

Antimicrobial Activity of Licochalcone E Against *Staphylococcus aureus* and Its Impact on the Production of Staphylococcal Alpha-Toxin

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Received: December 9, 2011 / Revised: January 30, 2012 / Accepted: January 31, 2012

Licochalcone E was firstly isolated from licorice root in 2005, which belongs to the retrochalcone family. Studies on the biological activities of licochalcone E were in the initial stage. In the study, we demonstrated that licochalcone E has potent antimicrobial property against *Staphylococcus aureus*. Furthermore, via hemolysis, Western blot, and real-time RT-PCR assays, we have shown that subinhibitory concentrations of licochalcone E dose-dependently reduces the production of α -toxin in both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). The data suggest that licochalcone E may deserve further investigation as a potential therapeutic against *S. aureus* infections, or the structure of licochalcone E may be used as a basis for chemical synthesis of novel anti-*S. aureus* compounds.

Keywords: *Staphylococcus aureus*, licochalcone E, alpha-toxin

Staphylococcus aureus is an important opportunistic pathogen that causes a number of infections, including cellulitis, fasciitis, food poisoning, toxic shock syndrome, sepsis, endocarditis, osteomyelitis, and pneumonia [16]. Nowadays, since many strains of this organism are resistant against clinically useful antibiotics like methicillin and vancomycin, the treatment of *S. aureus* infections has become sophisticated [7]. Moreover, with the rapid emergence of multidrug-resistant organisms, the problem is further aggravated. It is only a few years after the introduction of linezolid and daptomycin for the clinical use of methicillin-resistant *S. aureus* (MRSA) infections. However, new resistant strains against these drugs have already been reported [25].

Consequently, there is an urgent and continued demand for the discovery and development of novel antibacterial agents.

In addition to antimicrobial resistance, *S. aureus* synthesizes a number of extracellular and cell-wall-associated proteins, many of which play a major role in the pathogenesis of staphylococcal infections. α -Toxin is secreted as a 33.2 kDa water-soluble monomer by the majority of *S. aureus* strains [2]. Upon binding to the membrane of susceptible cells, the monomer permits a series of well-defined intermolecular interactions between neighboring monomers, resulting in the formation of a barrel-shaped 232.4 kDa membrane-inserted heptamer [6]. The channel-forming heptamer mediates the cytolytic properties of α -toxin, and a number of human cells including erythrocytes, monocytes, lymphocytes, macrophages, and epithelial cells are influenced by the toxin.

Licorice root, derived from the plant *Glycyrrhiza glabra* or *Glycyrrhiza radix*, has traditionally been used for the treatment of pulmonary diseases, rheumatic and other pain, gastric ulcers, and inflammatory processes in Northeast Asia [1, 22]. To date, six retrochalcones, licochalcone A–E and echinatin, have been isolated and characterized from the roots of *Glycyrrhiza glabra* [27]. The retrochalcones are structurally different from normal chalcones by its lack of oxygen functionalities at the C-2' and C-6' positions. The retrochalcones family possesses various biological properties. For example, licochalcone A–D have antiparasitic, antioxidative, antitumor, anti-inflammatory, antileishmanial, superoxide scavenging, and antibacterial effects [5, 8, 9, 17, 24, 28]. As a member of the retrochalcone family, licochalcone E was isolated in 2005 by Cheon's group [27]. The biological studies on licochalcone E are in the initial stage. Licochalcone E exhibits topoisomerase 1 inhibition and induces endothelial cell apoptosis by modulating NF- κ B and the Bcl-2 family [4].

The isolation yield of licochalcone E (Fig. 1) was very low (5 mg from 1 kg of powdered *Glycyrrhiza glabra*).

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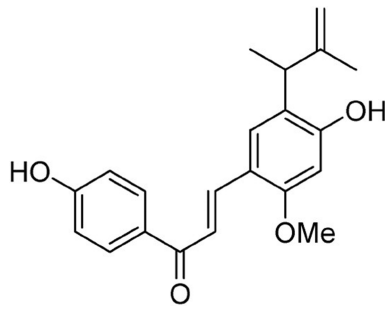


Fig. 1. Chemical structure of licochalcone E.

Therefore, the biological studies on licochalcone E have been limited, and chemical synthesis of licochalcone E was highly desirable. Recently, Li *et al.* [15] have introduced a short and efficient method to synthesize the licochalcone E [15].

In the study, we aimed to investigate the antimicrobial activity of licochalcone E against *S. aureus*, and further investigate the influence of subinhibitory concentrations of licochalcone E on the production of α -toxin.

MATERIALS AND METHODS

Bacterial Strains and Reagents

Licochalcone E was kindly provided by Professor Jizhen Li of Jilin University, and stock solutions were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). The bacterial strains used in the study are presented in Table 1. Strains ATCC 29213 and ATCC10832, producing high levels of α -toxin, were used to investigate the impact of licochalcone E on *S. aureus* α -toxin production.

Determination of Minimal Inhibitory Concentrations (MICs)

The MICs of licochalcone E for *S. aureus* were determined using the broth microdilution method in Mueller–Hinton broth (MHB)

(BD Biosciences, Sparks, MD, USA), following the recommendations of CLSI guidelines. Serial 2-fold dilutions of licochalcone E were prepared in sterile 96-well microplates over the concentrations range from 0.25 to 1,024 $\mu\text{g/ml}$. Following inoculation of 5×10^5 CFU/ml of overnight broth cultures in each well, the plates were incubated aerobically at 37°C for 24 h. The MIC was defined as the lowest concentration of licochalcone E at which the microorganism did not demonstrate visible growth. Oxacillin was used as a positive control.

Growth Curves

Overnight cultures of ATCC 29213 and 10832 were diluted in 600 ml of fresh MHB to obtain an initial $\text{OD}_{600\text{nm}}$ of 0.05 and grown at 37°C with 200 rpm shaking to an $\text{OD}_{600\text{nm}}$ of 0.3. The broth was divided equally into six 250 ml glass flasks. Each sample was treated with or without licochalcone E at a final concentration of 0, 1/16, 1/8, 1/4, 1/2, and 1 MIC. Bacteria were grown at 37°C with agitation at 200 rpm under aerobic conditions, and the growth rate of the cells was monitored by measuring the OD values at 600 nm at 1 h intervals.

Hemolysis Assay

S. aureus strains were cultured in MHB at 37°C with the indicated concentrations of licochalcone E until the bacteria reached the postexponential phase ($\text{OD}_{600\text{nm}}$ of 2.5 and 2.0 for strains ATCC 29213 and 10832, respectively). Hemolytic activity was assessed as described previously [26]. Briefly, 100 μl of washed rabbit erythrocytes ($5 \times 10^9/\text{ml}$) was added to 96-well V-bottom plates, filled with 100 μl of serially diluted bacterial culture supernatants and incubated for 20 min at 37°C. One percent saponin (Sigma) was used as a positive control, and PBS served as a negative control. Following centrifugation, the $\text{OD}_{450\text{nm}}$ of the supernatant fluid was determined. One unit of hemolytic activity was defined as the amount of test solution able to liberate half of the total hemoglobin from the erythrocytes.

Determination of Extracellular Protein Concentration and Western Blot Assay

S. aureus strains ATCC 29213 and 10832 were incubated with increasing concentrations of licochalcone E to the postexponential phase. The culture supernatants were precipitated by adding 100%

Table 1. Bacterial strains used in the study and their MICs to licochalcone E.

<i>S. aureus</i> strains	Description	Source	MIC ($\mu\text{g/ml}$)	
			Oxacillin	Licochalcone E
ATCC 29213	β -Lactamase-producing oxacillin-susceptible strain, α -toxin-producing strain	ATCC	0.25	2
ATCC 25923	A clinical isolate collected at Seattle in 1945	ATCC	0.25	4
ATCC 25904	Newman D2C	ATCC	0.5	4
ATCC 10832	Wood 46, a natural isolate that produces high levels of α -toxin	ATCC	0.125	2
8325-4	A high-level α -toxin-producing strain derived from NCTC 8325	Timothy J. Foster	0.125	1
BAA-1720	MRSA 252, a hospital-acquired strain isolated in the United Kingdom	ATCC	256	4
BAA-1717	USA300-HOU-MR, isolated from adolescent patient with severe sepsis syndrome in Texas Children's Hospital, α -toxin-producing strain	ATCC	128	4

trichloroacetic acid (Sigma) to a final concentration of 10%. After overnight incubation at 4°C, the precipitate was centrifuged at 15,000 ×g for 20 min at 4°C and finally washed three times with ice-cold (-20°C) ethanol. The aggregated proteins were dried by using a Speed-Vac for a few minutes. The protein extracts were dissolved in 0.5 ml of 0.1 M Tris. The protein concentrations were determined with a Bio-Rad (Munich, Germany) protein assay kit according to the instructions of the manufacturer.

Equal amounts of protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide (12%) gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membranes (Wako Pure Chemical Industries, Ltd, Osaka, Japan) using a semidry transfer cell (Bio-Rad). Membranes were incubated overnight at 4°C in 10% milk powder (a blocking reagent). An antibody to α -toxin was purchased from Sigma-Aldrich and diluted 1:8,000, and horseradish peroxidase-conjugated anti-rabbit antiserum (Sigma-Aldrich) diluted 1:4,000 was used as the secondary antibody. The blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

Real-Time RT-PCR

S. aureus ATCC 29213 was cultured with or without licochalcone E to the postexponential phase. The RNA was isolated as described previously [19]. Briefly, the cells were harvested by centrifugation (5,000 ×g for 5 min at 4°C) and resuspended in TES buffer containing 100 μ g/ml lysostaphin (Sigma-Aldrich). The samples were incubated at 37°C for 10 min, and total bacterial RNA was isolated using Qiagen RNeasy Maxi columns according to the manufacturer's instructions. RNase-free DNase I (Qiagen, Hilden, Germany) was used to remove contaminating DNA. The quality, integrity, and concentration of the purified RNA were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's protocol. The primer pairs used for real-time RT-PCR are listed in Table 2. cDNA was synthesized from total RNA using the Takara RNA PCR kit (AMV) Ver. 3.0 (Takara, Kyoto, Japan) according to the manufacturer's instructions. The PCRs were performed in 25 μ l reactions using SYBR Premix Ex Taq (Takara) as recommended by the manufacturer. PCR amplification was carried out using the 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). Reaction mixtures were initially incubated for 30 s at 95°C, followed by 35 cycles of 5 s at 95°C, 30 s at 55°C, and 20 s at 72°C. Melt-curve analysis was also performed to assess PCR specificity, resulting in single primer-specific melting temperatures. All samples were analyzed in triplicate, and the housekeeping gene 16S rRNA was used as an endogenous control. In this study, relative quantification based on the expression of the target gene relative to 16S rRNA was used to determine changes in transcription levels between samples.

Table 2. Primers used for real-time RT-PCR.

Primer	N315 ORF	Sequence (5'–3')
<i>16S rRNA</i> sense	SArRNA01	CGTGCTACAATGGACAATACAAA
<i>16S rRNA</i> antisense	SArRNA01	ATCTACGACTACTAGCGATTCCA
<i>hla</i> sense	SA1007	TGAATCCTGTCGCTAATG
<i>hla</i> antisense	SA1007	TATCATCCGACCTTTCACT
<i>agrA</i> sense	SA1844	TGAAATTCGTAAGCATGACCC
<i>agrA</i> antisense	SA1844	CATCGCTGCAACTTTGTAGAC

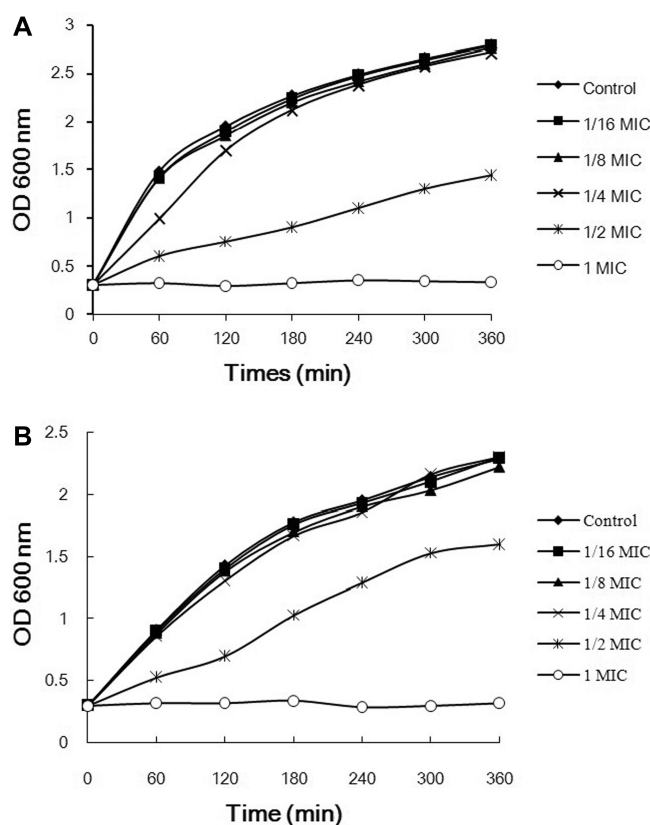


Fig. 2. Growth curves of *S. aureus* cultured with increasing concentrations of licochalcone E.

(A) *S. aureus* strain ATCC 29213. (B) *S. aureus* strain ATCC 10832.

Statistical Analysis

SPSS 13.0 statistical software was used to analyze experimental data. The independent Student's t-test was used to determine statistical differences, and a p value of less than 0.05 was considered to be statistically significant.

RESULTS

Licochalcone E Inhibits *S. aureus* Growth

In the study, the antibacterial activities of licochalcone E against eight *S. aureus* strains were determined. The data indicated that licochalcone E has potent antimicrobial activity against both methicillin-sensitive *S. aureus* (MSSA)

and methicillin-resistant *S. aureus* (MRSA) with MICs ranged from 1 to 4 µg/ml (Table 1).

The growth curves of *S. aureus* ATCC 29213 and 10832 cultured in the presence of increasing concentrations of licochalcone E are shown in Fig. 2. Clearly, the addition of 1/16, 1/8, and 1/4 MIC of licochalcone E had no significant influence on the growth of *S. aureus*. However, when 1/2 MIC of licochalcone E is added to the culture, the growth rate was markedly decreased. When exposed to 1 MIC of licochalcone E, the growth of *S. aureus* was completely inhibited.

Licochalcone E Reduces Hemolysis of *S. aureus* by Decreasing the Production of α-Toxin

Strains ATCC 29213 and 10832 were cultured with increasing concentrations of licochalcone E, and the bacterial culture supernatants were subjected to hemolysis assay. As shown in Fig. 3, the hemolytic units (HUs) in drug-free culture fluids were 65.1 and 120.3 for *S. aureus* ATCC 29213 and 10832, respectively. When cultured with 1/4 MIC of licochalcone E, the HUs were reduced to 7.2 and 5.4 for ATCC 29213 and 10832, respectively. As expected, a dose-dependent attenuation of hemolysis was observed in all the tested strains.

Among the secreted virulence factors of *S. aureus*, α-toxin is the major toxin that is responsible for the hemolytic activity of *S. aureus*. Therefore, the supernatants were also used for Western blot analysis to determine the α-toxin levels. As shown in Fig. 4, licochalcone E decreased, in a dose-dependent manner, the production of α-toxin. Addition of 1/32 MIC of licochalcone E resulted in a recognizable reduction in α-toxin secretion; when at 1/4 MIC, no immunoreactive protein could be detected in

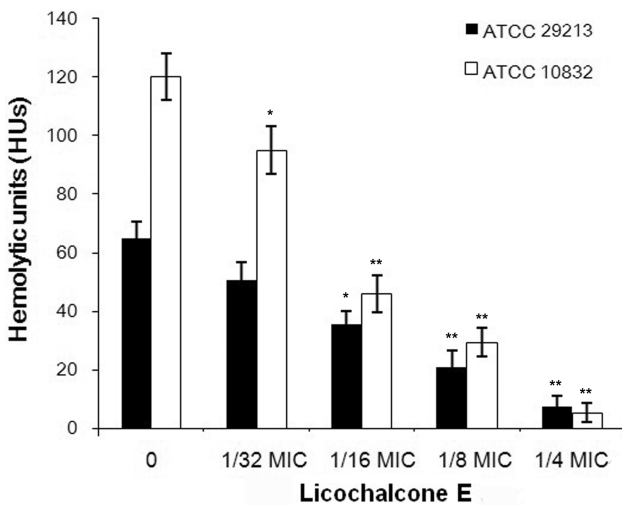


Fig. 3. Hemolytic activities of *S. aureus* culture supernatants grown in the presence or absence of licochalcone E. *represents p < 0.05 and **represents p < 0.01.

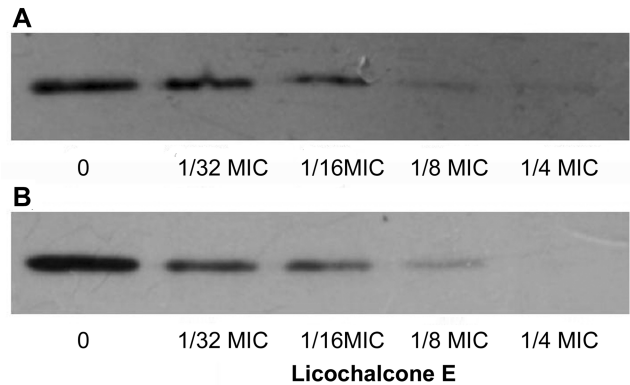


Fig. 4. α-Toxin production in *S. aureus* ATCC 29213 (A) and 10832 (B) after treatment with increasing concentrations of licochalcone E.

ATCC 29213 and 10832. Furthermore, the addition of 1/32, 1/16, 1/8, and 1/4 MIC of licochalcone E had no significant influence on *S. aureus* extracellular protein concentration (data not shown).

Licochalcone E inhibits *S. aureus hla* and *agrA* Transcription

To determine whether the decreased production of α-toxin in the licochalcone-E-treated culture supernatants was due to a reduction in *hla* (encoding α-toxin) transcription, real-time RT-PCR analysis was used to quantitate mRNA levels of the investigated gene in *S. aureus* cultures after treatment with different concentrations of licochalcone E. As the expression of *hla* is positively regulated by the *agr* locus [3], the transcription of *agrA* was also assessed. As expected, licochalcone E markedly decreased the transcription of these genes in *S. aureus* strain ATCC 29213 in a dose-dependent manner. When grown in the presence of 1/4

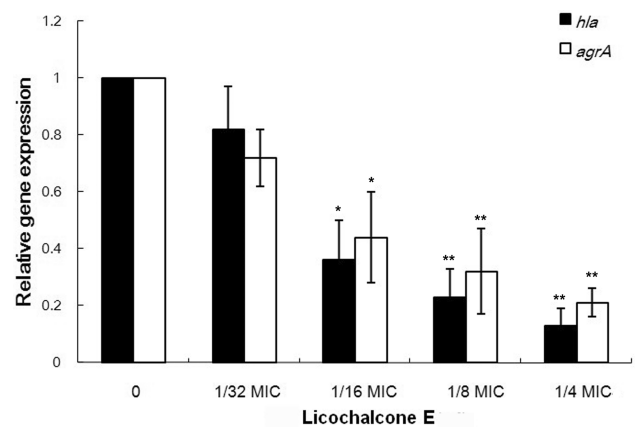


Fig. 5. Relative gene expression levels of *hla* and *agrA* in *S. aureus* ATCC 29213 following exposure to different doses of licochalcone E. *represents p < 0.05 and **represents p < 0.01.

MIC concentration of licochalcone E, the transcriptional levels of *hla* and *agrA* were decreased by 7.7- and 4.6-fold, respectively (Fig. 5).

DISCUSSION

The antibiotic resistance of bacteria is one of the most serious challenges in clinical medicine today. Therefore, development of novel drugs against the drug-resistant bacteria is highly desired. Previous studies have shown that many licorice phenolics have potent antibacterial activity on both methicillin-sensitive *S. aureus* (MSSA) and MRSA, for example, chalcones, isoflavones, isoflavanones, and isoflavans [10]. Licochalcone E is a phenolic compound of licorice root that was isolated in 2005 by Cheon's group [27]. Presently, no published reports on the biological activities of licochalcone E have referred to its antimicrobial property. In this study, we demonstrated that licochalcone E could markedly inhibit the growth of *S. aureus* with MICs ranged from 1 to 4 $\mu\text{g/ml}$. These data suggest that licochalcone E is a potentially effective antimicrobial against *S. aureus*, and it may warrant further investigation as a potential therapeutic against *S. aureus* infections. Furthermore, the licochalcone E structure could be used as the basis for development of novel anti-*S. aureus* drugs. When compared with the data of Hatano's report [10], the anti-*S. aureus* activity of licochalcone E was superior than other licochalcones (as reported by Hatano *et al.*, the MICs of licochalcones A, B, and echinatin were 16, 128, and greater than 64 $\mu\text{g/ml}$, respectively).

The growing problem of antibiotic-resistant microorganisms has necessitated alternative therapeutic strategies. The ability of *S. aureus* to cause disease, to a great extent, relies on the production of an impressive collection of virulence factors, including α -toxin, protein A, coagulase, enterotoxins and toxic shock syndrome toxin-1, among others [20]. For instance, α -toxin plays an essential role in *S. aureus* pneumonia, as strains lacking this toxin are avirulent in a murine model of disease [21]. Therefore, antivirulence therapy is an alternative strategy that is now gaining considerable interest for use in treating *S. aureus* infections.

It has been well-recognized that the production of virulence factors may be interfered with by certain antibiotics, especially when used at subinhibitory concentrations [23]. Therefore, besides bactericidal or bacteriostatic activities, the influence on toxin production is also a significant consideration in the selection of an antimicrobial agent to treat with *S. aureus* infections. For example, β -lactam antibiotics proved unfavorable for the management of toxin-related *S. aureus* infections, as even subinhibitory concentrations resulted in an increase in α -toxin production due to a stimulatory effect on exoprotein synthesis [14]. In contrast, protein-synthesis-inhibiting antibiotics such as

clindamycin, linezolid, and quinupristin/dalfopristin are recommended for the treatment of *S. aureus*-produced toxic syndromes since concentrations below the MIC have been demonstrated to decrease the secretion of exotoxins in *S. aureus* (e.g., α -toxin) [12, 13, 18]. Ingmer *et al.* [11] suggested the importance of searching for new agents that interfere with *S. aureus* virulence gene expression. In this study, we demonstrated, *via* phenotypic, expressional, and transcriptional analyses, that subinhibitory concentrations of licochalcone E result in a dose-dependent decrease in α -toxin expression in *S. aureus*. These data indicate that licochalcone E may potentially be useful to treat *S. aureus* infections when used in combination with β -lactam antibiotics, which increase α -toxin production at sublethal concentrations. In addition, the structure of licochalcone E may be applied as a basis for developing novel anti-*S. aureus* antibiotics targeting bacterial virulence factors.

The expression of α -toxin in *S. aureus* is co-regulated by a network of global regulators, such as Agr, Sar, Sae, among others [3]. In this study, we found that the transcription of *agrA* was inhibited by licochalcone E. Therefore, we infer that the reduced α -toxin production in *S. aureus* may be, in part, due to the licochalcone-E-induced inhibition of the agr two-component system, although other regulators may also be influenced.

Acknowledgments

We would like to thank Professor Jizhen Li of Jilin University for kindly providing licochalcone E, and Professor Timothy J. Foster of Trinity College for kindly providing *S. aureus* strains 8325-4. This work was supported by the Key Project of Agricultural Science and Technology Research of Liaoning Province (No. 2011214001) and Liaoning BaiQianWan Talents Program (No. 2010921043).

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