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Combination of Acacetin with Antibiotics against Methicillin Resistant *Staphylococcus aureus* Isolated from Clinical Specimens

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Abstract

Methicillin-restitant *Staphylococcus aureus* (MRSA) is very dangerous bacteria and one of the most feared nosocomial germs. In this study, acacetin was evaluated against 20 clinical isolates of MRSA, either alone or in combination with antibiotics. The acacetin exhibited a good activity against isolates MRSA with MICs/MBCs ranged between 10 - 80/20 - 160 μg/mL, for ampicillin 64 - 1024/128 - 2048 μg/mL, and for oxacillin 8 - 32/16 - 64 μg/mL. The combination of acacetin plus oxacillin or ampicillin was reduced by ≥4-fold against isolates MRSA tested, evidencing a synergistic effect as defined by a FICI of ≤0.5. Furthermore, a time-kill study evaluating the growth of the tested bacteria was completely attenuated after 2 - 5 h of treatment with the 1/2 MIC of acacetin, regardless of whether it was administered alone or with oxacillin (1/2 MIC) or ampicillin (1/2 MIC). In conclusion, acacetin exerted synergistic effects when administered with oxacillin or ampicillin and the antibacterial activity and resistant regulation of acacetin against clinical isolates of MRSA might be useful in controlling MRSA infections.

Keywords

Acacetin; Methicillin-Resistant *Staphylococcus aureus*; Minimum Inhibitory Concentrations; Minimum Bactericidal Concentrations; Time-Kill Curves; Fractional Inhibitory Concentration

1. Introduction

Staphylococcus aureus (*S. aureus*) is an important human pathogen, causing life-threatening systemic infections

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such as pneumonia, septicemia, endocarditis, and osteomyelitis [1] [2]. Furthermore, *S. aureus* can spread easily, and have been found in the noses of approximately 40% - 50% of healthy people, is the etiological agent more commonly associated to the disease, and is normally related to subclinical or chronic infections [3] [4]. Clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) have become the most common cause of infections among pathogenic bacteria around the Globe, and many life-threatening diseases such as endocarditis, pneumonia and toxin shock syndrome are ascribed to them [4] [5]. Contrary to methicillin-susceptible *S. aureus* (MSSA), MRSA tend to be multi-drug resistant (MDR), that is, resistant not only to β-lactam antibiotics but also to a wide range of different antibiotic classes, such as fluoroquinolones, tetracyclines, macrolides, lincosamides, and aminoglycosides, and even strains of vancomycin intermediate susceptible or full resistant (VISA and VRSA, respectively) have emerged [6] [7]. Antimicrobial drugs effective for treatment of patients infected with MRSA are limited. Thus, it is important and valuable to find compounds that potentiate antimicrobial activity of antibiotics.

Many plant-derived medicines used in traditional medicinal systems have been recorded in pharmacopeias as agents used to treat infections and a number of these have been recently investigated for their efficacy against MRSA [8] [9]. Flavonoids have also been shown to exhibit broader bioactivities such as protection of vascular integrity, antihepatotoxicity, anti-inflammatory activity, antitumor effect, antiallergic properties, and antimicrobial effects [9]-[13]. Acacetin (5,7-dihydroxy-4'-methoxyflavone), a flavone compound found in several plants, has been reported to show anti-peroxidative, anti-mutagenic, anti-cancer, anti-inflammatory, antibacterial, and anti-plasmodial activities [14]-[16]. Drug synergism between known antibiotic and bioactive plant extracts is a novel concept and could be benificial (synergistic or addition interaction) or deleterious (antagonistic or toxic outcome) [7] [17]. Although a broad range of biological and pharmacological activities of acacetin have been reported, the mechanism(s) behind its antibacterial effects are not fully understood.

In this study, the antimicrobial activities of acacetin against methicillin-resistant *Staphylococcus aureus* isolated in a clinic were assessed using broth microdilution method and the checkerboard and time-kill methods for synergistic effect of the combination with ampicillin or oxacillin.

2. Materials and Methods

2.1. Preparation of Bacterial Strains

20 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. Antibiotic susceptibility was determined in testing the inhibition zones (inoculums 0.5 McFarland suspension, 1.5×10^8 CFU/ml) and MIC/MBC (inoculums 5×10^5 CFU/ml) for strains, measured as described in the National Committee for Clinical Laboratory Standards (NCCLS, 1999). To rapidly identifying the methicillin-resistance, presence of *mecA* gene in MRSA isolates was detected using PCR method as the following [18].

2.2. Minimum Inhibitory Concentration/Minimum Bactericidal Concentration Assay

The antimicrobial activities of acacetin against clinical isolates MRSA 20 and reference strains were determined *via* the broth dilution method [17] [19]. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration of test samples resulting in the complete inhibition of visible growth. For clinical strains, MIC_{50}s and MIC_{90}s, defined as MICs at which, 50% and 90%, respectively of the isolates were inhibited, were determined. The minimum bactericidal concentration (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.

2.3. Checkerboard Dilution Test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, MSSA, and one clinical isolate strains via MIC determination [19] [20]. 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 FIC index was calculated as follows: FIC = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FIC indices (FICI) were interpreted as follows: ≤ 0.5, synergy; >0.5 - ≤ 1.0, additive; >1.0 - ≤ 2.0,

indifference; and >2.0, antagonism [20].

2.4. 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. Cultures with an initial cell density of 1×10^6 - 5×10^6 CFU/ml were exposed to the MIC of acacetin alone, or acacetin (1/2 MIC) plus oxacillin (1/2 MIC) or acacetin (1/2 MIC) plus ampicillin (1/2 MIC). Viable counts were conducted at 0, 0.5, 1, 2, 3, 4, 5, 6, 12, and 24 h by plating aliquots of the samples on agar and subsequent incubation for 24 hours at 37˚C. All experiments were repeated several times and colony counts were conducted in duplicate, after which the means were determined.

3. Results and Discussion

Many researchers are studying natural products that could be used as antibiotics against MRSA, and are employing novel dosing regimens and antimicrobials that would be advantageous for combating the therapeutic problems associated with *S. aureus* [9] [10] [21]-[23]. The results of the antibacterial activity showed that the acacetin exhibited inhibitory activities against isolates MRSA and reference stains, MRSA ATCC33591 and MSSA ATCC25923. In **Table 1**, the acacetin displayed varying degrees of activity against clinical isolated MRSA 1 - 20 with MIC in the range of 10 - 80 μg/mL and MBC in the range of 20 - 160 μg/mL. The MICs/MBCs for ampicillin were determined to be either 64/128 or 1024/2048 μg/mL; for oxacillin, either 4/16 or 32/64 μg/mL against MRSA 1 - 20 isolates. The range of MIC_{50} and MIC_{90} were 1.25 - 20 μg/mL and 10 - 80 μg/mL against MRSA 1 - 20 isolates, respectively. Flavonoid compounds constitute an important class of phytochemicals which possess diverse biological activities against MRSA [9] [19] [23] [24]. Some of these compounds, like polyphenols, have been shown to exert their antibacterial action through membrane perturbations [25] [26]. The acacetin is known to contain a number of antimicrobial compounds, such as polyphenols and flavonoids. The acacetin, one of main compounds of *A. afra* showed good inhibitory effects against Gram positive oral bacteria [16].

Table 1. Antibacterial activity of acacetin and antibiotics in isolated MRSA and some of reference bacteria.

Samples	Acacetin (μg/mL)			Ampicillin	Oxacillin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
MSSA ATCC 25923 [1]	1.25	5	5/20	8/16	0.25/1
MRSA ATCC 33591 [2]	5	20	20/40	1024/2048	8/16
MRSA 1 [3]	2.5	10	10/40	1024/2048	16/32
MRSA 2	5	20	20/80	128/256	8/16
MRSA 3	10	40	40/80	1024/2048	8/16
MRSA 4	5	20	20/80	256/512	16/32
MRSA 5	10	40	40/160	128/256	16/32
MRSA 6	20	80	80/160	256/256	8/16
MRSA 7	5	20	20/40	128/512	16/32
MRSA 8	2.5	10	10/20	128/256	8/32
MRSA 9	1.25	10	10/40	128/512	16/32
MRSA 10	10	40	40/80	64/128	8/16
MRSA 11	5	20	20/80	128/256	16/64
MRSA 12	10	80	80/160	256/256	32/64
MRSA 13	20	80	80/160	64/128	32/64
MRSA 14	5	40	40/80	128/256	16/32
MRSA 15	10	40	40/160	64/128	8/16
MRSA 16	20	80	80/160	128/256	16/32
MRSA 17	2.5	10	10/20	128/256	8/16
MRSA 18	20	80	80/160	64/128	8/16
MRSA 19	20	80	80/160	64/128	4/16
MRSA 20	5	40	40/80	128/512	16/32

[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 a clinic.

Combination antibiotic therapy has been studied to promote the effective use of antibiotics in increasing *in vivo* activity of antibiotics, in preventing the spread of drug-resistant strains, and in minimizing toxicity [7] [17] [20]. The combination of oxacillin and acacetin resulted in a reduction in the MICs/MBCs for all bacteria, with the MICs/MBCs of 1.25/5 or 20/80 μg/mL for oxacillin becoming 0.0625 - 8/0.25 - 16 μg/mL and reduced by ≥4-fold in most of *S. aureus* tested, evidencing a synergistic effect as defined by a FICI of ≤0.5 except clinic MRSA 3, 8, 9, and 10 at MIC and clinic MRSA 3, 5, 6, 10, 13, 15, 17, and 20 at MBC (**Table 2**). In

Table 2. Synergistic effects of the acacetin with oxacillin in isolated MRSA and some of reference bacteria.

Samples	Agent	MIC/MBC (μg/mL)		FIC/FBC	FICI/FBCI[2]	Outcome
		Alone	**Combination**[1]			
MSSA ATCC 25923[3]	Acacetin	5/20	1.25/5	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	0.25/1	0.0625/0.25	0.25/0.25		
MRSA ATCC 33591[4]	Acacetin	20/40	5/10	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 1[5]	Acacetin	10/40	2.5/10	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 2	Acacetin	20/80	5/10	0.25/0.125	0.5/0.375	Synergistic/Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 3	Acacetin	40/80	10/20	0.25/0.25	0.75/0.75	Additive/Additive
	Oxacillin	8/16	4/8	0.5/0.5		
MRSA 4	Acacetin	20/80	5/10	0.25/0.125	0.5/0.375	Synergistic/Additive
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 5	Acacetin	40/160	10/40	0.25/0.25	0.5/0.75	Synergistic/Additive
	Oxacillin	16/32	4/16	0.25/0.5		
MRSA 6	Acacetin	80/160	20/40	0.25/0.25	0.5/0.75	Synergistic/Additive
	Oxacillin	8/16	2/8	0.25/0.5		
MRSA 7	Acacetin	20/40	5/10	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 8	Acacetin	10/20	5/10	0.5/0.5	0.75/0.375	Additive/Synergistic
	Oxacillin	8/32	2/4	0.25/0.125		
MRSA 9	Acacetin	10/40	2.5/5	0.25/0.125	0.75/0.375	Additive/Synergistic
	Oxacillin	16/32	8/8	0.5/0.25		
MRSA 10	Acacetin	40/80	10/20	0.25/0.25	0.75/0.75	Synergistic/Synergistic
	Oxacillin	8/16	4/8	0.5/0.5		
MRSA 11	Acacetin	20/80	5/20	0.25/0.25	0.5/0.375	Synergistic/Synergistic
	Oxacillin	16/64	4/8	0.25/0.125		
MRSA 12	Acacetin	80/160	20/40	0.25/0.25	0.375/0.5	Synergistic/Synergistic
	Oxacillin	32/64	4/16	0.125/0.25		
MRSA 13	Acacetin	80/160	20/80	0.25/0.5	0.375/0.75	Synergistic/Additive
	Oxacillin	32/64	4/16	0.125/0.25		
MRSA 14	Acacetin	40/80	10/20	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 15	Acacetin	40/160	10/40	0.25/0.25	0.5/0.75	Synergistic/Additive
	Oxacillin	8/16	2/8	0.25/0.5		
MRSA 16	Acacetin	80/160	20/40	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	16/32	4/8	0.25/0.25		
MRSA 17	Acacetin	10/20	2.5/10	0.25/0.5	0.5/1.0	Synergistic/Additive
	Oxacillin	8/16	2/8	0.25/0.5		
MRSA 18	Acacetin	80/160	20/40	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Oxacillin	8/16	2/4	0.25/0.25		
MRSA 19	Acacetin	80/160	20/40	0.25/0.25	0.5/0.375	Synergistic/Synergistic
	Oxacillin	4/16	1/2	0.25/0.125		
MRSA 20	Acacetin	40/80	10/20	0.25/0.25	0.5/0.75	Synergistic/Additive
	Oxacillin	16/32	4/16	0.25/0.5		

[1]The MIC and MBC of acacetin with oxacillin; [2]The 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.

combination with acacetin, the MICs/MBCs for ampicillin were reduced by ≥4-fold in most of *S. aureus* tested, evidencing a synergistic effect as defined by a FICI of ≤0.5 except clinic MRSA 7, 10, 11, 16, and 19 at MIC and clinic MRSA 6, 8, 10, 11, 12, 13, 15, and 19 at MBC by FICI of >0.625 (**Table 3**). The effects of acacetin

Table 3. Synergistic effects of acacetin with ampicillin in isolated MRSA and some of reference bacteria.

Samples	Agent	MIC/MBC (μg/mL)		FIC/FBC	FICI/FBCI[2]	Outcome
		Alone	Combination[1]			
MSSA ATCC 25923[3]	Acacetin	5/20	1.25/5	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	8/16	2/4	0.25/0.25		
MRSA ATCC 33591[4]	Acacetin	20/40	5/10	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 1[5]	Acacetin	10/40	2.5/10	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 2	Acacetin	20/80	5/20	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 3	Acacetin	40/80	10/20	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	1024/2048	256/512	0.25/0.25		
MRSA 4	Acacetin	20/80	5/10	0.25/0.125	0.5/0.375	**Synergistic/Synergistic**
	Ampicillin	256/512	64/128	0.25/0.25		
MRSA 5	Acacetin	40/160	10/40	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 6	Acacetin	80/160	20/40	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Ampicillin	256/256	64/128	0.25/0.5		
MRSA 7	Acacetin	20/40	5/10	0.25/0.25	0.75/0.5	**Additive/Synergistic**
	Ampicillin	128/512	64/128	0.5/0.25		
MRSA 8	Acacetin	10/20	2.5/10	0.25/0.5	0.5/0.75	**Synergistic/Additive**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 9	Acacetin	10/40	2.5/10	0.25/0.25	0.5/0.375	**Synergistic/Synergistic**
	Ampicillin	128/512	32/64	0.25/0.125		
MRSA 10	Acacetin	40/80	10/20	0.25/0.25	0.75/0.75	**Additive/Additive**
	Ampicillin	64/128	32/64	0.5/0.5		
MRSA 11	Acacetin	20/80	5/10	0.25/0.125	0.75/0.625	**Additive/Additive**
	Ampicillin	128/256	64/128	0.5/0.5		
MRSA 12	Acacetin	80/160	20/40	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Ampicillin	256/256	64/128	0.25/0.5		
MRSA 13	Acacetin	80/160	20/40	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Ampicillin	64/128	16/64	0.25/0.5		
MRSA 14	Acacetin	40/80	10/20	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 15	Acacetin	40/160	10/40	0.25/0.25	0.5/0.75	**Synergistic/Additive**
	Ampicillin	64/128	16/64	0.25/0.5		
MRSA 16	Acacetin	80/160	20/40	0.25/0.25	0.75/0.5	**Additive/Synergistic**
	Ampicillin	128/256	64/64	0.5/0.25		
MRSA 17	Acacetin	10/20	2.5/5	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 18	Acacetin	80/160	20/40	0.25/0.25	0.5/0.5	**Synergistic/Synergistic**
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 19	Acacetin	80/160	20/40	0.25/0.25	0.75/0.75	**Additive/Additive**
	Ampicillin	64/128	32/64	0.5/0.5		
MRSA 20	Acacetin	40/80	10/20	0.25/0.25	0.5/0.375	**Synergistic/Synergistic**
	Ampicillin	128/512	32/64	0.25/0.125		

[1]The MIC and MBC of acacetin with ampicillin; [2]The 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.

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 (**Figures 1-4**). Cultures of each strain of bacteria with a cell density of 10^6 CFU/mL were exposed to the MIC of acacetin alone or acacetin (1/2 MIC) with oxacillin (1/2 MIC) or ampicillin (1/2 MIC). We observed that 30 minutes of acacetin treatment with ampicillin or oxacillin resulted in a rapidly increased rate of killing as compared to that observed with acacetin (MIC) alone (**Figures 1-4**).

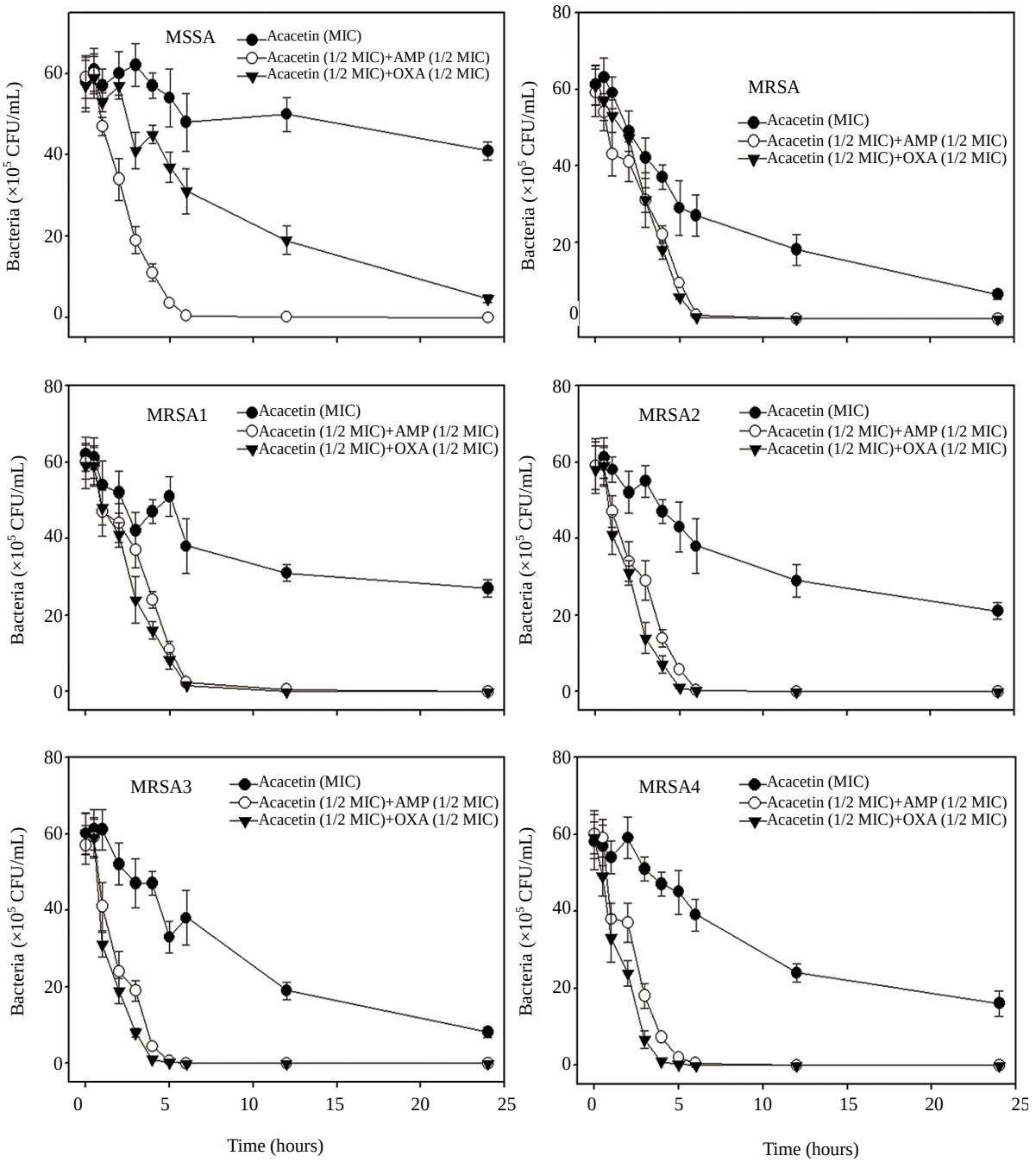

Figure 1. Time-kill curves of MIC of the acacetin alone and 1/2 MIC of acacetin with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (1-4) and methicillin-sensitive *S. aureus* (MSSA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591 strains. Bacteria were incubated with the acacetin alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

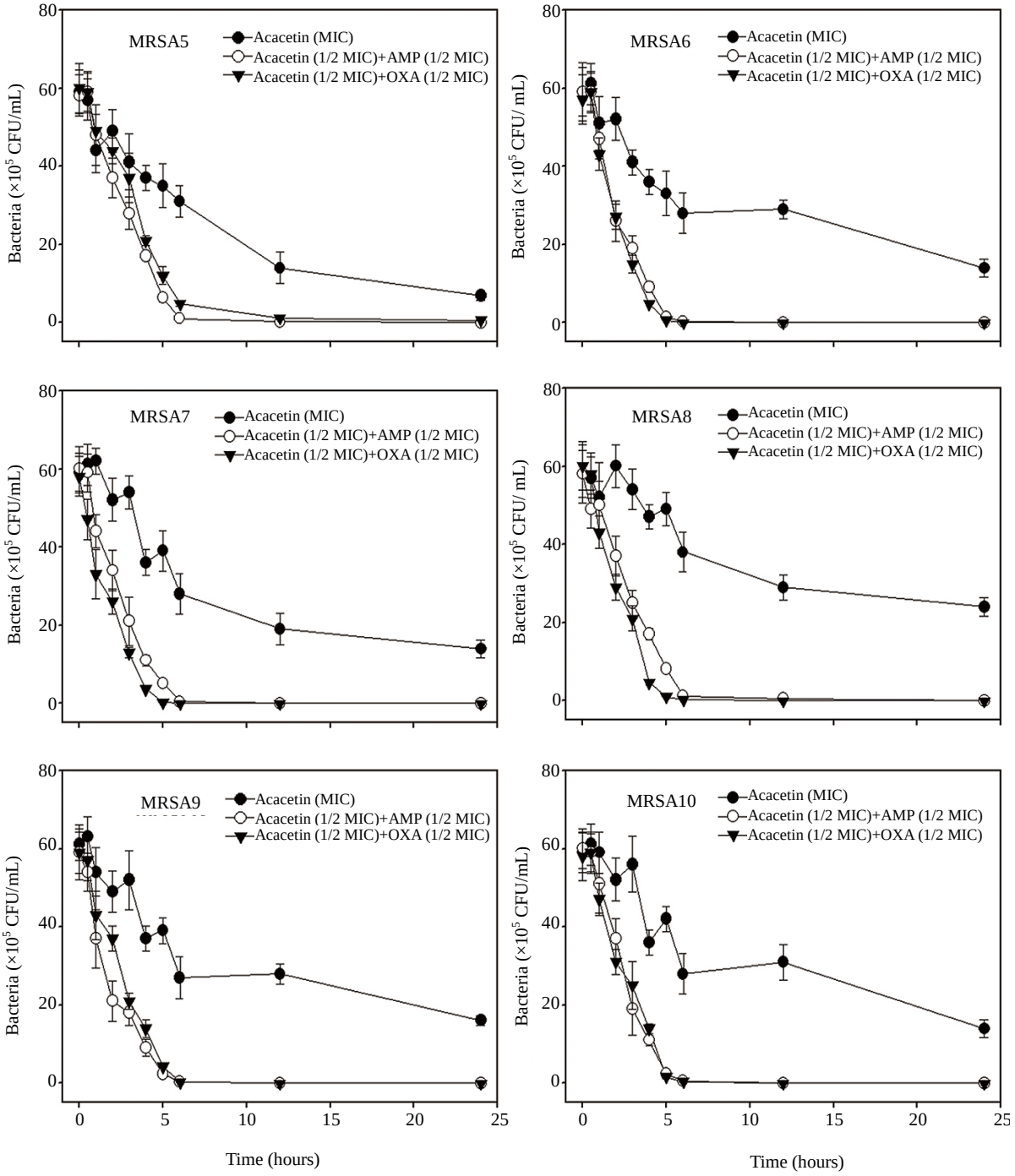

Figure 2. Time-kill curves of MIC of the acacetin alone and 1/2 MIC of acacetin with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (5 - 10). Bacteria were incubated with the acacetin alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

A profound bactericidal effect was exerted when a combination of drugs was utilized. The growth of the tested bacteria was completely attenuated after 2 - 6 h of treatment with the 1/2 MIC of acacetin, regardless of whether it was administered alone or with oxacillin (1/2 MIC) or ampicillin (1/2 MIC) except MRSA 15 and 17. Flavonoids affect bacterial membrane potential and cause permeability alteration within the inner microorganisms membrane [27]-[29]. This perturbation of the cell membrane coupled with the action of *β*-lactams on

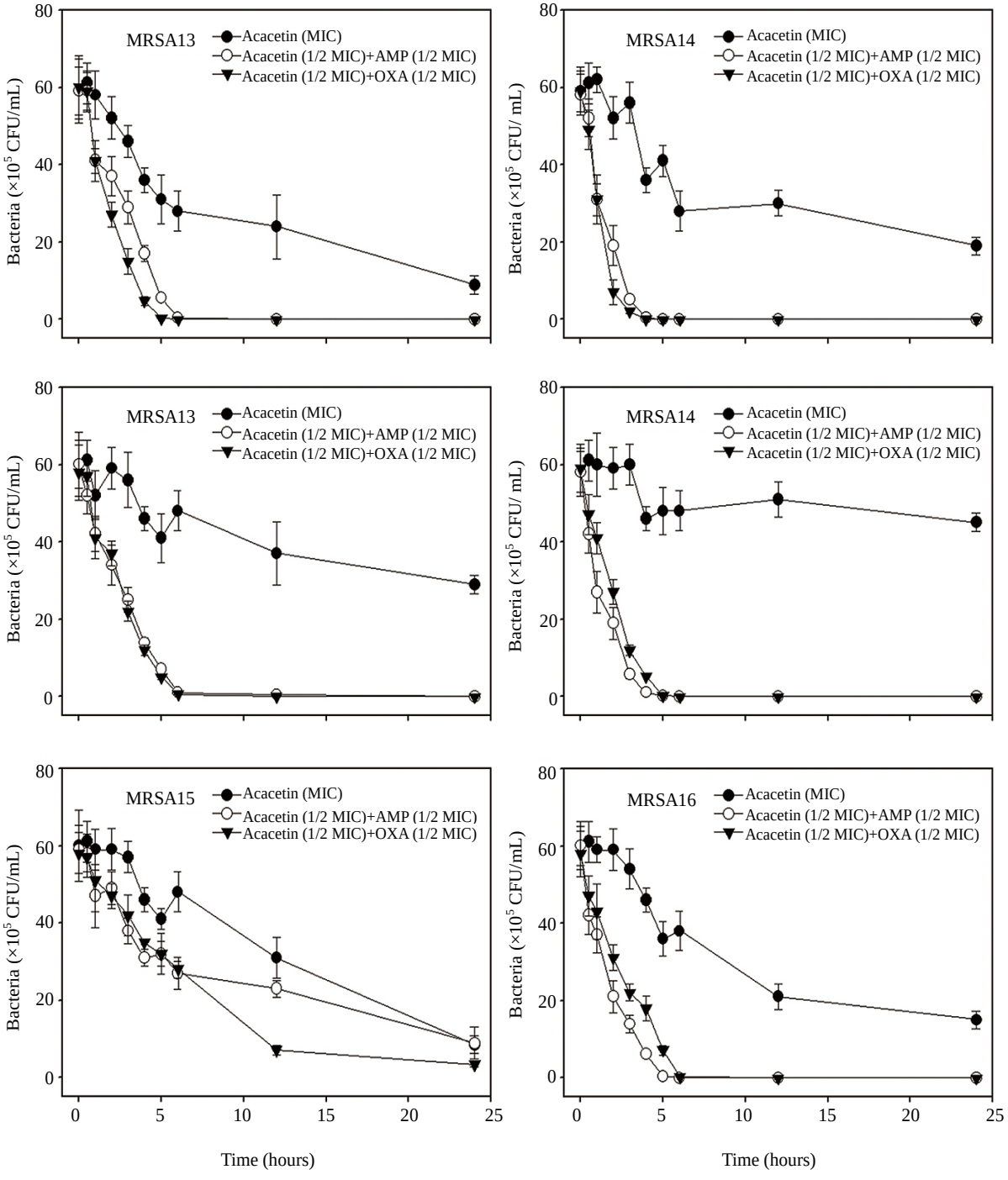

Figure 3. Time-kill curves of MIC of the acacetin alone and 1/2 MIC of acacetin with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (11 - 16). Bacteria were incubated with the acacetin alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

the transpeptidation of the cell membrane could lead to the enhanced antimicrobial effect [27] [29].

In conclusion, acacetin exerted synergistic effects when administered with oxacillin or ampicillin and the antimicrobial effect and resistant regulation of acacetin against MRSA might be useful for potential application as a natural product agent.

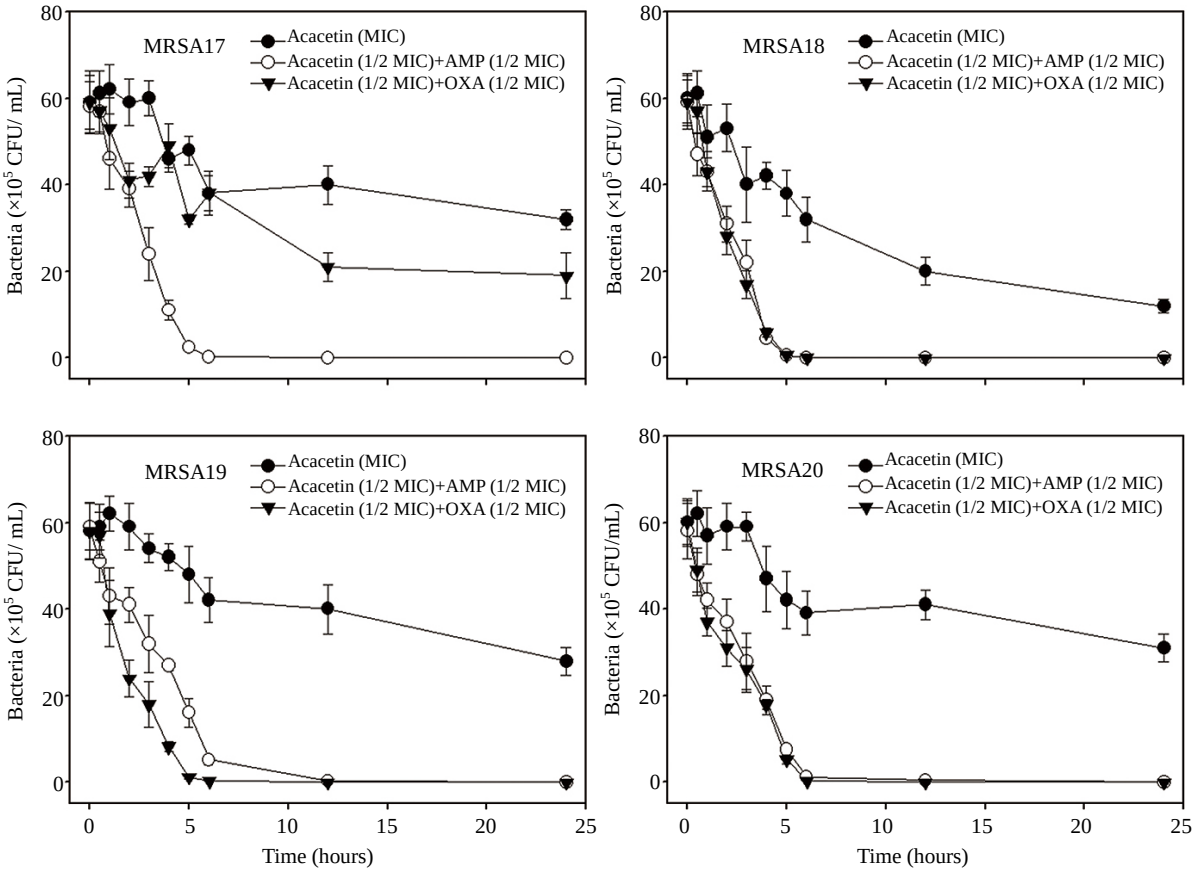

Figure 4. Time-kill curves of MIC of the acacetin alone and 1/2 MIC of acacetin with 1/2 MIC of oxacillin or ampicillin against isolates MRSA (17 - 20). Bacteria were incubated with the acacetin alone (●) and with ampicillin (○) or with oxacillin (▼) over time. CFU, colony-forming units.

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