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Synergistic Effect in Combination of *Danshen* (*Salvia miltiorrhiza*) Extracts with Antibiotics against Methicillin-resistant *Staphylococcus aureus*

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Abstract *Danshen* (*Salvia miltiorrhiza* Bunge) is a traditional Korean medicine that is commonly used for the treatment of inflammatory diseases such as edema, arthritis, hepatitis, and endangitis. The several extracts of *danshen* were tested for antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) isolated in clinic by broth microdilution method, the checkerboard, and time-kill methods to evaluate the synergistic effects of the combination of the extracts with antibiotics. The chloroform ($CHCl_3$) and *n*-hexane (HEX) extracts [minimum inhibitory concentration (MIC), 0.0078-0.3125 μg/mL; minimum bactericidal concentration (MBC), 0.019-0.625 μg/mL] were found to have strong antibacterial activity against MRSA. Additionally, when the $CHCl_3$ and HEX extracts were co-administered with ampicillin or oxacillin, a synergistic effect against MRSA was observed. Furthermore, a time-kill study evaluating the effects of the extracts against MRSA indicated that treatment with the $CHCl_3$ extract in combination with ampicillin or oxacillin produced rapid bactericidal activity. These results suggest that *danshen* extracts may have potently antimicrobial activity and thus, it can be a suitable phytotherapeutic agent for treating MRSA infections.

Keywords: *Salvia miltiorrhiza*, methicillin-resistant *Staphylococcus aureus*, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), time-kill curve, fractional inhibitory concentration

Introduction

Staphylococcus aureus is known to be a versatile pathogen causing a side variety of community and hospital acquired infections that are associated with high morbidity and mortality rates (1-3). Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are becoming increasingly widespread. MRSA normally possesses a multidrug-resistant genotype that causes it to be resistant to β-lactams, aminoglycosides, fluoroquinolones, and macrolides (3-5). As a result, there is an urgent need to develop anti-MRSA agents with novel mechanisms of action (6,7). Many studies have been conducted to evaluate natural products as novel antibiotic substances against MRSA and to develop antibiotics that would be advantageous for combating the therapeutic problems associated with MRSA (8-12).

Danshen (*Salvia miltiorrhiza* Bunge) (Labiatae) has long been used in traditional oriental herbal medicine for the treatment of a variety of diseases, including edema, arthritis, hepatitis, endangitis, coronary heart disease, cerebrovascular disease, hypertension, chronic renal failure, dysmenorrheal, insomnia, blood circulation diseases, angina pectoris, and cytotoxicity against human tumor cell lines (13-18). The aqueous extracts of the fresh leaves, dried leaves, and bark of *danshen* have been administered as a counteracting agent for the treatment of insecticide and ethyl alcohol poisoning, and the dried roots have been utilized as an anti-inflammatory agent and antipyretic (19-23). It has recently been reported that the extracts of *danshen* leaves and roots exert a protective effect against ethanol-induced hepatic lipid peroxidation, blood ethanol concentration, and alcohol dehydrogenase and aldehyde dehydrogenase activity (17,22,24). Over 50 chemical constituents have been isolated and identified from *danshen* (25-27). These constituents include 2 constituent groups of hydrophilic phenolic acids, salvianolic acid B, and lithospermic acid, as well as lipophilic tanshinones such as tanshinone I, tanshinone IIA, tanshinone IIB, and cryptotanshinone (25-29). These lipophilic tanshinones have been shown to inhibit platelet-aggregation and protect the myocardium against ischemia-induced derangement, as well as to protect liver microsomes, hepatocytes, and erythrocytes against oxidative damage (17,22,24,30,31). Specifically, the phenolic acids in *danshen* have been found to have significant bioactivities including antioxidant, antiblood coagulation, and cell protection, as well as a wide variety of other activities including anti-ischemia-reperfusion, antihypertension, anti-fibrosis, antivirus, and anti-tumor effects (32-34). Tanshinone I, tanshinone IIA, and cryptotanshinone, which are the major diterpene structures of *danshen*, are effective antioxidants that inhibit lipid peroxidation and improve metabolic disorders (35,36).

In the present study, the antimicrobial activities of several extracts of *danshen* roots against MASA isolated in a clinic were assessed using broth microdilution method for minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) and the checkerboard and time-kill methods to evaluate the synergistic effects of treatment with a combination of the extracts and ampicillin or oxacillin.

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Materials and Methods

Plant material and preparation of crude plant extracts *Danshen* (*S. miltiorrhiza*) was purchased from the Herbal Medicine Cooperative Association of Jeonbuk, Korea, in March 2005. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, Wonkwang University. Voucher specimens (DJ-JC05A) were deposited in the Herbarium of the College of Natural Science, Wonkwang University. The dried and powered roots of *danshen* were extracted by refluxing the samples with methanol (MeOH, Samchun Co., Pyeongtaek, Korea), chloroform ($CHCl_3$, Samchun), *n*-hexane (HEX, Samchun), ethyl acetate (EtOAc, Samchun), *n*-butanol (*n*-BuOH, Samchun), or water (H_2O) for 4 hr at 80°C 3 times. The solvents of each of the extracts were removed under vacuum on a rotary evaporator at 40°C. The extracts were then dissolved in 10% dimethyl sulfoxide (DMSO) for testing. All the extracts were kept at 4°C in the dark until further use.

Bacterial strains Twenty isolates of methicillin-resistant *Staphylococcus aureus* isolated from the Wonkwang University Hospital, as well as standard strains of methicillin-sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591 were used for antibacterial activity of *danshen* extracts. Previously, the clinical isolates, MRSA 20 were identified using the standard methods described for antibiotic susceptibility (12). In addition, the strains utilized were defined as MRSA based on the presence of the *mecA* gene and their resistance to oxacillin (12).

Minimum inhibitory concentration (MIC)/minimum bactericidal concentration assay (MBC) The antimicrobial activities of *danshen* extracts against clinical isolates MRSA 20 and reference strains were determined via the broth dilution method (37,38). The MIC was recorded as the lowest concentration of test samples resulting in the complete inhibition of visible growth. The MBC was determined based on the lowest concentration of the extracts required to kill 99.9% of bacteria from the initial inoculum as determined by plating on agar.

Checkerboard dilution test A combination of $CHCl_3$ or HEX extracts with antibiotics, oxacillin, or ampicillin was found to exert the greatest antibacterial effects (Table 2-5); therefore, these antibiotics were evaluated via the checkerboard test, as previously described (8,39). The antimicrobial combinations assayed herein included $CHCl_3$ or HEX extracts with ampicillin or oxacillin. The fractional inhibitory concentration index (FICI) is the sum of the FICs of each of the drugs, which were defined as the MIC of each drug when used in combination divided by the MIC of each drug when used alone. The interaction was defined as synergistic in cases in which the FIC was less than or equal to 0.5, additive in cases in which the FIC index was greater than 0.5 and less than or equal to 1.0, indifferent when the

Table 1. Minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) of *danshen* extracts for isolated MRSA and some of reference bacteria

Sample	*Danshen* extracts (μg/mL)							
	H_2O	MeOH	$CHCl_3$	BUOH	HEX	EA	Oxacillin	Ampicillin
MSSA ATCC 25923[1)]	n/n	128/256	8/8	2048/2048	8/16	2048/2048	0.25/0.5	0.125/0.25
MRSA ATCC 33591[2)]	n/n	64/128	8/16	2048/4096	4/8	2048/4096	8/16	32/64
MRSA 1[3)]	n/n	256/256	16/16	2048/2048	16/32	2048/2048	4/8	64/64
MRSA 2	n/n	128/256	4/8	2048/4096	8/8	2048/4096	8/16	32/64
MRSA 3	n/n	128/128	4/8	2048/2048	8/16	4096/4096	4/4	32/32
MRSA 4	n/n	256/256<	32/32	2048/4096	32/64	2048/2048	16/16	32/32
MRSA 5	n/n	128/256	8/16	2048/4096	8/16	2048/2048	16/32	64/64
MRSA 6	n/n	256/256	16/32	2048/2048	16/32	2048/4096	8/16	16/32
MRSA 7	n/n	128/128	8/16	2048/2048	8/16	2048/2048	16/32	32/64
MRSA 8	n/n	256/256<	32/64	2048/4096	16/32	2048/2048	4/8	16/32
MRSA 9	n/n	128/128	8/16	2048/2048	8/16	2048/2048	16/32	64/64
MRSA 10	n/n	256/256<	16/32	2048/2048	16/32	2048/4096	8/16	16/32
MRSA 11	n/n	256/256<	64/64	2048/4096	64/64	2048/4096	16/16	32/64
MRSA 12	n/n	256/256<	64/64	2048/4096	32/64	2048/4096	32/32	64/128
MRSA 13	n/n	256/256<	16/32	4096/4096	16/32	4096/4096	32/64	64/128
MRSA 14	n/n	256/256<	32/64	2048/4096	32/64	2048/2048	16/32	32/64
MRSA 15	n/n	64/128	8/16	2048/2048	4/8	2048/2048	8/16	32/64
MRSA 16	n/n	256/256	16/32	2048/4096	16/32	2048/2048	16/32	16/32
MRSA 17	n/n	256/256<	32/64	4096/4096	32/64	2048/4096	8/16	32/64
MRSA 18	n/n	256/256	32/32	2048/2048	16/32	2048/2048	4/16	32/64
MRSA 19	n/n	128/256	8/16	2048/2048	8/8	1024/2048	4/8	64/64
MRSA 20	n/n	256/256	16/32	2048/4096	8/16	1024/1024	16/16	64/128

[1)]MSSA (ATCC 25923): reference strain methicillin-sensitive *Staphylococcus aureus*.
[2)]MRSA (ATCC 33591): reference strain methicillin-resistant *Staphylococcus aureus*.
[3)]MRSA (1-20): methicillin-resistant *Staphylococcus aureus* isolated in clinic.

Table 2. Synergic effects of the $CHCl_3$ extract of *danshen* with oxacillin in isolated MRSA and some of reference bacteria

Sample	Agent	MIC/MBC (μg/mL)		FIC	FICI[2)]	Outcome
		Alone	Combination[1)]			
MSSA ATCC 25923[3)]	$CHCl_3$	8/8	2/4	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Oxacillin	0.25/0.5	0.0625/0.125	0.25/0.25		
MRSA ATCC 33591[4)]	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 1[5)]	$CHCl_3$	16/16	4/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 2	$CHCl_3$	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 3	$CHCl_3$	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	4/4	1/1	0.25/0.25		
MRSA 4	$CHCl_3$	32/32	8/16	0.25/0.5	0.375/1	Synergistic/ Additive
	Oxacillin	16/16	2/8	0.125/0.5		
MRSA 5	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 6	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	8/16	2/2	0.25/0.125		
MRSA 7	$CHCl_3$	8/16	2/4	0.25/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	2/8	0.125/0.25		
MRSA 8	$CHCl_3$	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 9	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	16/32	4/4	0.25/0.125		
MRSA 10	$CHCl_3$	16/32	4/8	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Oxacillin	8/16	4/4	0.5/0.25		
MRSA 11	$CHCl_3$	64/64	8/16	0.125/0.25	0.25/0.5	Synergistic/ Synergistic
	Oxacillin	16/16	2/4	0.125/0.25		
MRSA 12	$CHCl_3$	64/64	8/16	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	32/32	8/8	0.25/0.25		
MRSA 13	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	32/64	8/16	0.25/0.25		
MRSA 14	$CHCl_3$	32/64	8/8	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 15	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	8/16	2/2	0.25/0.125		
MRSA 16	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	16/32	4/4	0.25/0.125		
MRSA 17	$CHCl_3$	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 18	$CHCl_3$	32/32	8/8	0.25/0.25	0.375/0.375	Synergistic/ Synergistic
	Oxacillin	4/16	0.5/2	0.125/0.125		
MRSA 19	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 20	$CHCl_3$	16/32	4/4	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	16/16	4/4	0.25/0.25		

[1)]$CHCl_3$ extract with oxacillin.
[2)]FIC index.
[3)]MSSA (ATCC 25923): reference strain methicillin-sensitive *Staphylococcus aureus.*
[4)]MRSA (ATCC 33591): reference strain methicillin-resistant *Staphylococcus aureus.*
[5)]MRSA (1-20): methicillin-resistant *Staphylococcus aureus* isolated a clinic.

FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic in cases in which the FIC index was greater than 2.0 (39).

Time-kill curves The bactericidal activities of the drugs evaluated in this study were also evaluated using time-kill curves constructed using the isolated and reference strains.

Table 3. Synergic effects of the HEX extract of *danshen* with oxacillin in isolated MRSA and some of reference bacteria

Sample	Agent	MIC/MBC (μg/mL)		FIC	FICI[2)]	Outcome
		Alone	Combination[1)]			
MSSA ATCC 25923[3)]	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	0.25/0.5	0.0625/0.125	0.25/0.25		
MRSA ATCC 33591[4)]	HEX	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 1[5)]	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 2	HEX	8/8	2/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 3	HEX	8/16	2/2	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	4/4	1/2	0.25/0.5		
MRSA 4	HEX	32/64	4/8	0.125/0.125	0.25/0.625	Synergistic/ Additive
	Oxacillin	16/16	2/4	0.125/0.5		
MRSA 5	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 6	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 7	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 8	HEX	16/32	2/8	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 9	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 10	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 11	HEX	64/64	8/8	0.125/0.125	0.375/0.625	Synergistic/ Additive
	Oxacillin	16/16	4/8	0.25/0.5		
MRSA 12	HEX	32/64	8/8	0.25/0.125	0.375/0.375	Synergistic/ Synergistic
	Oxacillin	32/32	4/8	0.125/0.25		
MRSA 13	HEX	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	32/64	8/8	0.25/0.125		
MRSA 14	HEX	32/64	4/8	0.125/0.125	0.375/0.375	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 15	HEX	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 16	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 17	HEX	32/64	4/8	0.125/0.125	0.375/0.375	Synergistic/ Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 18	HEX	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	4/16	1/2	0.25/0.125		
MRSA 19	HEX	8/8	2/4	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Oxacillin	4/8	1/2	0.25/0.25		
MRSA 20	HEX	8/16	2/2	0.25/0.125	0.5/0.625	Synergistic/ Additive
	Oxacillin	16/16	4/8	0.25/0.5		

[1)]HEX extract with oxacillin.
[2)]FIC index.
[3)]MSSA (ATCC 25923): reference strain methicillin-sensitive *Staphylococcus aureus*.
[4)]MRSA (ATCC 33591): reference strain methicillin-resistant *Staphylococcus aureus*.
[5)]MRSA (1-20): methicillin-resistant *Staphylococcus aureus* isolated in clinic.

Tubes containing the $CHCl_3$ extract and bacteria were incubated at 37°C, and viable counts were conducted at 0, 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 hr after the addition of the antimicrobial agents by plating aliquots of the samples on agar and subsequent incubation for 24 hr at 37°C. Cultures with an initial cell density of 1×10^6 CFU/mL were exposed

Table 4. Synergic effects of the $CHCl_3$ extract of *danshen* with ampicillin in isolated MRSA and some of reference bacteria

Samples	Agent	MIC/MBC (μg/mL)		FIC	FICI[2)]	Outcome
		Alone	Combination[1)]			
MSSA ATCC 25923[3)]	$CHCl_3$	8/8	1/2	0.125/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	2/2	0.5/0.5	0.25/0.25		
MRSA ATCC 33591[4)]	$CHCl_3$	8/16	2/8	0.25/0.5	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	1024/2048	128/256	0.125/0.25		
MRSA 1[5)]	$CHCl_3$	16/16	4/8	0.25/0.5	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 2	$CHCl_3$	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 3	$CHCl_3$	4/8	1/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	1024/2048	256/256	0.25/0.125		
MRSA 4	$CHCl_3$	32/32	8/8	0.25/0.25	0.375/1	Synergistic/ Additive
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 5	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 6	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 7	$CHCl_3$	8/16	2/4	0.25/0.25	0.375/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 8	$CHCl_3$	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 9	$CHCl_3$	8/16	2/8	0.25/0.5	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 10	$CHCl_3$	16/32	4/8	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Ampicillin	64/64	16/16	0.25/0.25		
MRSA 11	$CHCl_3$	64/64	8/16	0.125/0.25	0.25/0.5	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 12	$CHCl_3$	64/64	16/32	0.25/0.5	0.375/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 13	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/64	16/16	0.25/0.25		
MRSA 14	$CHCl_3$	32/64	8/8	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 15	$CHCl_3$	8/16	2/4	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 16	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 17	$CHCl_3$	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 18	$CHCl_3$	32/32	8/8	0.25/0.25	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 19	$CHCl_3$	8/16	4/8	0.25/0.5	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 20	$CHCl_3$	16/32	4/8	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	128/128	32/64	0.25/0.5		

[1)]$CHCl_3$ extract with ampicillin.
[2)]FIC index.
[3)]MSSA (ATCC 25923): reference strain methicillin-sensitive *Staphylococcus aureus*.
[4)]MRSA (ATCC 33591): reference strain methicillin-resistant *Staphylococcus aureus*.
[5)]MRSA (1-20): methicillin-resistant *Staphylococcus aureus* isolated in clinic.

to the MIC of the $CHCl_3$ extract alone, or with ampicillin or oxacillin. All experiments were repeated several times and colony counts were conducted in duplicate, after which the means were determined.

Table 5. Synergic effects of the HEX extract of *danshen* with ampicillin in isolated MRSA and some of reference bacteria

Sample	Agent	MIC/MBC (μg/mL)		FIC	FICI[2)]	Outcome
		Alone	Combination[1)]			
MSSA ATCC 25923[3)]	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	2/2	0.5/0.5	0.25/0.25		
MRSA ATCC 33591[4)]	HEX	4/8	1/2	0.25/0.25	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	1024/2048	128/256	0.125/0.125		
MRSA 1[5)]	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 2	HEX	8/8	2/2	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 3	HEX	8/16	2/4	0.25/0.25	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	1024/2048	128/256	0.125/0.125		
MRSA 4	HEX	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 5	HEX	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 6	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	32/32	0.25/0.25		
MRSA 7	HEX	8/16	2/4	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 8	HEX	16/32	4/4	0.25/0.125	0.5/0.625	Synergistic/ Synergistic
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 9	HEX	8/16	2/4	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 10	HEX	16/32	4/8	0.25/0.25	0.5/0.75	Synergistic / Additive
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 11	HEX	64/64	16/32	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 12	HEX	32/64	8/16	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/128	32/64	0.25/0.5		
MRSA 13	HEX	16/32	4/16	0.25/0.5	0.5/1	Synergistic/ Additive
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 14	HEX	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 15	HEX	4/8	1/2	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 16	HEX	16/32	8/16	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 17	HEX	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 18	HEX	16/32	4/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/64	16/16	0.25/0.25		
MRSA 19	HEX	8/8	2/4	0.25/0.5	0.5/1	Synergistic/ Additive
	Ampicillin	64/64	16/32	0.25/0.5		
MRSA 20	HEX	8/16	2/4	0.25/0.25	0.375/0.5	Synergistic/ Synergistic
	Ampicillin	128/128	16/32	0.125/0.25		

[1)]HEX extract with ampicillin.
[2)]FIC index.
[3)]MSSA (ATCC 25923): reference strain methicillin-sensitive *Staphylococcus aureus*.
[4)]MRSA (ATCC 33591): reference strain methicillin-resistant *Staphylococcus aureus*.
[5)]MRSA (1-20): methicillin-resistant *Staphylococcus aureus* isolated in clinic.

Results and Discussion

The antibacterial activity of the MeOH, $CHCl_3$, HEX, EtOAc, *n*-BuOH, and H_2O extracts are shown in Table 1. With the exception of the H_2O extracts, each of the extracts exhibited antibacterial effects against all of the tested

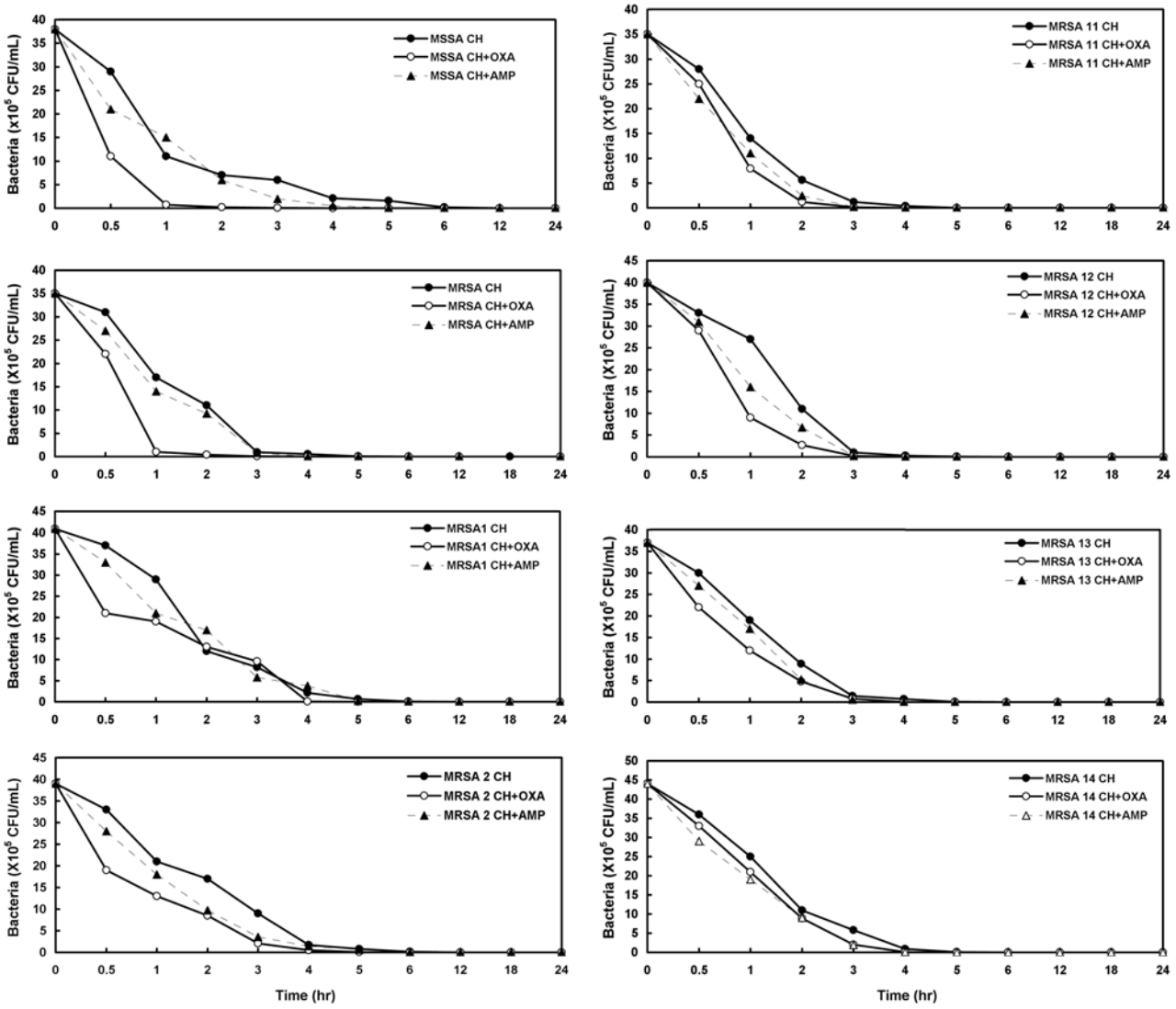

Fig. 1. Time-kill curves of MBC of the $CHCl_3$ extract alone and its combination with MIC of oxacillin or ampicillin against isolates MRSA (1-6) and reference strains. Bacteria were incubated with the $CHCl_3$ extract alone (●) and with ampicillin (○) or with oxacillin (▼) over time. Data points are the mean values±SEM of 6 experiments. CFU, colony-forming units.

bacteria (MIC, 4 to 2,048 μg/mL; MBC, 8 to 4,096 μg/mL). The $CHCl_3$ and HEX extracts showed stronger antibacterial activity against isolated MRSA and some of reference bacteria (MIC, 4 to 64 μg/mL; MBC, 8 to 64 μg/mL) than the other extracts. The MIC/MBC for ampicillin was found to be either 0.125/0.25 or 64/128 μg/mL, while that of oxacillin was either 0.25/0.5 or 32/64 μg/mL.

The synergic effects of the $CHCl_3$ or HEX extracts administered in conjunction with ampicillin or oxacillin are shown in Table 2-5. When administered in combination with the $CHCl_3$ extract, the MIC for oxacillin was reduced ≥4-8 fold when tested against all of the bacteria evaluated in this study, with the exception of MRSA 10. These findings indicate that a synergistic effect based on a FICI of ≤0.25-0.5 (Table 2). The administration of the HEX extract in conjunction with oxacillin induced a reduction of ≥4-8 fold in all tested bacteria, which was considered to be synergistic based on a FICI of ≤0.25-0.5 (Table 3). The administration of ampicillin with $CHCl_3$ extract also induced a ≥4-8 fold reduction against all tested bacteria 95.45%. Furthermore, the effects of these compounds were found to be synergistic (FICI ≤0.25-0.5) against all of the bacteria evaluated here, with the exception of MRSA 10, for which the effects were additive (FICI ≤0.75) (Table 4). Finally, administration of the HEX extract and ampicillin produced a synergistic effect against all of the bacteria tested in this study 100% (FICI ≤0.25-0.5) (Table 5).

The effects of the $CHCl_3$ extract administered in combination with oxacillin or ampicillin against standard (MSSA and MRSA) and clinical isolates of MRSA (MRSA 1-20) were confirmed by time-kill curve experiments (Fig. 1-3). Cultures of each strain of bacteria with a cell density of 10^6 CFU/mL were exposed to the MBC of $CHCl_3$ extract alone or combined with oxacillin or ampicillin. We found that treatment with a combination of $CHCl_3$ extract and oxacillin or ampicillin produced a more rapid decrease in the concentration of bacteria CFU/mL than treatment with $CHCl_3$ alone, and that these effects

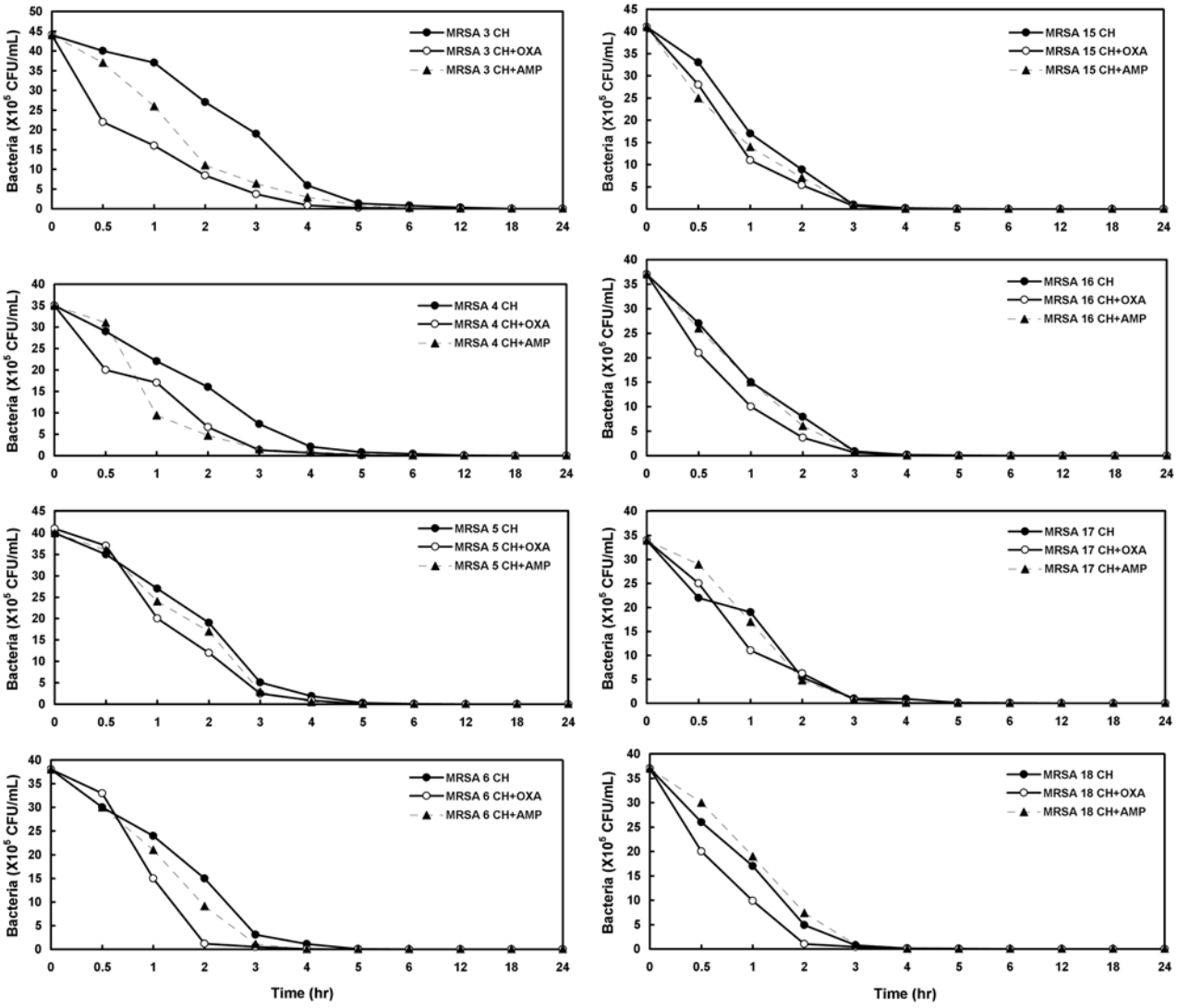

Fig. 2. Time-kill curves of MBC of the $CHCl_3$ extract alone and its combination with MIC of oxacillin or ampicillin against isolates MRSA (7-14). Bacteria were incubated with the $CHCl_3$ extract alone (●) and with ampicillin (○) or with oxacillin (▼) over time. Data points are the mean±SEM of 6 experiments. CFU, colony-forming units.

occurred in a time dependant manner. The growth of the tested bacteria was completely attenuated after 2-5 hr of treatment with the MBC of the $CHCl_3$ extract, regardless of whether it was administered alone or with oxacillin or ampicillin.

MRSA is resistant to methicillin and other β-lactams, as well as to a host of other antibacterial agents, including macrolide (2,5). MRSA is very dangerous, and can produce serious medical problems because it causes many common infectious diseases and often acquires multi-drug resistance (3,40). Several studies have demonstrated that phenolic compounds produced by plants exert antibacterial activity against MRSA (9-11); therefore, it is likely that the phenolic compounds in *danshen* may be related, in part, to the antibacterial effects observed in the present study. Various pharmacological studies conducted *in vitro* and *in vivo* have concentrated on *danshen* components such as hydrophilic phenolic compounds, danshensu, salvianolic acid B, and the lipophilic diterpene compounds known as tanshinones (25,26,28). These compounds are known to have bioactivities that make them useful for the treatment of coronary heart disease, myocardial infection, hypertension, chronic hepatitis, liver fibrosis, and bone loss, as well as to possess antioxidant, antiinflammation, antitumor, antivirus, and antibacterial activity (17,21,22, 24,41). Some tanshinones in *danshen* exert strong antibacterial activity against a wide range of Gram-positive bacteria including *Bacillus subtilis* (42). Furthermore, the flavonoid derivative from the *Sophora flavescens* known as sophoraflavanone G has been found to induce antimicrobial activity against oral bacteria and MRSA (8,43).

In conclusion, we verified the antibacterial activity of *danshen* chloroform and hexane extracts against MRSA isolated in clinic. Furthermore, these extracts exerted synergistic effects when administered with oxacillin or ampicillin and the antimicrobial effect and resistant regulation of *danshen* against MRSA might be useful for potential application as a natural product agent.

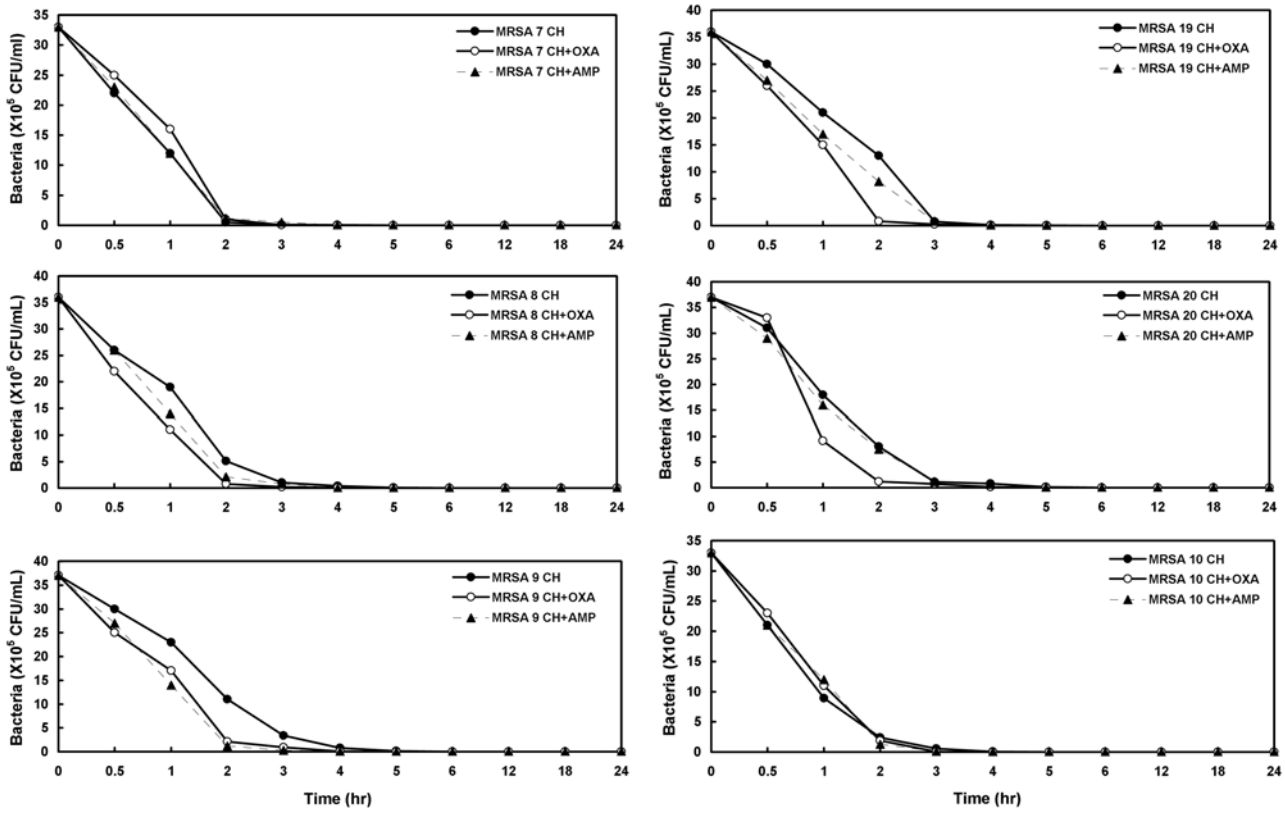

Fig. 3. Time-kill curves of MBC of the $CHCl_3$ extract alone and its combination with MIC of oxacillin or ampicillin against isolates MRSA (15-20). Bacteria were incubated with the $CHCl_3$ extract alone (●) and with ampicillin (○) or with oxacillin (▼) over time. Data points are the mean±SEM of 6 experiments. CFU, colony-forming units.

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