

# Synergistic effect of rhein in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus*

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**Abstract.** Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for a number of infections in humans that are difficult to treat, and as a result, is a substantial contributor to morbidity and mortality. In the present study, in search of natural products capable of inhibiting this multidrug-resistant bacterium, we investigated the antimicrobial activity of rhein isolated from *Rheum palmatum* L. (Polygonaceae) against 16 different strains of the bacterium. New antimicrobial activity was found using the paper disc diffusion method [minimal inhibitory concentrations (MICs)], MTT test and checkerboard dilution test. Against the 16 strains, the disc diffusion test was in the range of 20-29 mm and the MICs of rhein were in the range of 7.8-31.25 µg/ml. From these results we performed the checkerboard test to determine the synergism of rhein in combination with ampicillin (AM) or oxacillin (OX) against all strains. The combined activity of rhein and the two antimicrobial agents (AM and OX) against all strains resulted in a fractional inhibitory concentration index ranging from 0.28-1 and from 0.18-1, respectively. The effect of rhein with AM and OX was found to be synergistic or partially synergistic. We found that rhein reduced the MICs of AM and OX. Rhein in combination with AM or OX could lead to the development of new combinations of antibiotics against MRSA infection.

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious and urgent clinical problem worldwide. Few new drugs are available against MRSA, since it has the ability to acquire resistance to most antibiotics. An effective method for treating MRSA may be to develop antibiotics from natural products which are likely to have lower toxicity and fewer side effects. *Rheum palmatum*, popularly known as Dahuang, has traditionally been used as an oriental folk medicine. *Rhein* naturally occurs in anthraquinone (1,3,8-trihydroxy-6-methyl anthraquinone) (Fig. 1), which is found in *Rheum palmatum* and related plants such as rhubarb. Anthraquinone components include aloe-emodin, rhein, emodin, chrysophanol and physcion (1). Rhein is used for treating numerous complaints, including constipation, jaundice, gastrointestinal hemorrhage and ulcers (2). It has also been shown to have good antitumor (3-6) and anti-inflammatory properties (7,8), anticancer (9), purgative (10), nephric protection (11,12), liver protection (13), antimicrobial and hemostatic properties (14,15). As such, it has been widely used for the treatment of gastrointestinal diseases, hepatitis and chronic renal failure. However, little is known about its antimicrobial effects on MRSA. Thus, we present the current study demonstrating the antimicrobial activity of rhein against MRSA and methicillin-sensitive *S. aureus* (MSSA) strains, as well as its synergistic effect.

## Materials and methods

**Test microorganisms.** Among the 16 *S. aureus* strains used in this study, 14 clinical isolates of MRSA were obtained from 14 different patients at Wonkwang University Hospital (Iksan, Republic of Korea). The other two strains used were *S. aureus* ATCC 33591 (methicillin-resistant strain) and *S. aureus* ATCC 25923 (methicillin-susceptible strain). ATCC 25923 (American Type Culture Collection, Manassas, VA, USA) and ATCC 33591 were commercially purchased. Before use, all

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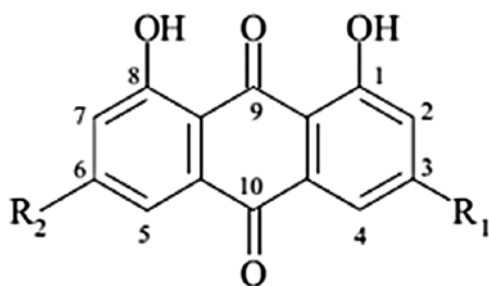


Figure 1. Structures of important anthraquinone derivatives. Rhein:  $R_1=\text{COOH}$ ;  $R_2=\text{H}$ . Aloe-emodin:  $R_1=\text{CH}_2\text{OH}$ ;  $R_2=\text{H}$ . Emodin:  $R_1=\text{CH}_3$ ;  $R_2=\text{OH}$ . Chrysophanol:  $R_1=\text{CH}_3$ ;  $R_2=\text{H}$ . Physcion:  $R_1=\text{CH}_3$ ;  $R_2=\text{OCH}_3$ .

bacteria were stored in 30% glycerol and frozen at  $-70^\circ\text{C}$ . The bacteria were cultured in Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) (Difco Laboratories, Baltimore, MD, USA). Bacteria were suspended in MHB and then incubated at  $37^\circ\text{C}$  for 24 h.

**Disc diffusion method.** The disc diffusion method was performed as described by the Clinical and Laboratory Standards Institute (CLSI) and by using a modified agar well diffusion method (CLSI, 2001) (16). Bacterial strains grown on MHA at  $37^\circ\text{C}$  for 18 h were suspended in MHB and adjusted to a turbidity of 0.5 on the McFarland standard scale (approximately  $1.5 \times 10^8$  CFU/ml). The MHA was poured into Petri dishes and inoculated with 100  $\mu\text{l}$  of the suspension. Sterile paper discs (diameter, 6 mm; Tokyo Roshi Kaihsa, Japan) were punched into the agar and each well was filled with 500 and 250  $\mu\text{g}$  of each drug. The dissolution of the ampicillin (AM), oxacillin (OX) and rhein was facilitated by the addition of 50% (v/v) DMSO (50% DMSO was not active against all strains) (DMSO; Sigma, St. Louis, MO, USA). AM and OX were used as the positive controls, and the discs treated with DMSO were used as the negative control. The plates were placed in an incubator at  $37^\circ\text{C}$  for 18 h in the dark. The inhibition zone diameter around each of the discs was measured and recorded at the end of the incubation period.

**Minimum inhibitory concentration.** The minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method according to the CLSI guidelines, 2000 (17). Briefly, the inoculation of the microorganisms was carried out on 24 h broth cultures, and the suspensions were adjusted to a 0.5 McFarland standard turbidity (approximately  $1.5 \times 10^8$  CFU/ml). Final inoculum size was adjusted to  $1.5 \times 10^6$  CFU/ml. These serially diluted cultures were then incubated at  $37^\circ\text{C}$  for 18 h. MIC was defined at the lowest concentration of AM, OX and rhein. At the end of the incubation period, the well plates were visually examined for turbidity. Cloudiness indicated that bacterial growth had not been inhibited by the concentration of the antimicrobial agent contained in the medium.

**Checkerboard dilution test.** The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA and MSSA strains, and one clinical isolate strain via MIC determination, according to

Table I. The *S. aureus* strains used in the experiments.

<i>S. aureus</i> strain	Class	mecA gene	Antibiotic resistance pattern
ATCC 25923	MSSA	-	-
ATCC 33591	MRSA	+	AM, OX
DPS-1 <sup>a</sup>	MRSA	+	AM, OX
DPS-2	MRSA	+	AM, OX
DPS-3	MRSA	+	AM, OX
DPS-4	MRSA	+	AM, OX
DPS-5	MRSA	+	AM, OX
DPS-6	MRSA	+	AM, OX
DPS-7	MRSA	+	AM, OX
DPS-8	MRSA	+	AM, OX
DPS-9	MRSA	+	AM, OX
DPS-10	MRSA	+	AM, OX
DPS-11	MRSA	+	AM, OX
DPS-12	MRSA	+	AM, OX
DPS-13	MRSA	+	AM, OX
DPS-14	MRSA	+	AM, OX

<sup>a</sup>*Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital, Iksan, Korea. (+), positive; (-), negative; AM, ampicillin; OX, oxacillin.

the CLSI guidelines (18). The MIC was defined as the lowest concentration of drug alone or in combination that inhibited the visible growth. The *in vitro* interaction was quantified by determining the fractional inhibitory concentration (FIC). The FIC index was calculated as follows:  $\text{FIC} = (\text{MIC of drug A in combination} / \text{MIC of drug A alone}) + (\text{MIC of drug B in combination} / \text{MIC of drug B alone})$ . FIC indices (FICIs) were interpreted as follows:  $<0.5$ , synergy;  $0.5-0.75$ , partial synergy;  $0.76-1.0$ , additive effect;  $>1.0-4.0$ , indifference; and  $>4.0$ , antagonism. Finally, the varying rates of synergy between the two agents were determined. All experiments were independently repeated three times.

**Colorimetric assay using MTT test.** A colorimetric assay based on MTT for the rapid detection of the presence of bacteria was performed as previously described (19-21). Briefly, a stock solution of 5 mg/ml MTT (Sigma) was prepared in phosphate-buffered saline and kept at  $-70^\circ\text{C}$ . A final concentration of 1 mg/ml of MTT was used in the assay. Following 24 h of incubation at  $37^\circ\text{C}$ , 20  $\mu\text{l}$  of the yellow MTT was added to the 96-well microtiter plate (0.3 ml volume) and incubated for an additional 20 min. The presence of a blue color indicated the presence of bacteria.

## Results

Table I shows the *S. aureus* strains used in the experiments. Table II shows the means of inhibition zones produced against the tested bacteria ranged between 20 and 29 mm. The growth of all the tested strains of MRSA and MSSA was inhibited at 500 and 250  $\mu\text{g}/\text{disc}$ . As shown in Table III, rhein demon-

Table II. The antimicrobial activity (as inhibitory zone diameters) of rhein, AM and OX against the *S. aureus* strain.

<i>S. aureus</i> strain	Zone of inhibition (mm)					
	Rhein ( $\mu\text{g/ml}$ )		Ampicillin ( $\mu\text{g/ml}$ )		Oxacillin ( $\mu\text{g/ml}$ )	
	500	250	500	250	500	250
ATCC 25923	25	22	43	41	37	35
ATCC 33591	29	23	16	15	18	15
DPS-1 <sup>a</sup>	19	14	18	14	ND	ND
DPS-2	25	20	18	17	17	15
DPS-3	21	17	21	16	ND	ND
DPS-4	21	18	20	15	ND	ND
DPS-5	22	18	17	14	ND	ND
DPS-6	20	16	17	14	ND	ND
DPS-7	21	18	11	10	ND	ND
DPS-8	21	17	12	11	ND	ND
DPS-9	23	19	13	12	ND	ND
DPS-10	25	20	12	11	ND	ND
DPS-11	25	21	11	9	ND	ND
DPS-12	24	20	12	10	ND	ND
DPS-13	24	19	18	14	ND	ND
DPS-14	20	16	15	11	ND	ND

<sup>a</sup>*Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital, Iksan, Korea. ND, no detected activity at this concentration. AM, ampicillin; OX, oxacillin.

Table III. The MICs of rhein, AM and OX against the *S. aureus* strain.

<i>S. aureus</i> strain	MICs ( $\mu\text{g/ml}$ )		
	Rhein	Ampicillin	Oxacillin
ATCC25923	15.62	7.8	7.8
ATCC33591	15.62	1,000	250
DPS-1 <sup>a</sup>	15.62	31.25	500
DPS-2	15.62	1,000	500
DPS-3	15.62	31.25	500
DPS-4	31.25	31.25	500
DPS-5	7.8	31.25	500
DPS-6	7.8	31.25	250
DPS-7	31.25	250	500
DPS-8	31.25	250	500
DPS-9	31.25	125	500
DPS-10	31.25	250	500
DPS-11	31.25	250	500
DPS-12	31.25	250	500
DPS-13	7.8	31.25	1,000
DPS-14	7.8	250	500

<sup>a</sup>*Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital, Iksan, Korea. MIC, minimal inhibitory concentration; AM, ampicillin; OX, oxacillin.

strated antimicrobial activity against all the tested strains of MRSA as well as the MSSA strain. The MICs of rhein against *S. aureus* ranged from 7.8-31.25  $\mu\text{g/ml}$ . As shown in Tables IV

and V, tests were performed to determine the action of rhein alone as well as its synergistic action with AM or OX against the MRSA clinical isolate, the standard MRSA strain or the standard MSSA strain. Rhein lowered the MICs of AM and OX against the MRSA strains. For the standard MSSA strain, rhein lowered the MICs of both AM and OX. The combined activity of rhein and the two antimicrobial agents (AM and OX) against all strains resulted in a FICI ranging from 0.28-1 and from 0.18-1, respectively. In conclusion, the combination effect of rhein with AM and OX was found to be synergistic or partially synergistic.

## Discussion

In the present study, we investigated the antimicrobial activity of rhein against clinical isolates of MRSA and a standard MSSA strain. We report that the rhein agent also shows synergistic activity with AM and OX against MRSA. When combined, these antibiotic effects were dramatically increased. While the results obtained in the present study cannot currently be applied in clinical practice, we consider that the combination treatment of rhein isolated with AM or OX will prove to be helpful in treating MRSA and MSSA. Further medicinal, clinical and mechanism studies are required to verify the mechanisms by which rhein enhances the antibacterial activity. At present, the continued emergence of multi-drug resistant bacteria and the infectious diseases caused by them are serious global problems. It is hoped that it will be possible to reduce the use of existing antibacterial drugs and increase the use of natural product drugs, such as rhein. At this point in time, the product is still under investigation. In our study, rhein mark-

Table IV. Result of the combined effect of rhein and AM against *S. aureus*.

<i>S. aureus</i> strain	MICs ( $\mu\text{g/ml}$ )				FICI
	Rhein alone	With AM	AM alone	With rhein	
ATCC 25923	15.62	3.9	7.8	1.95	0.5
ATCC 33591	15.62	0.97	1,000	250	0.31
DPS-1 <sup>a</sup>	15.62	3.5	31.25	7.8	0.5
DPS-2	15.62	3.9	1,000	250	0.5
DPS-3	15.62	7.8	31.25	0.97	0.53
DPS-4	31.25	15.62	31.25	0.97	0.53
DPS-5	7.8	3.9	31.25	15.62	1
DPS-6	7.8	3.9	31.25	7.9	0.75
DPS-7	31.25	7.8	250	62.5	0.5
DPS-8	31.25	7.8	250	7.8	0.28
DPS-9	31.25	7.8	125	3.9	0.28
DPS-10	31.25	7.8	250	7.8	0.28
DPS-11	31.25	7.8	250	15.62	0.31
DPS-12	31.25	7.8	250	62.5	0.5
DPS-13	7.8	3.9	31.25	15.62	1
DPS-14	7.8	0.48	250	125	0.56

<sup>a</sup>*Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital, Iksan, Korea. ND, no detected activity at this concentration; MIC, minimal inhibitory concentration; AM, ampicillin; FICI, fractional inhibitory concentration index.

Table V. Result of the combined effect of rhein and OX against *S. aureus*.

<i>S. aureus</i> strain	MICs ( $\mu\text{g/ml}$ )				FICI
	Rhein alone	With OX	OX alone	With rhein	
ATCC 25923	15.62	1.95	7.8	1.95	0.37
ATCC 33591	15.62	1.95	250	15.62	0.18
DPS-1 <sup>a</sup>	15.62	7.8	500	31.25	0.53
DPS-2	15.62	3.9	500	125	0.5
DPS-3	15.62	7.8	500	15.62	0.53
DPS-4	31.25	7.8	500	125	0.5
DPS-5	7.8	1.95	500	125	0.5
DPS-6	7.8	1.95	250	7.8	0.53
DPS-7	31.25	7.8	500	125	0.5
DPS-8	31.25	7.8	500	62.5	0.37
DPS-9	31.25	7.8	500	125	0.5
DPS-10	31.25	7.8	500	62.5	0.37
DPS-11	31.25	7.8	500	125	0.5
DPS-12	31.25	7.8	500	250	0.75
DPS-13	7.9	0.48	1,000	500	0.56
DPS-14	7.9	3.9	500	250	1

<sup>a</sup>*Staphylococcus aureus* strains from the Department of Plastic Surgery, Wonkwang University Hospital, Iksan, Korea. ND, no detected activity at this concentration; MIC, minimal inhibitory concentration; OX, oxacillin; FICI, fractional inhibitory concentration index.

edly lowered the MICs of AM and OX against the two MRSA strains and one MSSA strain. While the product is still under investigation, the present results are promising and may help to promote the use of natural products rather than antibiotics.

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