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Potentiating activity of rhein in targeting of resistance genes in methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Objective: To investigate the synergistic effect between rhein (RHE) and oxacillin against *Staphylococcus aureus* (MRSA) at the gene level. **Method:** A minimum inhibitory concentration and checkerboard dilution test were conducted to evaluate antibacterial activity. Reverse transcriptase polymerase chain reaction was conducted to investigate the gene expressions. **Results:** RHE exhibited a minimum inhibitory concentration of 62.5–250.0 µg/mL against various MRSA strains and the reference strain, respectively. As revealed by the checkerboard assay, a combination of RHE and oxacillin exhibited synergistic or partially synergistic effects against MRSA strains. RHE decreased the expressions of *mecA/blaZ* in a dose-dependent manner. RHE also decreased the expressions of the regulator genes *mecI/blaI* and *mecRI/blaRI*. **Conclusions:** We suggest that RHE affects the activity of *mecRI/blaRI*, which is located in the cell membrane of MRSA and results in the suppression of *mecA/mecI/mecRI* and *blaZ/blaI/blaRI* gene expressions.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) causes infections ranging from skin and soft tissue infection to more severe diseases, including bacteremia, osteomyelitis, arthritis, pneumonia, sepsis, and pericarditis[1]. β-Lactams, which are antibiotics that disrupt cell wall synthesis by targeting the four *S. aureus* penicillin-binding proteins involved in the transpeptidation of peptidoglycan, were commonly used to treat bacterial infection[2]. However, MRSA has recently developed two major resistance mechanisms against β-lactam antibiotics. First, bacteria produce β-lactamases, a family of enzymes that inactivate β-lactam

antibiotics, such as penicillins, carbapenems, and monobactams by hydrolyzing their β-lactam rings[3]. Second, the production of penicillin-binding protein 2A (PBP2a) is a major determinant of β-lactam resistance. PBP2a has a low affinity for β-lactams, thereby allowing it to maintain its transpeptidation activity in the presence of β-lactam antibiotics[4]. β-lactamase and PBP2a are encoded by *blaZ* and *mecA*, respectively; these are located in a mobile genetic element known as the Staphylococcal cassette chromosome *mec* (SCC*mec*). The transcription of *mecA* and *blaZ* is regulated by the *mecI-mecRI* and *blaI-blaRI* systems, respectively. The *mec* and *bla* operons are controlled by two-component systems comprising the repressors *mecI* and *blaI* and the sensors *mecRI* and *blaRI*. The

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genes for *mecA/blaZ*, its repressors *mecI/blaI*, and the transducer-sensors *mecR1/blaR1* are clumped together, either on a plasmid or within the bacterial chromosome. In the absence of β -lactam, the DNA repressor MecI/BlaI suppresses *mecA/blaZ* by binding to the conserved DNA motif TACA/TGTA, which is located in the promoter region of *mecA/blaZ*. However, when exposed to β -lactam antibiotics, acylation of *BlaR1/MecR1*, which is a transmembrane and signal transduction protein, is subsequently followed by autoproteolytic cleavage on the cytoplasmic side of the cell membrane. The cleaved intracellular fragment of *MecR1/BlaR1* then moves to the bacterial chromosome where it removes its cognate repressor MecI/BlaI by proteolysis. Transcription of the *mecA/mecR1/mecI* and *blaZ/blaR1/blaI* genes begins once the repressor dissociates from the promoter region[5-7].

To overcome β -lactam resistance, antibiotics with different modes-of-action or agents that inhibit drug resistance should be used concurrently. Therefore, antibacterial agent combination therapy appears useful because it can enhance antibacterial activity and reduce drug resistance[8]. Rhein (RHE) is a lipophilic anthraquinone compound found in rhubarb (*Rheum palmatum* L.), *Reynoutria japonica* (Houtt.), and *Fallopia multiflora*, and is extensively used in treating various clinical disorders, such as hepatic disease, osteoarthritis, atherosclerosis, various cancers, and diabetic nephropathy (Figure 1). RHE was identified as a major metabolite of diacerein, a prodrug used in treating osteoarthritis[9-12]. In addition, RHE has been reported to possess antibacterial activity and synergistic effects against MRSA when combined with antibiotics such as ampicillin (AM) or oxacillin (OX)[13].

However, there is no reports which state that RHE inhibits the drug resistance mechanism of MRSA at the molecular level. Here, we investigated the synergistic effect of RHE combined with antibiotics on MRSA drug resistance at the gene level.

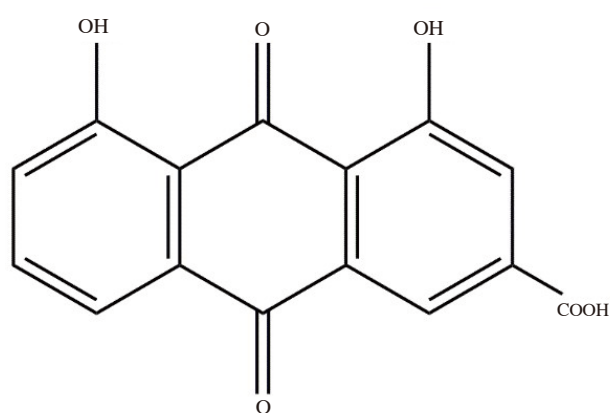


Figure 1. Chemical structure of rhein.

2. Materials and methods

2.1. Chemicals

RHE (4,5-dihydroxyanthraquinone-2-carboxylic acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

dimethyl sulfoxide (DMSO), AM, and OX were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mueller-Hinton (MH) broth and MH agar were purchased from Difco Laboratories (Baltimore, MD, USA).

2.2. Bacterial strains and growth conditions

Strains 25923 and 33591 were purchased from the American Type Culture Collection (ATCC). Clinical isolates of bacterial strains (DHCR 1-15) were obtained from the Department of Plastic Surgery of Wonkwang University Hospital (Iksan, Korea). Before experimentation, all bacteria were stored in 30% glycerol and were frozen at -70°C . All strains were maintained on MH agar plates or nutrient agar, and antibacterial assays were conducted using MH broth. Bacterial growth was monitored as a function of turbidity by measuring optical density (OD) at 600 nm[14,15].

2.3. Determination of minimum inhibitory concentration (MIC)

MICs were determined using the method described in the Clinical and Laboratory Standards Institute (CLSI)[16]. The RHE MIC determinations with MRSA were performed using 96-well microtiter plates. Briefly, RHE was dissolved in DMSO followed by MH broth (final DMSO concentration=15% v/v). The suspension was serially diluted using 15% DMSO in MH broth after which 10^8 CFU/mL of an overnight bacterial culture was inoculated into each well. The final volume in each well was 150 μL , and the DMSO concentration was 5% (which has no influence on bacterial growth). The DMSO concentration used is extremely important, because high concentrations can influence the activity of RHE. The samples were then incubated at 37°C for 24 h. Twenty microliters of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1 mg/mL) was added to the suspension and incubated at 37°C for 30 min. Clear yellowish-colored wells indicated inhibition of microbial growth, whereas dark blue-colored wells indicated the absence of inhibition. The MIC was defined as the lowest concentration producing no visible growth, as observed through the naked eye[17].

2.4. Checkerboard dilution test

The checkerboard dilution test was used to measure the synergistic effects of RHE and OX. OX was mixed in MH broth and serially diluted with RHE. The bacterial concentration was adjusted to 1.5×10^7 CFU/mL. Suppression of microbial growth was checked for after incubation for 24 h at 37°C . Each experiment was conducted in triplicates. The interaction between RHE and OX was determined using the fractional inhibitory concentration index.

2.5. Reverse transcriptase polymerase chain reaction (PCR)

MRSA (ATCC 33591) was cultured in MH broth until an OD₆₀₀ of 0.35–0.45 was reached; the culture was then treated with various concentrations of OX and RHE and incubated for 20 min at 37 °C. RNA was extracted using the E.Z.N.A.® bacterial RNA kit (Omega Bio-tek, Norcross, GA, USA), in accordance with the manufacturer's instructions. Single-strand cDNA synthesis was performed using the Quantitact Reverse Transcription Kit, in accordance with the manufacturer's instructions. PCR was performed using the Maxime PCR PreMix (20 µL; iNtRon Biotechnology, Inc., Seongnam, Korea), primers, cDNA sample, and sterile distilled water. Primer sequences of *mecA*, *mecI*, *mecR1*, *blaZ*, *blaI*, *blaR1*, and *16s rRNA* are presented in Table 1. After PCR, equal amounts of the PCR products (12 µL) were subjected to 1.5% agarose gel electrophoresis; bands were visualized under ultraviolet light.

Table 1
RT-PCR primer sequences.

Primer	Sequence (5' → 3')
<i>blaZ</i> -F	GATAAGAGATTTGCCTATGC
<i>blaZ</i> -R	GCATATGTTATTGCTTGACC
<i>blaI</i> -F	GCAAGTTGAAATATCTATGG
<i>blaI</i> -R	GAAAGGATCCATTTTCTGTACTCTCATC
<i>blaR1</i> -F	CATGACAATGAAGTAGAAGC
<i>blaR1</i> -R	CTTATGATTCCATGACATACG
<i>mecA</i> -F	ATGAGATTAGGCATCGTTCC
<i>mecA</i> -R	TGGATGACAGTACCTGAGCC
<i>mecI</i> -F	CTGCAGAATGGGAAGTTATG
<i>mecI</i> -R	ACAAGTGAATTGAAACCGCC
<i>mecR1</i> -F	AAGCACCGTACTATCTGCACA
<i>mecR1</i> -R	GAGTAAATTTGGTTCGAATGCC
<i>16s rRNA</i> -F	CGTGCCTAATACATGCAAGTC
<i>16s rRNA</i> -R	CCGTCTTTCACITTTTGACCA

2.6. Statistical analyses

The statistical analysis was assessed using one-way analysis of variance, followed by Scheffe's test for multiple comparisons. Data were presented as means±standard deviations. All calculations were assessed using SPSS statistics 23 software (SPSS Inc.Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of antibacterial activity

To examine the antibacterial activity of RHE, AM, and OX, antibacterial susceptibility testing was conducted on MRSA using the CLSI method. AM and OX, which are β -lactam antibiotics capable of inhibiting bacterial cell wall synthesis, were used as positive controls. RHE exhibited similar MICs in all tested strains. The RHE MIC values of all tested strains (ATCC and clinical isolates) ranged from 62.5 to 250.0 µg/mL (62.5 µg/mL for ATCC 33591), whereas AM MICs ranged from 0.97 to 125.00 µg/mL. The MIC for OX against ATCC 33591 was 1 000 µg/mL; MIC values ranged from 250 to 1 000 µg/mL for other strains. The results of the MIC assay are presented in Table 2.

Table 2

MICs of ampicillin, oxacillin, and rhein against various *S. aureus* strains (µg/mL).

<i>S. aureus</i> strains	RHE	AM	OX
ATCC 33591	62.5	62.50	1 000.00
ATCC 25923	62.5	0.97	1.95
DHCR 1	62.5	31.25	1 000.00
DHCR 2	62.5	31.25	1 000.00
DHCR 3	62.5	31.25	500.00
DHCR 4	62.5	31.25	1 000.00
DHCR 5	125.0	31.25	1 000.00
DHCR 6	250.0	31.25	500.00
DHCR 7	250.0	62.50	500.00
DHCR 8	250.0	125.00	500.00
DHCR 9	250.0	125.00	500.00
DHCR 10	125.0	62.50	500.00
DHCR 11	125.0	125.00	500.00
DHCR 12	62.5	125.00	500.00
DHCR 13	250.0	125.00	500.00
DHCR 14	250.0	62.50	1 000.00
DHCR 15	250.0	125.00	500.00

DHCR: Staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.

3.2. Synergistic effects of RHE/antibiotic combinations against MRSA

A checkerboard assay was conducted to investigate the antibacterial effect of RHE combined with OX against MRSA. The combination of RHE and OX indeed exhibited synergistic effects against ATCC33591 and DHCR-1 and partial synergistic effects against DHCR-2 (Table 3).

Table 3

Synergy effects of rhein combined the β -lactam antibiotic oxacillin against *S. aureus* strains.

<i>S. aureus</i> strains	MIC of RHE (µg/mL)		MIC of antibiotics (µg/mL)		FICI	Overcome
	RHE alone	With OX	OX alone	With RHE		
ATCC 33591	62.5	15.60	1 000	125.0	0.375 0	Synergistic
DHCR-1	62.5	15.60	1 000	62.5	0.312 5	Synergistic
DHCR-2	62.5	31.25	1 000	250.0	0.750 0	Partial synergy

Synergistic combinations were investigated using the checkerboard method. The fractional inhibitory concentration indices (FICI) were interpreted as follows: synergy < 0.5; partial synergy 0.50–0.75; additive effect 0.76–1.0; indifference > 1.0–4.0; and antagonism > 4.0. MIC: minimum inhibitory concentration.

3.3. Polymerase chain reaction (PCR) analysis

RT-PCR was performed to evaluate the inhibitory effects of RHE against the MRSA resistance genes. RHE decreased the expression of *mecA/blaZ* in a dose-dependent manner. In contrast, their expression was increased by OX and again decreased with a combination of RHE and OX. In addition, the expression of the regulator genes *mecI/blaI* and *mecR1/blaR1* also decreased in the presence of RHE or RHE combined with OX (Figure 2 and 3).

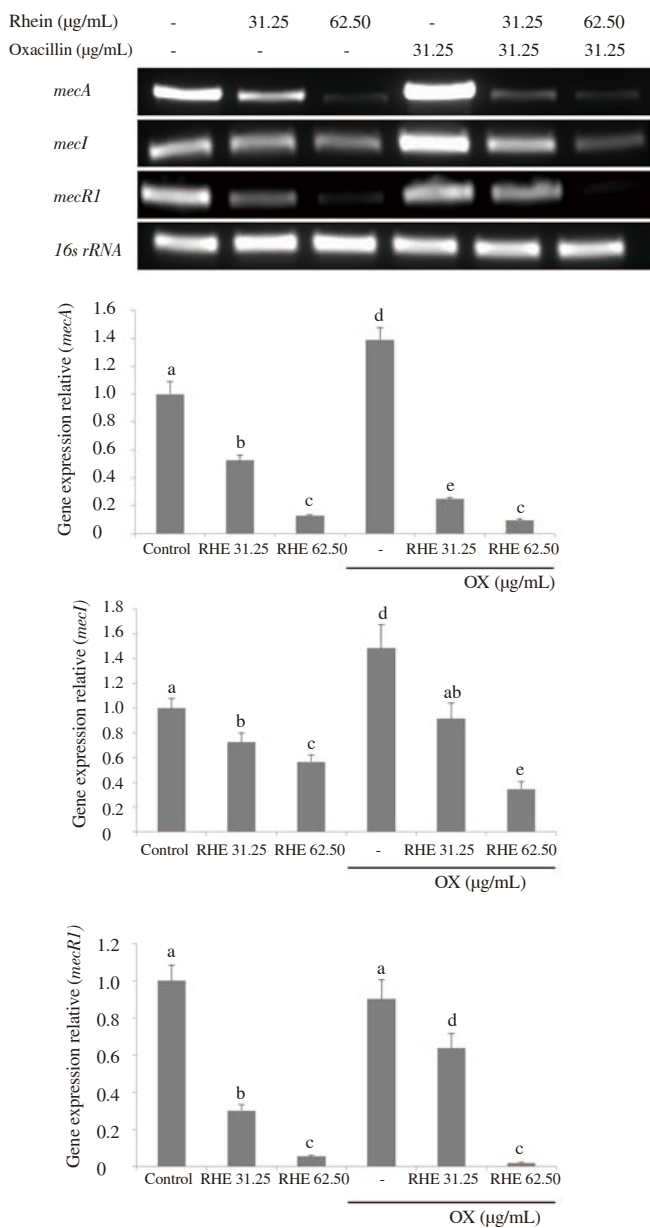


Figure 2. Expression of PBP2a-related genes.

PCR showing the expression of *mecA*, *mecI*, and *mecR1* of MRSA exposed to various concentrations of rhein alone or in combination with 31.25 μg/mL oxacillin. *16s rRNA* was used as a loading control, and expression levels were normalized to *16s rRNA* levels. Different letters (a-e) indicate significant differences at $P < 0.05$ based on analysis of variance followed by the Scheffe's test for multiple comparisons. Values are presented as mean ± SD.

4. Discussion

Drug resistant bacteria are a menace to the healthcare system, because they limit remedies to infections worldwide. Resistance can occur through the acquisition of the resistance gene by horizontal gene transfer or through spontaneous mutation. Both *mecA* and *blaZ* play an important role in the β-lactam resistance of MRSA. The chromosomal gene *mecA* encodes PBP2a, an alternative penicillin-binding protein having low affinity for β-lactams; PBP2a takes over the function of other PBPs that have been inactivated by β-lactam antibiotics[18]. The *blaZ* gene encodes β-lactamase, which inactivates

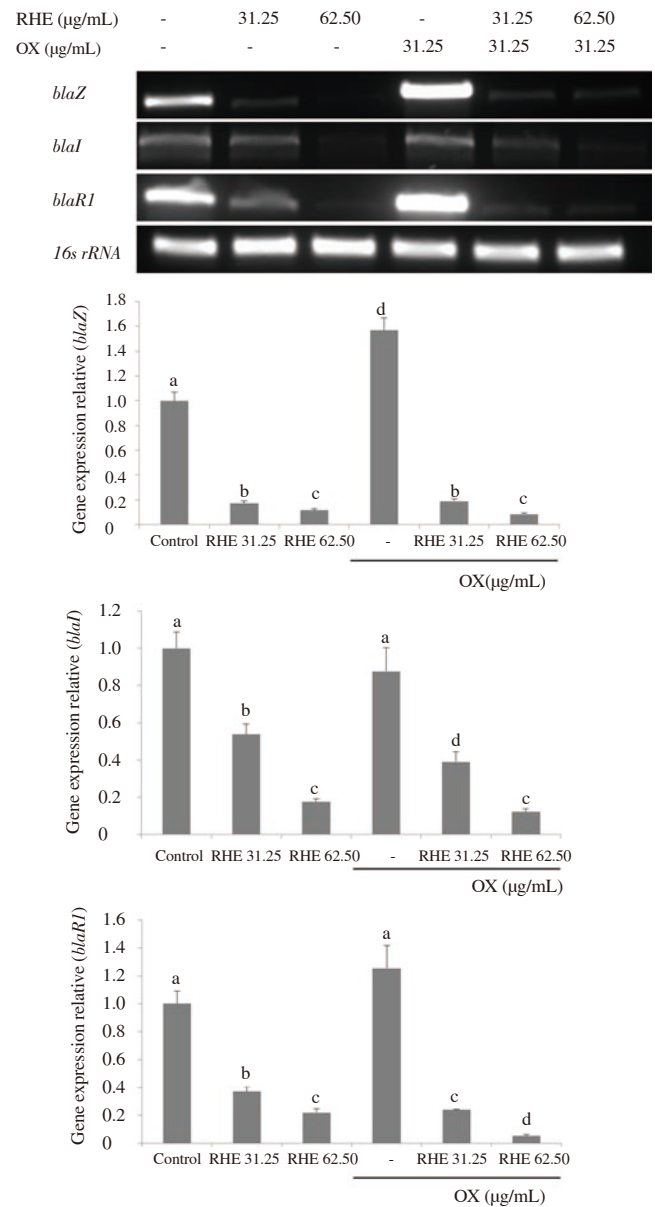


Figure 3. Expression of β-lactamase-related genes.

PCR showing the expression of *blaZ*, *blaI*, and *blaR1* of MRSA exposed to various concentrations of rhein alone or in combination with 31.25 μg/mL oxacillin. *16s rRNA* was used as a loading control and expression levels were normalized to *16s rRNA* levels. Different letters (a-d) indicate significant differences at $P < 0.05$, based on analysis of variance followed by the Scheffe's test for multiple comparisons. Values are presented as mean ± SD.

penicillin by hydrolysis of the β-lactam ring[19]. Given restricted remedy in the research of novel antibacterial agent, there is notable study in targeting resistance genes to help re-establish susceptibility to commercial antibiotics[20]. Several researchers have previously reported that some antibacterial substances target these two β-lactam resistance genes, *mecA* and *blaZ* genes[21,22]. Therefore, we performed the study on antibacterial compound targeting β-lactam resistance genes. A previous study described the synergistic effect of RHE combined with AM or OX against MRSA[13]. We hypothesized that RHE produces a synergistic effect with OX in MRSA, because RHE suppresses β-lactam resistance genes. The role of PBP2a and β-lactamase are important in β-lactam resistance, and these proteins

are expressed by the transcription of *mecA* and *blaZ*, respectively. In the presence of OX, acylation of *BlaR1/MecR1*, a sensor protein located in the cell membrane, is followed by the autoprolytic cleavage of its cytoplasmic domain. The intracellular fragment of *BlaR1/MecR1* travels to the SCCmec and proteolytically cleaves the *BlaI/MecI* repressor bound to the conserved TACA/TGTA DNA motif, thereby leading to the transcription of the *mec/bla* system[5-7]. When RHE was added to the media containing MRSA, the gene expressions of *mecA/mecI/mecR1* and *blaZ/blaI/blaR1* reduced. This suggests that RHE decrease β -lactam resistance of MRSA, because it inhibits *mec* and *bla* genes located in SCCmec. When MRSA senses the presence of β -lactam antibiotics by *MecR1/BlaR1*, it produces resistance factors. The *mec/bla* genes expressions were increased by OX, again decreased with a combination of RHE and OX. It showed that RHE influences the *MecR1/blaR1* located in the cell membrane of MRSA, thereby ultimately inhibiting the *mec/bla* system by interrupting signal transduction for transcription. Similar findings have been reported in a previous study wherein MRSA strains were treated with thioridazine, which affects membrane stability and induces conformational changes in the *MecR1* and *BlaR1* protein, thereby resulting in a subsequent decrease in the level of PBP2a activity via the inhibition of *mecA/mecI/mecR1* and *blaZ/blaI/blaR1* gene expression[21]. Chlorpromazine also was reported to have a similar effect. Chlorpromazine inhibits the expression of *mecA/mecI/mecR1* and *blaZ/blaI/blaR1* by affecting the cell membrane of MRSA[23]. In conclusion, RHE inhibited the gene expressions of *mec/bla* system because of affecting the *MecR1/BlaR1* located in cell membrane of MRSA, which manifested itself as a synergistic effect between RHE and OX against MRSA. These results indicate that RHE was targeted to the expression of the resistance gene of MRSA. This study therefore demonstrates a new pharmacological activity for RHE, which could be a good candidate for clinical development as an antibacterial drug. The promising results of this study are expected to enhance the use of natural products as drugs in the future.

Conflicts of interest statement

The authors declare that there is no conflict of interest.

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