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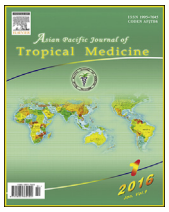

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Potentiating activity of luteolin on membrane permeabilizing agent and ATPase inhibitor against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the mechanism of antibacterial activity of luteolin (LUT) against methicillin-resistant *Staphylococcus aureus* (MRSA).
Methods: The mechanism of anti-MRSA activity of LUT was analyzed by the viability assay in membrane permeabilizing agent, ATPase inhibitors, and peptidoglycan (PGN) derived from *Staphylococcus aureus* (*S. aureus*). Also, transmission electron microscopy was used to monitor survival characteristics and changes in *S. aureus* morphology.
Results: Compared to the LUT alone, the optical density of suspensions treated with the combination of 125 μg/mL Tris and 250 μg/mL *N*,*N*′-dicyclohexylcarbodiimide were reduced to 60% and 46% of the control, respectively. PGN (15.6 μg/mL) gradually impeded the activity of LUT, and PGN (62.5 μg/mL) completely blocked the activity of LUT on *S. aureus*.
Conclusions: Increased susceptibility to LUT with the Tris-dicyclohexylcarbodiimide combinations is evident in all tested MRSA isolates. The results indicate LUT synergy in increasing cytoplasmic membrane permeability and inhibiting ATPase. *S. aureus* PGN directly blocks the antibacterial activity of LUT, suggesting the direct binding of LUT with PGN. These findings may be validated for the development of antibacterial agent for low MRSA resistance.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961, soon after the introduction of methicillin into clinical use. MRSA is resistant to most β-lactam antibiotics including penicillins and cephalosporins [1]. MRSA accounts for nearly 70 percent of *Staphylococcus aureus* (*S. aureus*) infections, and is the main cause of community-acquired and healthcare-associated infections [2]. The mechanisms of bacterial resistance against antibiotics are inactivation of antibiotics by enzymes, change in the target site, change of membrane permeability, and antibiotic efflux out of cells [3,4]. Therefore, alternative therapeutic strategies involving effective antimicrobial agents that minimize bacterial resistance to antibiotics are needed.

Luteolin (LUT), a well-known flavonoid polyphenolic compound (Figure 1), is found in many plant groups including Bryophyta, Pteridophyta, Pinophyta, and Magnoliophyta. LUT has diverse biological benefits that include cardioprotection, antioxidantion, anti-inflammation, anti-cancer, and antimicrobial effects [5–9]. Qio *et al*. reported the impact of LUT on the alpha-

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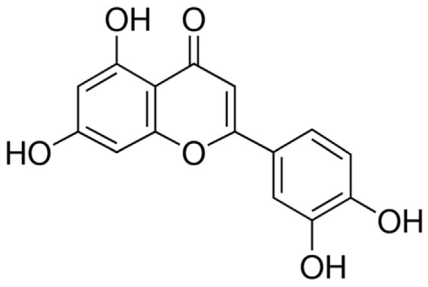

Figure 1. Structure of LUT.

toxin produced by *S. aureus*; LUT decreased production of the toxin [10]. In the present study, we investigated the anti-MRSA activity of LUT on the membrane-binding agent and ATPase-inhibiting agents. We also confirmed that binding effect of LUT on the bacterial cell wall. Bacterial ultrastructural changes following treatment with LUT were assessed by transmission electron microscopy (TEM).

2. Materials and methods

2.1. Reagents

N,N′-dicyclohexylcarbodiimide (DCCD) and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mueller-Hinton broth (MHB) was purchased from Difco (Baltimore, MD, USA). Tris-(hydroxymethyl) aminomethane (Tris) was obtained from AMRESCO (San Francisco, CA, USA). Peptidoglycan (PGN) was purchased fromFluka (Basel, Switzerland).

2.2. Bacterial strains and growth conditions

S. aureus ATCC 33591 (methicillin-resistant strain) and *S. aureus* ATCC 25923 (methicillin-susceptible strain) were stored in 30%glycerol and frozen at −70 °C. They were cultured in MHA and MHB, and incubated at 37 °C for 24 h for each experiment.

2.3. Minimum inhibitory concentration (MIC)

MIC was determined using the broth micro dilution method according to the Clinical and Laboratory Standards Institute 2006 guideline [11]. LUT was diluted in MHB in 96-well plate (0.5% w/v stock concentration). Preparation of the microorganism suspension was prepared by growing *S. aureus* in broth for 24 h, and adjusting the suspensions to 0.5 McFarland standard turbidity (approximately 1.5×10^8 CFU/mL). The final inoculum was adjusted to 1.5×10^6 CFU/mL. Inoculated broth in wells was incubated at 37 °C for 18 h. At the end of each incubation period, turbidity indicated that bacterial growth had not been inhibited by the concentration of antimicrobial agent in the medium. MIC was defined at the lowest concentration of antibiotics and LUT that inhibited growth.

2.4. Effect of LUT on membrane-permeabilizing agent or ATPase inhibitors

To elucidate whether the antibacterial activity of LUT was associated with either altered membrane permeability or ATP synthase inhibit, the antibacterial activity of LUT was examined in the presence of membrane-permeabilizing agent, Tris and adenosine triphosphatase (ATPase)-inhibiting agents, N,N-DCCD respectively. Tris was used to increase membrane permeability of cell membranes [1]. DCCD, a metabolic inhibitor that could decrease ATP levels by disrupting electrochemical proton gradients in a bacterial environment, was used as an inhibitor of ATPase [12]. The effect of LUT on the membrane-permeabilizing agent (125 μg/mL Tris) or ATPase inhibitor (250 μg/mL DCCD) was determined.

2.5. Effect of PGN on LUT activity

To determine the activity of PGN in the presence of LUT, a LUT + PGN combination assay was performed [13]. This assay indicated whether LUT bound to PGN, the major constituent of the *S. aureus* cell wall. LUT (31.25 μg/mL) was added to PGN by serial dilution. LPS, a constituent of the outer membrane of Gram-negative bacteria [14], was used as negative control.

Table 1
MIC of LUT against two strains of *S. aureus* used in this study.

Strains	MIC (μg/mL)	
	LUT	Oxacillin
MSSA (ATCC 25923)	62.5	62.5
MRSA (ATCC 33591)	62.5	500.0

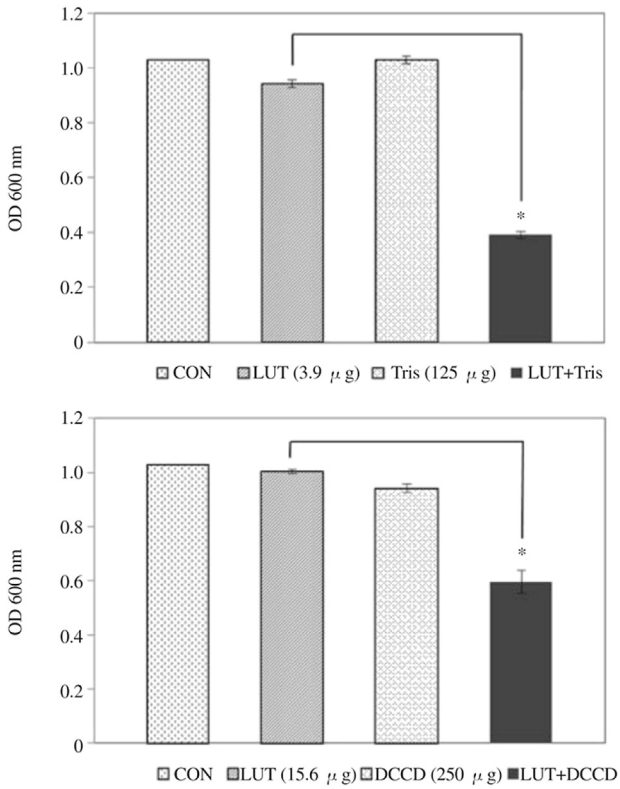

Figure 2. Effect of membrane-permeabilizing agent Tris and ATPase-inhibitor *N,N′*-DCCD on susceptibility of *S. aureus* (ATCC 33591) to LUT. The viability of bacteria was determined spectrophotometrically (optical density at 600 nm, OD 600 nm) after incubation for 36 h with 3.9 μg/mL LUT with 125 μg/mL Tris and 250 μg/mL DCCD. The data was the average of three independent experiments. *$P < 0.01$ as compared to LUT alone, were determined.

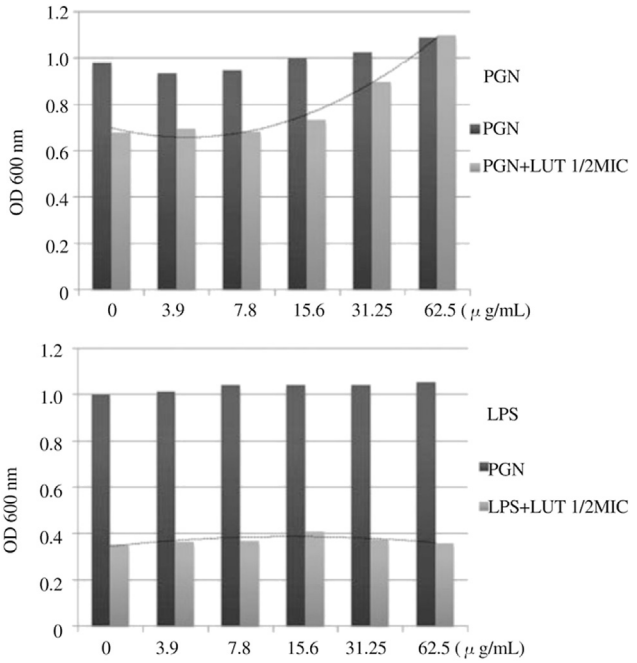

Figure 3. Direct binding of LUT with PGN in cell wall of *S. aureus* (ATCC 25923).
PGN was added to MHB containing LUT. LPS was used as a control.

2.6. TEM

MRSA exponential-phase cultures were prepared by diluting cultures into MHB overnight, which was continued at 37 °C until the cultures reached the mid-logarithmic phase of growth. MHB-grown exponential-phase MRSA was treated with 1/2 MIC and the MIC of LUT for 30 min. Following the treatment, 2 mL of the culture was collected by centrifugation at 10000 *g* for 10 min. After removal of the supernatant, pellets were fixed with modified Karnovsky's fixative. The specimens were examined with an energy-filtering LIBRA 120 TEM (CarlZeiss, Oberkochen, Germany) at an accelerating voltage of 120 kV. Transmitted electron signals were recorded using anUltrascan4000 SP 4 k × 4 k slow-scan charge-coupled device camera (Gatan, Pleasanton, CA, USA) attached to the electron microscope.

2.7. Statistical analyses

All experiments were performed more than three times. Data from the experiments are presented as the mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's *t* test (SPSS software version 19.0; IBM SPSS, Armonk, NY, USA). $P < 0.01$ was considered statistically significant difference.

3. Results

3.1. Potentiated effects of LUT by Tris and DCCD

MIC values of LUT against two strains of *S. aureus* were presented in Table 1. Compared to the optical density at 600 nm (OD_{600}) value of LUT alone (3.9 g/mL), the OD_{600} of suspensions treated with the combination of 125 μg/mL Tris was reduced to 60%.Bacterial viability in the presence of LUT with 250 μg/mL DCCD, a metabolic inhibitor, as reduced to 46% compared to LUT alone (3.9 g/mL) (Figure 2).

3.2. Binding of LUT and PGN

The binding of LUT with PGN was confirmed by the addition of PGN (0.0–62.5 μg/mL) derived from *S. aureus* into MHB containing LUT. As shown in Figure 3, LUT (31.25 μg/mL) inhibited *S. aureus* growth by over 25%. A 62.5 μg/mL concentration of PGN disturbed the activity of LUT and 15.6 μg/mL concentration of PGN impeded the activity of LUT on *S. aureus*, while 62.5 μg/mL PGN completely blocked the antibacterial activity of LUT.

3.3. TEM

Antibiotic drugs induced other cellular changes such as separation of cytoplasmic contents [15]. The antibacterial activity of LUT on MRSA might be due to the ability of LUT to disrupt the cell wall of MRSA. LUT induced membrane disruption and cell lysis. LUT-free cultured cells had a normal morphology of *S. aureus* with distinct septa and smooth surfaces (Figure 4A). Cells treated with LUT (31.25 μg/mL) appeared to have damaged cytoplasmic membrane and had a rougher surface (Figure 4B). MRSA cells

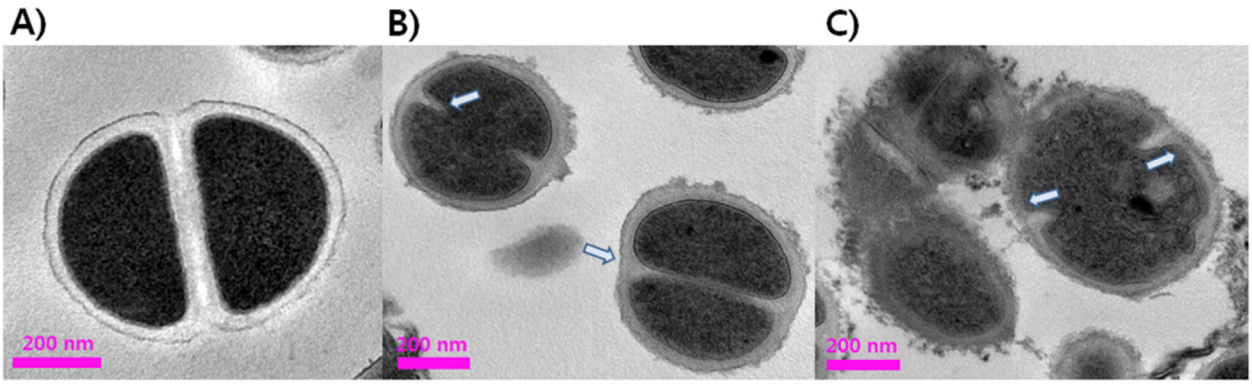

Figure 4. TEM images of MRSA (ATCC 33591) after 24 h of LUT treatment.
(A) MRSA in the untreated control appeared to have intact membrane. (B) MRSA treatment with 1/2 MIC of LUT (31.25 μg/mL). Arrows indicate hampered membrane integrity and caused membrane damage. (C) MRSA treatment with MIC LUT (62.5 μg/mL). Arrows indicate cytoplasmic membrane disruption and separated cell.

treated with LUT (62.5 μg/mL) were disrupted with reduced intracellular contents (Figure 4C).

4. Discussion

There is a clear need for development of new antibiotic that are widely effective against multidrug-resistant pathogens. We examined the effects of the membrane permeabilizing agent and ATP-binding cassette (ABC) transporter inhibiting agent on antibacterial activity of LUT. Most bacteria produce ABC transporter that is an essential uptake system for amino acids in the bacterial membrane. This is a determinant of bacterial antibiotic resistance [15–17]. ABC transporters have ATP-dependent transporting activity; DCCD inhibits the H^+ translocation activity of the F_0 domain of F_0F_1-ATPase [14]. In MRSA, LUT showed synergistic activity by increasing cytoplasmic membrane permeability and inhibiting ATPase. Gram-positive bacteria including *S. aureus* contain numerous layers (up to 30) of PGN. In *S. aureus* and other Gram-positive and Gram-negative bacteria, PGN is essential in osmotic protection and cell division [18]. Wall teichoic acids of *S. aureus* are anionic glycopolymers cross-linked to the thick PGN network, serving as a primer for cell wall biosynthesis [14,15]. The direct binding of PGN and LUT completely interrupts LUT-induced damage of the bacterial cell wall. The focus of the present study was on the development of natural antimicrobial agents to directly address the multidrug resistant of *S. aureus*. These findings can be important indication in study on mechanism of antimicrobial activity against MRSA *in vitro*. Further, *in vivo* experiments are needed for clinical application in MRSA-infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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