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RESEARCH ARTICLE

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

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a substantial contributor to morbidity and mortality. In search of a natural products capable of inhibiting this multidrug resistant bacteria, we have investigated the antimicrobial activity of emodin (EM) isolated from *Rheum palmatum* L. (Polygonaceae) against 17 different strains of the bacterium. New antimicrobial activity was found using the paper disc diffusion method, agar dilution as well as checkerboard method. Against the 17 strains, the disc diffusion test was in the range of 18–30 mm, and the minimum inhibitory concentrations (MICs) of EM were in the range of 1.5–25 μg/mL. From those results we performed the checkerboard test to determine the synergism of EM in combination with ampicillin (AM) or oxacillin (OX) against all strains. The combined activity of EM and two antimicrobial agents (AM, OX) against all strains resulted in a fractional inhibitory concentrations index (FICI) ranging from 0.37–0.5 and from 0.37–0.75, respectively. The effect of EM with AM and OX was found to be synergistic or partially synergistic. We found that EM reduced the MICs of AM and OX. EM and in combination with AM or OX could lead to the development of new combination antibiotics against MRSA infection.

Keywords: *Antimicrobial; plant extract; Rheum palmatum*

Introduction

Staphylococcus aureus (*S. aureus*) is a public, fatal pathogen associated with a variety of infections (Baltch et al., 2007). It is the principal cause of several infections, such as skin, soft tissue, surgical site and catheter, pneumonia, bacteremia, and osteoarticular infections (Denis et al., 2006). Moreover, because *S. aureus* generally is an intracellular pathogenic organ, infections caused by this microbe can be difficult for medical treatment and can survive and relapse. Also, the overuse of drugs causes resistance (Baltch et al., 2007). Infections caused by methicillin or other β-lactam antibiotic-resistant *S. aureus* are generally nosocomial and are reported from most countries (Patel, 2009).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged worldwide and has become one of the most important hospital and community pathogens (Patel, 2009; Schito, 2006; Baltch et al., 2007). The pharmacological tools available to cure MRSA being limited today, new agents to treat MRSA-associated infections are greatly needed (Liu et al., 2009). First, positive detection of the mecA gene for MRSA, or methicillin-resistant gene product, namely penicillin binding protein PBP2a, is an advance preparation for demonstration of MRSA (Witte et al., 2007). Second, the treatment guidelines for these infections recommend combinations of antibiotics against these pathogenic organisms (Koga et al., 2008). To meet this need, various natural products were screened

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for new antibiotic substances. During the screening for antimicrobial agents against MRSA, extraction from *Rheum palmatum* L. (Polygonaceae) was identified as a candidate for further studies.

Rheum palmatum, popularly known as Dahuang, has traditionally been used as an oriental folk medicine. The emodin (EM) is biologically active and naturally occurs in anthraquinone (1,3,8-trihydroxy-6-methyl anthraquinone) found in *Rheum palmatum* and related plants such as rhubarb (Shuangsuo et al., 2006; Yan et al., 2009). The anthraquinone components including EM, aloe-emodin, rhein, chrysophanol and physcion, have been reported (Yan et al., 2009). It is an active compound known for the medicinal and therapeutic specific of many plant-derived drugs (Basu et al., 2005). The anthraquinones have shown anti-inflammatory and anti-cancer effects and since ancient times, EM has been an active component of herbal extracts used in medical treatments. Previous work has shown that EM inhibits the growth of certain bacteria (Basu et al., 2005; Li et al., 2006). However, little is known about its antimicrobial effects on MRSA. The aloe-emodin was reported, with different EM structure (Hatano et al., 2005). The screening of *Rheum palmatum* roots for antibacterial activity, showed EM to have antibacterial activity against MRSA.

Thus, here we present a study that shows the antimicrobial activity of EM against MRSA and Methicillin-sensitive Staphylococcus aureus (MSSA) strains, as well as its ability to lower the MICs of β-lactam antibiotics.

Materials and methods

Plant material and isolation

The extraction was performed with minor modification of the method previously described by Kang et al. (2008). The dried roots of *Rheum palmatum* (500 g), purchased from the oriental drug store Daehak Hanyak Kuk (Iksan, Korea), was authenticated by Dong-Yeul Kwon. A voucher specimen was also deposited in the Laboratory of Herbology, College of Pharmacy, Wonkwang University, Iksan, Korea. The EtOH extracts (60 g) were partitioned using organic solvents with different polarities to yield *n*-hexane (2.5 g), CH_2Cl_2 (1.2 g), EtOAc (30 g), *n*-BuOH (10 g) and H_2O (13.5 g) fractions in sequence. The CH_2Cl_2 (800 mg) fraction was separated by Sephadex LH-20 column chromatography (3 × 15 cm, $CHCl_3$:MeOH, 10:1) to obtain two fractions (A, B). Each fraction was tested for bioassay. The inactive fraction A (50-100 ml, 160 mg) was chromatographed on silica gel (200–300 mesh, 10 g) column with *n*-hexane-EtOAc (lower layers, by volume, 15:1, 3:1) to obtain chrysophanol (40–50 mL, 25 mg). The active fraction B (287 mg, 200–300 mL) was chromatographed on a Sephadex LH-20 column (20 × 10 cm) with $CHCl_3$:MeOH (10:1, 800 mL) to give two fractions (B1, B2). Fraction B2 (215 mg, 300–400 mL) was separated by a silica gel chromatography (*n*-hexane:EtOAc, 5:1–1:1, each volume, 500 mL) to afford emodin (50–80 mL, 20 mg). The structure of the compound was determined by its physico-chemical and spectral data, which agreed with those previously reported (Kang et al., 2008). Emodin was deposited at the Standardized Material Bank for New Botanical Drugs (No. NNMBP004), Wonkwnag University (Republic of Korea).

Bacterial strains and growth conditions

Among the 17 *S. aureus* strains used in this study, 15 clinical isolates (MRSA) were obtained from 15 different patients at Wonkwang University Hospital (Iksan, South Korea). The other two strains were *S. aureus* ATCC 33591 (methicillin-resistant strain) and *S. aureus* ATCC 25923 (methicillin-susceptible strain). ATCC 25923 (American Type Culture Collection, Manassas, VA) and ATCC 33591 were commercially purchased. Before use, all bacteria were stored in 30% glycerol and frozen at –70°C. The bacteria were cultured in Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) (Difco, Baltimore, MD). The 17 *S. aureus* strains were used to test the antibacterial activity of ampicillin (AM), oxacillin (OX) and EM.

Determination of the mecA gene

Detection of the mecA gene in the MRSA strains was performed by PCR (Polymerase chain reaction) amplification (Table 1-2). Prior to the DNA extraction, bacteria stock cultures were subcultured twice on to MHA plates. For rapid extraction, one to five bacterial colonies were suspended in 300 μL of cell lysis buffer and heated at 100°C for 20 min. After centrifugation at 12 000 rpm for 10 min, 2 μL of the supernatant was used for the DNA extraction. PCR reactions were performed using a MRSA Primer Mix Kit (Genotek, Daejeon, Korea). The PCR amplification consisted of 30 cycles (94°C, 60 s; 55°C, 60 s; 72°C, 60 s). The final PCR products were separated on 2% agarose gel. We have previously reported on these primers (Lee et al., 2008).

Determination of antibacterial activity by the disc diffusion method

The paper disc diffusion method was used to determine antibacterial activity (Veljic et al., 2008; Witte et al., 2007). Sterile paper discs (6 mm; Toyo Roshi Kaisha, Tokyo) were loaded with 20 μL of EM (varying concentrations: 10 and 50 μg/disc, Figure 1) dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO), and were left to dry for 12 h at 37°C in a sterile room. Before the experiment the stock solution was diluted with distilled water to obtain a 50% DMSO stock solution (Veljic

et al., 2008). The bacterial suspensions were diluted to match the 0.5 McFarland standard scale (approximately 1.5×10^8 cfu/mL), and were further diluted to obtain the final inoculum. The MHA was poured into Petri dishes and inoculated with 100 µL of the suspension containing 1.5×10^8 cfu/mL of bacteria. AM and OX were used as positive control, and the discs treated with 50% DMSO were used as negative controls (50% DMSO was not active against all strain). The plates were placed in an incubator (Vision, Bucheon-city, Korea) at 37°C for 24 h. The inhibition zone diameter around each of the discs was measured and recorded at the end of the incubation period. The experiment was done in triplicate.

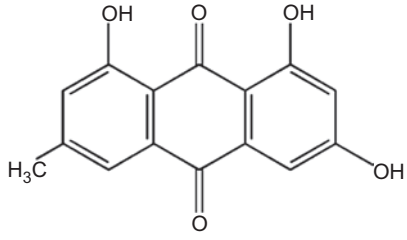

Figure 1. Determination of antibacterial activity by the disc diffusion method.

Table 1. Sequences of the primers used in PCR.

Lens		Sequence (5′ to 3′)
mecA	forward primer	ATGAGATTAGGCATCGTTTC
	reverse primer	TGGATGACAGTACCTGAGCC

Determination of minimal inhibitory concentrations (MICs)

The EM and the two antimicrobial agents were dissolved in MHA with 10% DMSO. Agar dilution method was used to determine the MICs of ampicillin, oxacillin or emodin. Samples were diluted in MHA to a final volume of 25 mL using microtiter plates. The 17 strains were suspended in MHB from MHA plates incubated for 48 h. All

Table 2. The *S. aureus* strains used in the experiments.

S. aureus strain		mecA gene*	Antibioticresistance pattern†
MSSA	ATCC 25923	–	–
MRSA	ATCC 33591	+	AM, OX
Clinical isolates	DPS 1‡	+	AM, OX
	DPS 2	+	AM, OX
	DPS 3	+	AM, OX
	DPS 4	+	AM, OX
	DPS 5	+	AM, OX
	DPS 6	+	AM, OX
	DPS 7	+	AM, OX
	DPS 8	+	AM, OX
	DPS 9	+	AM, OX
	DPS 10	+	AM, OX
	DPS 11	+	AM, OX
	DPS 12	+	AM, OX
	DPS 13	+	AM, OX
	DPS 14	+	AM, OX
	DPS 15	+	AM, OX

†AM, ampicillin; OX, oxacillin.
‡DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Table 3. The antimicrobial activity (as inhibition zone diameters) of EM, AM and OX against *S. aureus* strains.

		Zone of inhibition (mm)			
		Emodin		Ampicillin†	Oxacillin
S.aureus strain		10 µg	50 µg	10 µg	1 µg
MSSA	ATCC 25923	12	19	30	16
MRSA	ATCC 33591	26	30	20	–
Clinical isolates	DPS 1*	15	18	13	–
	DPS 2	18	23	14	7
	DPS 3	18	23	14	–
	DPS 4	18	22	13	–
	DPS 5	18	19	11	–
	DPS 6	16	20	8	–
	DPS 7	19	20	12	–
	DPS 8	14	21	15	–
	DPS 9	24	24	11	–
	DPS 10	18	26	16	8
	DPS 11	17	22	12	–
	DPS 12	19	22	19	–
	DPS 13	17	20	13	–
	DPS 14	20	23	17	–
	DPS 15	21	23	18	–

*DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.
†AM resistance ≤ 28 mm, OX resistance ≤ 10 mm; –, absence of inhibition.

strains suspensions were adjusted to the 0.5 McFarland standards (approximately 1×10^8 cfu/mL). Final inoculums were adjusted to the 10^6 cfu/spot. The MHA was supplemented with EM concentrations ranging from 1.56–25 μg/mL, with AM at concentrations from 0.9–125 μg/mL and with OX at concentrations from 1.9 to 1 mg/mL in plates. These serially diluted cultures were then incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that completely suppressed colony growth. The MIC of AM and OX were also determined, and similarly defined as the lowest antibiotic concentration at which no visible bacterial growth was observed.

Determination of in vitro *combinations*

The antimicrobial effects that resulted from combination inhibiting the two antimicrobial agents were assessed by the checkerboard test. The antimicrobial combination assayed included EM plus AM or OX. Serial dilutions of the EM with AM or EM with OM antimicrobial agents were mixed in cation-supplemented MHA. The inocula were prepared from colonies that had been grown on MHA overnight. The final bacterial concentration after inoculation was 5×10^6 cfu/mL. The MIC was determined after 24 h of incubation at 37°C. The MIC was defined as the lowest concentration of drug alone or in combination inhibiting the visible growth. Each experiment was repeated three times. The *in vitro* interaction was quantified by determining the fractional inhibitory concentration (FIC). The FIC index was determined using the following formula:

$$\text{FIC index} = \text{FIC}_A + \text{FIC}_B = [A]/\text{MIC}_A + [B]/\text{MIC}_B$$

where [A] is the concentration of drug A, MIC_A is its MIC, and FIC_A is the FIC of drug A for the organism, and [B], MIC_B, and FIC_B are defined in the same way for drug B. The FIC index thus obtained was interpreted as follows: <0.5, synergy; 0.5–0.75, partial synergy; 0.76–1, additive effect; >1–4, indifference; and >4, antagonism (Choi et al., 2008). Finally, the varying rates of synergy between the two agents were determined.

Results

The antimicrobial efficacy of EM against the 16 MRSA strains and the single MSSA strain was evaluated by the disc diffusion method via determination of the surrounding inhibition zones, as well as by evaluating the MIC using the agar dilution method. Table 3 shows the antimicrobial activity of EM. The mean of inhibition zones produced against the tested bacteria ranged between 12 and 30 mm. The growth of all the tested strains of MRSA and MSSA was inhibited at 10 and 50 μg/disc.

The MICs for EM, AM and OX against the the 17 strains; 15 strains of clinically-isolated of MRSA, on standard MRSA, and one standard MSSA are shown in Table 4. 15 strains of MRSA, one standard MRSA, and one standard MSSA strain are shown in Table 4. The MICs we determined using the agar dilution method confirmed the antimicrobial effects we found by the disc diffusion method. EM showed antimicrobial activity against all the tested strains of MRSA as well as the MSSA strain. The MICs of EM against *S. aureus* ranged from 1.56–25 μg/mL.

For the determination of *in vitro* combinations test we selected a clinical MRSA isolate, the standard MRSA strain, and the standard MSSA strain. This test was performed to determine the action of EM 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. The EM markedly lowered the MICs of AM and OX against the MRSA strains. For the standard MSSA strain the EM lowered the MICs of both AM and OX. The combined activity of EM and two antimicrobial agents (AM, OX) against all strains resulted in fractional inhibitory concentrations index (FICI) ranging from 0.37–0.5 and from 0.37–0.75, respectively (Table 5-6). So the combination effect of EM with AM and OX was found to be synergistic or partially synergistic. We found that EM reduced MICs of AM and OX.

Discussion

Since MRSA bacteria possess multi-drug resistance, MRSA infections are dangerous and present serious

Table 4. The MICs of EM, AM, OX against *S. aureus* strain.

S.aureus strain		MIC (μg/mL)		
		Emodin	Ampicillin	Oxacillin
MSSA	ATCC 25923	25	0.9	1.9
MRSA	ATCC 33591	25	62.5	1000
Clinical isolates	DPS 1 *	25	31.25	500
	DPS 2	1.56	31.25	1000
	DPS 3	1.56	31.25	1000
	DPS 4	25	31.25	1000
	DPS 5	6.25	31.25	1000
	DPS 6	1.56	31.25	250
	DPS 7	1.56	62.5	500
	DPS 8	1.56	125	500
	DPS 9	1.56	125	500
	DPS 10	1.56	62.5	500
	DPS 11	1.56	125	500
	DPS 12	1.56	125	500
	DPS 13	1.56	125	500
	DPS 14	12.5	62.5	1000
	DPS 15	1.56	125	500

*DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Table 5. Result of the combined effect of EM and AM against *S. aureus*.

		MIC (μg/mL)				
S.aureus strain		EM Alone[¶]	With AM[§]	AM Alone	With EM	FICI
MSSA	ATCC 25923	25	6.25	0.9	0.22	0.5
MRSA	ATCC 33591	25	6.25	62.5	15.62	0.5
Clinical isolates	DPS 1[*]	25	6.25	31.25	7.81	0.5
	DPS 2	1.56	0.39	31.25	7.81	0.5
	DPS 3	1.56	0.39	31.25	7.81	0.5
	DPS 4	25	3.12	31.25	7.81	0.37
	DPS 5	6.25	1.56	31.25	7.81	0.5
	DPS 6	1.56	0.39	31.25	7.81	0.5
	DPS 7	1.56	0.39	62.5	15.31	0.5
	DPS 8	1.56	0.39	125	31.25	0.5
	DPS 9	1.56	0.39	125	15.31	0.37
	DPS 10	1.56	0.39	62.5	15.31	0.5
	DPS 11	1.56	0.39	125	15.31	0.37
	DPS 12	1.56	0.39	125	15.31	0.37
	DPS 13	1.56	0.39	125	15.31	0.37
	DPS 14	12.5	3.12	62.5	15.31	0.5
	DPS 15	1.56	0.39	125	15.31	0.37

[*]DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.
MIC, Minimum Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index: <0.5, synergy; 0.5–0.75, partial synergy; 0.76–1, additive effect; >1–4, indifference; and >4, antagonism (Choi et al., 2008).
[¶]Alone, not combinated with EM or AM.
[§]With AM, EM + AM.

Table 6. Result of the combined effect of EM and OX against *S. aureus*.

		MIC (μg/mL)				
S.aureus strain		EM Alone[¶]	With OX[§]	OX Alone	With EM	FICI
MSSA	ATCC 25923	25	6.25	1.9	0.47	0.5
MRSA	ATCC 33591	25	6.25	1000	250	0.5
Clinical isolates	DPS 1[*]	25	6.25	500	125	0.5
	DPS 2	1.56	0.39	1000	250	0.5
	DPS 3	1.56	0.39	1000	250	0.5
	DPS 4	25	6.25	1000	250	0.5
	DPS 5	6.25	1.56	1000	250	0.5
	DPS 6	1.56	0.39	250	125	0.75
	DPS 7	1.56	0.39	500	125	0.5
	DPS 8	1.56	0.39	500	125	0.5
	DPS 9	1.56	0.39	500	125	0.5
	DPS 10	1.56	0.39	500	125	0.5
	DPS 11	1.56	0.39	500	125	0.5
	DPS 12	1.56	0.39	500	125	0.5
	DPS 13	1.56	0.39	500	250	0.5
	DPS 14	12.5	0.39	1000	125	0.37
	DPS 15	1.56	0.39	500	125	0.5

[*]DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.
MIC, Minimum Inhibitory Concentration; FICI, Fractional Inhibitory Concentration Index: <0.5, synergy; 0.5–0.75, partial synergy; 0.76–1, additive effect; >1–4, indifference; and >4, antagonism (Choi et al., 2008).
[¶]Alone, alone compound.
[§]With OX, EM + OX.

problems for hospitals. Many researchers are studying natural products that could potentially be used against MRSA. In the present study, we have investigated the antimicrobial activity of EM against clinical isolates of MRSA and a standard MSSA strain.

To our knowledge this is the first report showing that EM can lower the MICs of β-lactam antibiotics. Others have reported that β-lactam antibiotics inhibit bacterial cell wall biosynthesis. In this study EM exhibited antimicrobial activity and lowered the MICs of

β-lactam antibiotics against MRSA and a standard MSSA strain.

The isolated compound was characteristic of EM by its structure. The presence of two peri-hydroxy and three hydroxyl groups are an indication. In addition, the presence of the methoxy group confirmed to be EM. More importantly, the three phenol groups are on 1, 3 and 8 positions (Shia et al., 2009). This EM has been used as a traditional oriental medicine for the treatment of skin burn, infection, gallstone, hepatitis, inflammation, and osteomyelitis. Moreover, this is a safe agent even in long exposure in mice (Muto et al., 2007). Hatano et al. (1999) reported that EM, aloe-emodin, and the others, showed strong antibacterial activity against MRSA. Here we report that EM agent also showed synergistic activity with AM and OX against MRSA. While these results obtained here cannot be applied currently in clinical treatment, we consider that the combination treatment of EM isolated with AM or OX will prove to be helpful to treat MRSA. Further medicinal, clinical and mechanism studies are needed to verify how EM enhances the antibacterial activity. Today, the ongoing emergence of multi-drug resistant bacteria and the infectious diseases caused by them are serious global problems.

Therefore we will be able to reduce the use of existing antibacterial drugs and increase the use of natural product drugs such as EM. At this stage, the product is still under investigation.

The emodin markedly lowered the MICs of AM and OX against the two MRSA strains and one MSSA. While the product is still under investigation, the present results are promising and may enhance the use of natural products instead of antibiotics.

Declaration of interest

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