly, a subinhibitory concentration of clindamycin inhibited transcription of exoprotein genes differently in *S. aureus*, in part through regulation of the *sar* locus, suggesting that the primary effect must be differential inhibition of the synthesis of one or more regulatory proteins (Herbert, Barry and Novick 2001). Consequently, our findings suggest that the influence of punicalagin on staphylococcal α-toxin and enterotoxin production may be involved with the inhibition of the *Agr* regulatory system; and punicalagin at subinhibitory concentrations potently reduces the secretion of virulence factors produced by *S. aureus*, suggesting that a punicalagin-based structure may be used for the development of drugs aimed at staphylococcal virulence-related exoproteins. Therefore, our next challenge is to establish molecular target of a broad-spectrum antimicrobial agent punicalagin against MRSA.

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Conflict of interest. None declared.

REFERENCES

Aqil F, Munagala R, Vadhanam MV *et al*. Anti-proliferative activity and protection against oxidative DNA damage by punicalagin isolated from pomegranate husk. *Food Res Int* 2012;**49**:345–53.

Bantel H, Sinha B, Domschke W *et al*. alpha-Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *J Cell Biol* 2001;**155**:637–48.

Bernardo K, Pakulat N, Fleer S *et al*. Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob Agents Ch* 2004;**48**:546–55.

Bien J, Sokolova O, Bozko P. Characterization of virulence factors of *Staphylococcus aureus*: novel function of known virulence factors that are implicated in activation of airway epithelial proinflammatory response. *J Pathog* 2011;**2011**:601905.

Cheung AL, Bayer AS, Zhang G *et al*. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol Med Micr* 2004;**40**:1–9.

Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 2000;**13**:16–34.

Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* 2008;**46**:S350–9.

Herbert S, Barry P, Novick RP. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect Immun* 2001;**69**:2996–3003.

Leng BF, Qiu JZ, Dai XH *et al*. Allicin reduces the production of α;-toxin by *Staphylococcus aureus*. *Molecules* 2011;**16**:7958−68.

Ler SG, Lee FK, Gopalakrishnakone P. Trends in detection of warfare agents. Detection methods for ricin, staphylococcal enterotoxin B and T-2 toxin. *J Chromatogr A* 2006;**1133**:1–12.

Melo MC, Teixeira LR, Pol-Fachin L *et al*. Inhibition of the hemolytic activity caused by *Staphylococcus aureus* alpha-hemolysin through isatin-Schiff copper(II) complexes. FEMS Microbiol Lett 2016;**363**, DOI: 10.1093/femsle/fnw207.

Omoe K, Ishikawa M, Shimoda Y *et al*. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus isolates* harboring *seg*, *seh*, or *sei* genes. *J Clin Microbiol* 2002;**40**:857–62.

Oogai Y, Matsuo M, Hashimoto M *et al*. Expression of virulence factors by *Staphylococcus aureus* grown in serum. *Appl Environ Microb* 2011;**77**:8097–105.

Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. *Toxins* 2010;**2**:2177–97.

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Qiu J, Feng H, Xiang H *et al*. Influence of subinhibitory concentrations of licochalcone A on the secretion of enterotoxins A and B by *Staphylococcus aureus*. *FEMS Microbiol Lett* 2010;**307**:135–41.

Qiu J, Wang J, Luo H *et al*. The effects of subinhibitory concentrations of costus oil on virulence factor production in *Staphylococcus aureus*. *J Appl Microbiol* 2011a;**110**:333–40.

Qiu J, Zhang X, Luo M *et al*. Subinhibitory concentrations of perilla oil affect the expression of secreted virulence factor genes in *Staphylococcus aureus*. *PLoS One* 2011b;**6**:e16160.

Stevens DL, Ma Y, Salmi DB *et al*. Impact of antibiotics on expression of virulence-associated exotoxin genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2007;**195**:202–11.

Taguri T, Tanaka T, Kouno I. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biol Pharm Bull* 2004;**27**:1965–9.

Varshney AK, Mediavilla JR, Robiou N *et al*. Diverse enterotoxin gene profiles among clonal complexes of *Staphylococcus aureus* isolates from the Bronx, New York. *Appl Environ Microb* 2009;**75**:6839–49.

Xiang H, Qiu JZ, Wang DC *et al*. Influence of magnolol on the secretion of alpha-toxin by *Staphylococcus aureus*. *Molecules* 2010;**15**:1679–89.

Xu X, Li H, Hou X *et al*. Punicalagin induces Nrf2/HO-1 expression via upregulation of PI3K/AKT pathway and inhibits LPS-induced oxidative stress in RAW264.7 macrophages. *Mediat Inflamm* 2015;**2015**:380218.

Xu X, Yin P, Wan C *et al*. Punicalagin inhibits inflammation in LPS-induced RAW264.7 macrophages via the suppression of TLR4-mediated MAPKs and NF-κB activation. *Inflammation* 2014;**37**:956–65.

Yang Y, Xiu J, Zhang L *et al*. Antiviral activity of punicalagin toward human enterovirus 71 *in vitro* and *in vivo*. *Phytomedicine* 2012;**20**:67–70.

Yoh K, Kobayashi M, Yamaguchi N *et al*. Cytokines and T-cell responses in superantigen-related glomerulonephritis following methicillin-resistant *Staphylococcus aureus* infection. *Nephrol Dial Transpl* 2000;**15**:1170–4.

You YO, Choi NY, Kim KJ. Ethanol extract of *Ulmus pumila* root bark inhibits clinically isolated antibiotic-resistant bacteria. *Evid-Based Compl Alt* 2013;**2013**:269874.

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