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Synergistic Effect between Baicalein and Antibiotics against Clinic Methicillin and Vancomycin-Resistant *Staphylococcus aureus*

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Abstract

Baicalein is one of the major flavonoids in Scutellaria baicalensis Georgi, which has long been used in several biological effects, such as antiviral, anti-inflammation, anti-hepatotoxicity, and anti-tumor properties, have been reported. In this study, baicalein demonstrated strong antibacterial activity against clinic isolated methicillin and vancomycin-resistant Staphylococcus aureus (MRSA and VRSA) in this experiment. Baicalein was determined against clinic isolated MRSA 1-16 with MIC and MBC values ranging from 64 to 256 and 64 to 512 μg/ml; for MSSA 1-2 from 128 and 256 μg/ml and 128 and 512 μg/ml; for VRSA 1-2 from 64 and 128 μg/ml and 64 and 512 μg/ml, respectively. The range of MIC50 and MIC90 of baicalein were 16-64 μg/ml and 64-256 μg/ml, respectively. The combination effects of baicalein with antibiotics were synergistic (FIC index <0.5) against most of tested clinic isolated MRSA, MSSA, and VRSA except additive, MRSA 7 in oxacillin and MRSA 8 and 15 in vancomycin (FIC index <0.625-0.75). Furthermore, a time-kill study showed that the growth of the tested bacteria was completely attenuated after 2-6 h of treatment with the ½ MIC of baicalein, regardless of whether it was administered alone or with ampicillin, oxacillin, or vacomycin. The results suggest that baicalein could be employed as a natural antibacterial agent against multidrug-resistant pathogens infection.

Keywords: Baicalein, Antibacterial activity, Methicillin-resistant Staphylococcus aureus (MRSA), Vacomycin-Resistant *Staphylococcus aureus* (VRSA), Checker board method, Time-kill method

Introduction

Staphylococcus aureus (S. aureus) is an important human pathogen, causing life-threatening systemic infections such as pneumonia, septicemia, endocarditis, and osteomyelitis [1,2]. By the end of the 1990s the relatively few multidrug-resistant and highly epidemic clones of Methicillin-Resistant Staphylococcus aureus (MRSA) had become the most frequent causative agents of S. aureus disease in both hospitals and communities [3]. In spite of the availability of several structurally different antibacterial agents, the therapy most frequently used for treatment of MRSA infections has remained the glycopeptides antibiotics, primarily vancomycin [4,5]. From 1980 on, there was an abrupt and continued increase in the use of vancomycin in the United States and several countries, which seems to parallel the increasing frequency of MRSA infections in hospitals [5,6]. This illustrates the enormous selective pressure highly focused on MRSA strains worldwide. 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, tetracycline's, macrolides, lincosamides, and aminoglycosides, and even strains of vancomycin intermediate susceptible or full resistant (VISA and VRSA, respectively) have emerged [5-7]. Antimicrobial drugs effective for treatment of patients infected with MRSA are limited. Hence, search for novel antimicrobial compounds or alternative therapy for these infections is inevitable.

Plant medicines are used on a worldwide scale to prevent and treat infectious diseases. They are of great demand both in the developed as well as developing countries for the primary health care needs due to their wide biological and medicinal activities, higher safety margin and lesser costs [8,9]. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, terpenoids and flavonoids having been found in vitro since they have antimicrobial properties and may serve as an alternative, effective, cheap and safe antimicrobial for the treatment of microbial infections [10-14]. Baicalein (5,6,7-trihydroxyflavone), a flavonoid originally isolated from the root of Scutellaria baicalensis Georgi, has numerous pharmacological activities, such as anti-fibrotic, anti-virus, anti-bacterial, anti-fungal, anti-oxidant, anti-cancer, and anti-inflammatory activities [15-20]. It has been shown to significantly restore the effectiveness of β-lactam antibiotics and tetracycline against methicillin-resistant Staphylococcus aureus (MRSA) [21]. With multiple therapeutic benefits, the antibacterial actions of baicalein also are involved in overcoming other bacterial resistance mechanisms [21,22]. In gram-negative bacteria, baicalein is shown to reverse the resistance in TetK overexpressing Escherichia coli by inhibiting tetracyclin efflux by TetK [21].

In this study, the antimicrobial activities of baicalein against methicillin and vancomycin-resistant Staphylococcus aureus isolated in a clinic were assessed using broth micro-dilution method and the checkerboard and time-kill methods for synergistic effect of the combination with antibiotics.

Material

Preparation of bacterial strains

16 isolates of methicillin-resistant Staphylococcus aureus, 2 isolates of methicillin-sensitive S. aureus (MSSA), and 2 isolates of Vancomycin-Resistant S. Aureus (VRSA) purchased from the Culture Collection of Antimicrobial Resistant Microbes (CCARM), as well as standard strains of methicillin-sensitive S. aureus (MSSA) ATCC 25923 and methicillin-resistant S. aureus (MRSA) ATCC 33591 were used (Table 1). 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). Briefly, the growth of bacteria was examined at 37°C in 0.95 ml of BHI broth containing various concentrations of baicalein. These tubes were inoculated with 5 × 105 colony-forming units (CFU)/ml of an overnight culture grown in BHI broth, and incubated at 37°C. After 24 h of incubation, the optical density (OD) was measured spectro-photometrically at 600 nm. Three replicates were measured for each concentration of tested drugs.

Samples	Baicalein (μg/ml)			Ampicillin	Oxacillin	Vancomycin
	MIC50<	MIC90<	MIC/MBC	MIC/MBC (μg/ml)		
MSSA ATCC 25923 1	32	128	128/256	16-Aug	0.25/0.5	0.5/0.5
MRSA ATCC 33591 2	64	256	256/512	512/2048	16/32	4-Feb
VRSA 3A0633	32	128	128/512	1024/2048	512/1024	32/64
VRSA 3A0664	16	64	64/64	64/256	256/1024	32/64
MSSA 15	32	128	128/128	512/1024	0.5/0.5	2-Jan
MSSA2	32	256	256/512	256/512	0.5/2	2-Jan
MRSA 1	64	256	256/512	128/256	128/256	2-Jan
MRSA 2	32	128	128/512	128/256	16/32	4-Jan
MRSA 3	16	64	64/256	128/512	128/512	4-Feb
MRSA 4	16	64	64/128	256/512	512/2048	1-Jan
MRSA 5	32	256	256/512	128/512	512/1024	2-Jan
MRSA 6	64	256	256/512	64/128	128/256	2-Jan
MRSA 7	16	64	64/128	128/256	512/1024	2-Jan
MRSA 8	16	128	128/256	256/512	1024/2048	4-Jan
MRSA 9	64	256	256/512	64/128	512/1024	2-Jan
MRSA 10	64	256	256/256	128/256	512/512	0.5/2
MRSA 11	16	64	64/128	64/128	64/128	2-Jan
MRSA 12	16	64	64/64	256/256	128/256	0.5/4
MRSA 13	32	256	256/256	128/256	64/128	2-Jan
MRSA 14	32	256	256/512	128/256	16/32	4-Jan
MRSA 15	32	128	128/128	64/128	128/256	4-Jan
MRSA 16	64	256	256/512	128/512	256/512	0.5/1

Table 1: Antibacterial activity of baicalein and antibiotics in isolated MRSA, VRSA, MSSA, and some of reference bacteria; [1]MSSA (ATCC 25923): reference strain Methicillin-sensitive Staphylococcus aureus;2 MRSA (ATCC 33591): reference strain Methicillin-resistant Staphylococcus aureus;[3]VRSA 3A063: Vancomycin-resistant Staphylococcus aureus isolated a clinic;4VRSA 3A066 : Vancomycin-resistant Staphylococcus aureus isolated a clinic;[5]MSSA (1, 2): Methicillin-sensitive Staphylococcus aureus isolated a clinic;6MRSA (1-16): Methicillin-resistant Staphylococcus aureus isolated a clinic.

Methods

Minimum inhibitory concentration/minimum bactericidal concentration assay

The antimicrobial activities of baicalein against clinical isolates MRSA 16, MSSA 2, VRSA 2, and reference strains were determined via the broth dilution method [23]. 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, MIC50s and MIC90s, 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.

Checkerboard dilution test

The synergistic combinations were investigated in the preliminary checkerboard method performed using the MRSA, MSSA, and VRSA of clinical isolate strains via MIC and MBC determination [24]. The Fractional Inhibitory Concentration Index (FICI) and Fractional Bactericidal Concentration Index (FBCI) are the sum of the FICs and FBCs of each of the drugs, which were defined as the MIC and MBC of each drug when used in combination divided by the MIC and MBC of each drug when used alone. The FIC and FBC 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) and FBC=(MBC of drug A in combination/MBC of drug A alone)+(MBC of drug B in combination/MBC of drug B alone). FIC and FBC indices were interpreted as follows: the FIC index was interpreted as follows: synergy, <0.5; partial synergy, 0.5-0.75; additive effect, 0.76-1.0; indifference, >1.0-4.0; and antagonism, >4.0 [23,24].

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 1-5 × 10^6 CFU/ml were exposed to the MIC of baicalein alone, or baicalein (1/2 MIC) plus oxacillin or ampicillin or vancomycin (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.

Results

Antibacterial activity

Our results of the antibacterial activity showed that the baicalein exhibited inhibitory activities against isolates MSSA, MRSA, VRSA, and reference stains. The MICs and MBCs values of baicalein against MSSA ATCC25923 in the range of 128 μg/ml and 256 μg/ml, MRSA ATCC33591 in the range of 256 μg/ml and 512 μg/ml, and isolates MSSA 1 and 2 in the range of 128-256 μg/ml and 128-512 μg/ml, MRSA 1-16 in the ranges of 64-256 μg/ml and 64-512 μg/ml, and VRSA 1 and 2 in the range of 64-128 μg/ml and 64-512 μg/ml, respectively (Table 1). The baicalein showed the strongest activity against VRSA 3A063, MRSA 3, 4, 7, and 12 (MICs values 64 μg/ml and MBCs values 64-128 μg/ml). The ampicillin showed antibacterial activity against all tested bacteria by the MICs and MBCs ranges of 8-1024 μg/ml and 16-2048 μg/ml, oxacillin by MICs values 0.25-1024 μg/ml and MBCs values 0.5-2048 μg/mL, and vancomycin by MICs values 0.5-32 μg/ml and MBCs values 0.5-64 μg/mL (Table 1). The MIC50 and MIC90 values of baicalein for MRSA 1-16 isolates were 16-64 μg/ml and 64-256 μg/ml, respectively.

Synergistic effect of baicalein against VRSA and MRSA

The combination of oxacillin and baicalein resulted in a reduction in the MICs/MBCs for isolates VRSA 1-2 and MSSA 1-2, with the MICs/MBCs of 4/8 or 32/64 μg/ml and 16/32 or 64/128 μg/ml, for oxacillin becoming 0.125-64/0.125-256 μg/mL and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 (Table 2).

Samples	Agent	MIC/MBC (μg/ml)		FIC/FBC	FICI/FBCI[2]	Outcome
		Alone	Combination[1]			
MSSA ATCC 25923 3	Baicalein	128/256	32/64	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	Baicalein	256/512	64/64	0.25/0.125	0.5/0.625	Synergistic/ Additive
	Oxacillin	16/32	16-Apr	0.25/0.5		
VRSA 3A0635	Baicalein	128/512	32/64	0.25/0.125	0.375/0.375	Synergistic/ Synergistic
	Oxacillin	512/1024	64/256	0.125/0.25		
VRSA 3A0666	Baicalein	64/64	8-Apr	0.0625/0.125	0.3125/0.25	Synergistic/ Synergistic
	Oxacillin	256/1024	64/128	0.25/0.125		
MSSA 17	Baicalein	128/128	16/32	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	0.5/0.5	0.125/0.125	0.25/0.25		

MSSA 2	Baicalein	256/512	64/128	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	0.5/2	0.125/0.25	0.25/0.125		
MRSA 18	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/
	Oxacillin	128/256	32/64	0.25/0.25		Synergistic
MRSA 2	Baicalein	128/512	32/64	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	16/32	8-Apr	0.25/0.25		
MRSA 3	Baicalein	64/256	16/64	0.25/0.25	0.5/0.375	Synergistic/
	Oxacillin	128/512	32/64	0.25/0.125		Synergistic
MRSA 4	Baicalein	64/128	16/32	0.25/0.25	0.375/0.3125	Synergistic/ Synergistic
	Oxacillin	512/2048	64/128	0.125/0.0625		
MRSA 5	Baicalein	256/512	32/64	0.125/0.125	0.25/0.375	Synergistic/ Synergistic
	Oxacillin	512/1024	64/256	0.125/0.25		
MRSA 6	Baicalein	256/512	32/128	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	128/256	32/64	0.25/0.25		
MRSA 7	Baicalein	64/128	32/64	0.5/0.5	0.625/0.625	Additive/ Additive
	Oxacillin	512/1024	64/128	0.125/0.125		
MRSA 8	Baicalein	128/256	32/64	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Oxacillin	1024/2048	256/256	0.25/0.125		
MRSA 9	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	512/1024	128/256	0.25/0.25		
MRSA 10	Baicalein	256/256	64/128	0.25/0.5	0.5/1.0	Synergistic/ Additive
	Oxacillin	512/512	128/256	0.25/0.5		
MRSA 11	Baicalein	64/128	16/32	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	64/128	16/32	0.25/0.25		
MRSA 12	Baicalein	64/64	16/32	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Oxacillin	128/256	32/64	0.25/0.25		
MRSA 13	Baicalein	256/256	64/128	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Oxacillin	64/128	16/32	0.25/0.25		
MRSA 14	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Oxacillin	16/32	8-Apr	0.25/0.25		
MRSA 15	Baicalein	128/128	32/64	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Oxacillin	128/256	32/64	0.25/0.25		
MRSA 16	Baicalein	256/512	64/128	0.25/0.25	0.375/0.5	Synergistic/ Synergistic
	Oxacillin	256/512	32/128	0.125/0.25		

Table 2: Synergistic effects of the baicalein with oxacillin in isolated MRSA, VRSA, MSSA, and some of reference bacteria;[1]The MIC and MBC of baicalein 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]VRSA 3A063: Vancomycin-resistant Staphylococcus aureus isolated a clinic; [6]VRSA 3A066 : Vancomycin-resistant Staphylococcus aureus isolated a clinic;[7]MSSA (1, 2): Methicillin-sensitive Staphylococcus aureus isolated a clinic;[8]MRSA (1-16): Methicillin-resistant Staphylococcus aureus isolated a clinic.

The combination of oxacillin and baicalein resulted in a reduction against isolates MRSA 1-16, with the MICs/MBCs values of 16-64/32-128 μg/ml, for oxacillin becoming 4-256/8-256 μg/ml and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 7 of additive (FICI ≥ 0.625) and MRSA 10, 12, 13, and 15 of additive (FBCI ≥ 0.75). The combination of ampicillin and baicalein resulted in a reduction in the MICs/MBCs for isolates VRSA 1-2 and MSSA 1-2, with the MICs/MBCs of 8/16 or 32/64 μg/ml and 16/32 or 64/128, for ampicillin becoming 16/32 or 256/256 μg/ml and 64/128 or 128/256 μg/ml and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5. The combination of ampicillin and baicalein resulted in a reduction against isolates MRSA 1-16, with the MICs/MBCs values of 8-64/16-128 μg/ml, for oxacillin becoming 16-64/32-128 μg/mL and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except and in most of MRSA tested were reduced by ≥4-fold videncing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 10, 13, and 16 (FBCI ≥ 0.75), respectively (Table 3).

Samples	Agent	MIC/MBC (μg/ml)		FIC/FBC	FICI/FBCI[2]	Outcome
		Alone	Combination[1]			
MSSA ATCC 25923 3	Baicalein	128/256	32/64	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Ampicillin	16-Aug	4-Apr	0.5/0.25		
MRSA ATCC 33591 4	Baicalein	256/512	64/128	0.25/0.25	0.375/0.3125	Synergistic/ Synergistic
	Ampicillin	512/2048	64/128	0.125/0.0625		
VRSA 3A0635	Baicalein	128/512	32/64	0.25/0.125	0.5/0.25	Synergistic/ Synergistic
	Ampicillin	1024/2048	256/256	0.25/0.125		
VRSA 3A0666	Baicalein	64/64	16-Aug	0.125/0.25	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	64/256	16/32	0.25/0.125		
MSSA 17	Baicalein	128/128	16/32	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Ampicillin	512/1024	128/256	0.25/0.25		
MSSA 2	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	256/512	64/128	0.25/0.25		
MRSA 18	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 2	Baicalein	128/512	32/64	0.25/0.125	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	128/256	16/64	0.125/0.25		
MRSA 3	Baicalein	64/256	16/32	0.25/0.125	0.5/0.25	Synergistic/ Synergistic
	Ampicillin	128/512	32/64	0.25/0.125		
MRSA 4	Baicalein	64/128	16/32	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	256/512	64/128	0.25/0.25		
MRSA 5	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/512	32/128	0.25/0.25		
MRSA 6	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/
	Ampicillin	64/128	16/32	0.25/0.25		Synergistic
MRSA 7	Baicalein	64/128	Aug-32	0.125/0.25	0.375/0.5	Synergistic/

	Ampicillin	128/256	32/64	0.25/0.25		Synergistic
MRSA 8	Baicalein	128/256	32/64	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	256/512	64/128	0.25/0.25		
MRSA 9	Baicalein	256/512	32/128	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 10	Baicalein	256/256	64/128	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 11	Baicalein	64/128	16/32	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	64/128	16/32	0.25/0.25		
MRSA 12	Baicalein	64/64	16/16	0.25/0.25	0.375/0.5	Synergistic/
	Ampicillin	256/256	32/64	0.125/0.25		Synergistic
MRSA 13	Baicalein	256/256	64/128	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 14	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	128/256	32/64	0.25/0.25		
MRSA 15	Baicalein	128/128	32/32	0.25/0.25	0.5/0.5	Synergistic/
	Ampicillin	64/128	16/32	0.25/0.25		Synergistic
MRSA 16	Baicalein	256/512	64/128	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	128/512	32/64	0.25/0.125		

Table 3: Synergistic effects of baicalein with ampicillin in isolated MRSA, VRSA, MSSA, and some of reference bacteria;[1]The MIC and MBC of baicalein 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]VRSA 3A063: Vancomycin-resistant Staphylococcus aureus isolated a clinic; [6]VRSA 3A066 : Vancomycin-resistant Staphylococcus aureus isolated a clinic;[7]MSSA (1, 2): Methicillin-sensitive Staphylococcus aureus isolated a clinic;[8]MRSA (1-16): Methicillin-resistant Staphylococcus aureus isolated a clinic.

The combination of vancomycin and baicalein resulted in a reduction against isolates MRSA 1-16, with the MICs/MBCs values of 16-64/32-128 μg/ml, for vancomycin becoming 0.125-0.5/0.25-1 μg/ml and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 8 and 15 of additive (FICI ≥ 0.75) and MRSA 4, 10, 12, 13, and 15 of additive (FBCI ≥ 0.625) and for isolates VRSA 1-2 and MSSA 1-2, with the MICs/MBCs of 16/32 or 32/64 μg/ml and 32/64 or 64/128, for vancomycin becoming 8/16 μg/ml and 0.25/0.5 μg/ml and reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except VRSA3A066 and MSSA 1 of additive (FBCI ≥ 0.75) (Table 4).

Samples	**Agent**	**MIC/MBC (μg/ml)**		**FIC/FBC**	**FICI/FBCI[2]**	**Outcome**
		Alone	**Combination[1]**			
MSSA ATCC 25923 3	Baicalein	128/256	32/64	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Vancomycin	0.5/0.5	0.125/0.25	0.25/0.5		
MRSA ATCC 33591 4	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	4-Feb	0.5/1	0.25/0.25		
VRSA 3A0633	Baicalein	128/512	32/64	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Vancomycin	32/64	16-Aug	0.25/0.25		
VRSA 3A0664	Baicalein	64/64	16/32	0.25/0.5	0.5/0.75	Synergistic/ Additive

	Vancomycin	32/64	16-Aug	0.25/0.25		
MSSA 1	Baicalein	128/128	32/64	0.25/0.5	0.5/0.75	Synergistic/
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		Additive
MSSA 2	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 1	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		Synergistic
MRSA 2	Baicalein	128/512	32/64	0.25/0.125	0.5/0.25	Synergistic/ Synergistic
	Vancomycin	4-Jan	0.25/0.5	0.25/0.125		
MRSA 3	Baicalein	64/256	16/32	0.25/0.125	0.5/0.375	Synergistic/
	Vancomycin	4-Feb	0.5/1	0.25/0.25		Synergistic
MRSA 4	Baicalein	64/128	16/32	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Vancomycin	1-Jan	0.25/0.5	0.25/0.5		
MRSA 5	Baicalein	256/512	32/64	0.125/0.125	0.375/0.375	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 6	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 7	Baicalein	64/128	16/32	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 8	Baicalein	128/256	32/64	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Vancomycin	4-Jan	0.5/1	0.5/0.25		
MRSA 9	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 10	Baicalein	256/256	64/128	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Vancomycin	0.5/2	0.125/0.25	0.25/0.125		
MRSA 11	Baicalein	64/128	16/32	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 12	Baicalein	64/64	16/32	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Vancomycin	0.5/4	0.125/0.5	0.25/0.125		
MRSA 13	Baicalein	256/256	64/128	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Vancomycin	2-Jan	0.25/0.5	0.25/0.25		
MRSA 14	Baicalein	256/512	64/128	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Vancomycin	4-Jan	0.25/0.5	0.25/0.125		
MRSA 15	Baicalein	128/128	32/64	0.25/0.5	0.75/0.75	Additive/ Additive
	Vancomycin	4-Jan	0.25/1	0.5/0.25		
MRSA 16	Baicalein	256/512	64/128	0.25/0.25	0.5/0.5	Synergistic/ Synergistic

	Vancomycin	0.5/1	0.125/0.25	0.25/0.25		

Table 4: Synergistic effects of baicalein with vancomycin in isolated MRSA, VRSA, MSSA, and some of reference bacteria;[1]The MIC and MBC of baicalein with vancomycin; [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]VRSA 3A063: Vancomycin-resistant Staphylococcus aureus isolated a clinic; [6]VRSA 3A066 : Vancomycin-resistant Staphylococcus aureus isolated a clinic;[7]MSSA (1, 2): Methicillin-sensitive Staphylococcus aureus isolated a clinic;[8]MRSA (1-16): Methicillin-resistant Staphylococcus aureus isolated a clinic.

Time-kill curves

The effects of baicalein administered in combination with oxacillin and/or ampicillin and/or vancomycin against standard (MSSA and MRSA) and clinical isolates of MSSA (1, 2), VRSA (1,2), and MRSA (MRSA 1-16) were confirmed by time-kill curve experiments (Figures 1-4). Cultures of each strain of bacteria with a cell density of 106 CFU/mL were exposed to the MIC of baicalein and antibiotics alone or baicalein (1/2 MIC) with oxacillin (1/2 MIC) or ampicillin (1/2 MIC), and vancomycin (1/2 MIC). We observed that 30 minutes of baicalein treatment with ampicillin or oxacillin, vancomycin resulted in an increased rate of killing as compared to that observed with baicalein (MIC) alone. A profound bactericidal effect was exerted when a combination of drugs was utilized. The growth of the tested bacteria was completely attenuated after 2-5 h of treatment with the 1/2 MIC of baicalein, regardless of whether it was administered alone or with oxacillin (1/2 MIC) or ampicillin (1/2 MIC), or vancomycin (1/2 MIC) (Figures 1-4).

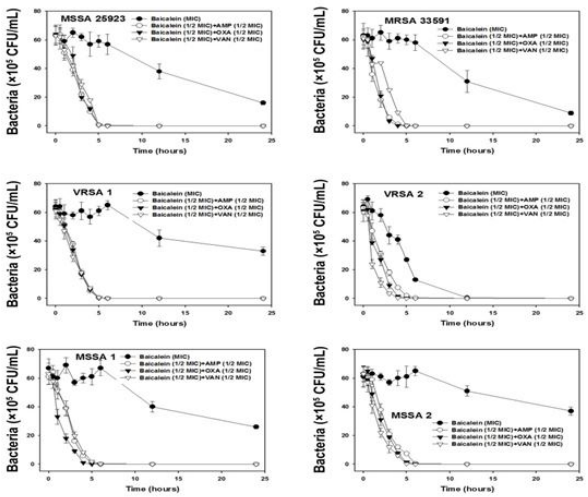

Figure 1: Time-kill curves of MIC or 1/2 MIC of bicalein, ampicillin (AMP), oxacillin (OXA), and vancomycin (VAN) alone and its combination with MIC50 of AMP or OXA, and VAN against MSSA 1, MSSA 2, VRSA 1, and VRSA 2 isolates and reference stains, MSSA ATCC25923 and MRSA ATCC33591. Bacteria were incubated with MIC of baicalein (), AMP, OXA, and VAN, and 1/2 MIC of baicalein+ 1/2 MIC of AMP (○), 1/2 MIC of baicalein + 1/2 MIC of OXA (), and 1/2 MIC of baicalein + 1/2 MIC of VAN () over time. CFU: Colony-Forming Units.

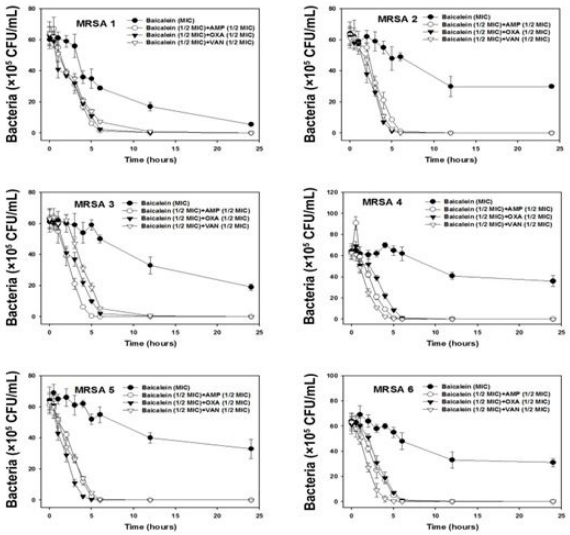

Figure 2 : Time-kill curves of MIC or 1/2 MIC of bicalein, ampicillin (AMP), oxacillin (OXA), and vancomycin (VAN) alone and its combination with 1/2 MIC of AMP or OXA, and VAN against MRSA 1, 2, 3, 4, 5, and 6 isolates. Bacteria were incubated with MIC of bicalein (), AMP, OXA, and VAN, and 1/2 MIC of bicalein + 1/2 MIC of AMP (○), 1/2 MIC of bicalein + 1/2 MIC of OXA (), and 1/2 MIC of bicalein + 1/2 MIC of VAN () over time. CFU: Colony-Forming Units.

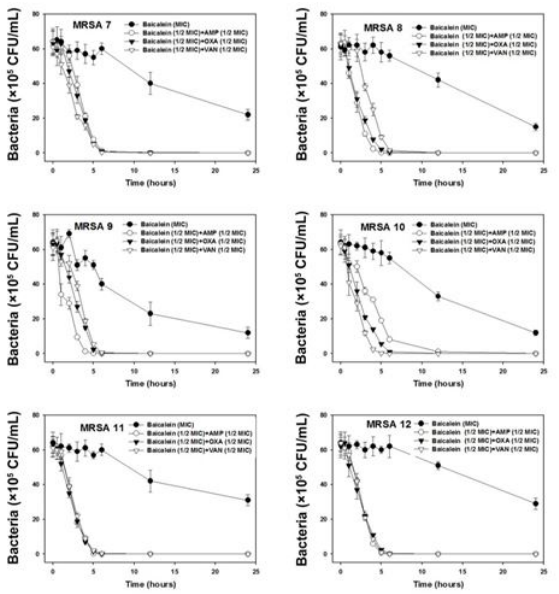

Figure 3: Time-kill curves of MIC or 1/2 MIC of baicalein, ampicillin (AMP), oxacillin (OXA), and vancomycin (VAN) alone and its combination with 1/2 MIC of AMP or OXA, and VAN against MRSA 7, 8, 9, 10, 11, and 12 isolates. Bacteria were incubated with MIC of bicalein (), AMP, OXA, and VAN, and 1/2 MIC of bicalein + 1/2 MIC of AMP (○), 1/2 MIC of bicalein + 1/2 MIC of OXA (), and 1/2 MIC of bicalein + 1/2 MIC of VAN () over time. CFU: Colony-Forming Units.

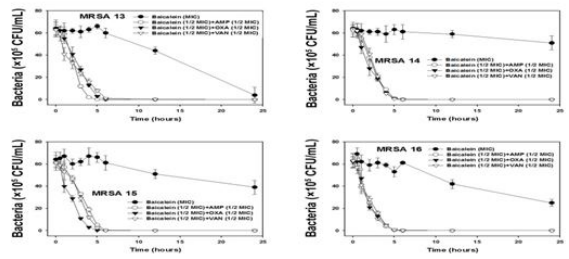

Figure 4: Time-kill curves of MIC or 1/2 MIC of bicalein, ampicillin (AMP), oxacillin (OXA), and vancomycin (VAN) alone and its combination with 1/2 MIC of AMP or OXA, and VAN against MRSA 13, 14, 15, and 16 isolates. Bacteria were incubated with MIC of bicalein (), AMP (○), OXA (), and VAN (), and 1/2 MIC of bicalein + 1/2 MIC of AMP (), 1/2 MIC of bicalein + 1/2 MIC of OXA (○), and 1/2 MIC of bicalein + 1/2 MIC of VAN () over time. CFU: Colony-Forming Units.

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 [8,11,12,21,24]. The baicalein exhibited inhibitory activities against isolates MSSA, MRSA, VRSA, and reference stains. The baicalein showed antibacteial activity against isolates MRSA 1-16 in MICs range of 64-256 μg/ml and MBCs range of 64-512 μg/mL, and VRSA 1 and 2 in MICs range of 64-128 μg/ml and in MBCs range of 64-512 μg/ml, respectively. The baicalein showed the strongest activity against VRSA 3A063, MRSA 3,4,7 and 12 (MICs values 64 μg/mL and MBCs values 64-128 μg/ml). The MIC50 and MIC90 values of baicalein for MRSA 1-16 isolates were 16-64 μg/ml and 64-256 μg/ml, respectively.

One potential choice is a flavone called baicalein, an active ingredient found in Scutellaria baicalensis Georgi., which is one of the most popular and multi-purpose herb used for the treatment of bacterial and viral infections [16,18,20,21,25]. In this study, the baicalein showed strong bacterial activity on MRSA and VRSA.

Evaluation of in vivo effectiveness of the antimicrobial combinations is necessary to generate data that can be extrapolated to the clinical situation as well as to predict relevant concentration of optimal dosing regimens for both agents of the combinations [26,27]. That combination therapy proceeds by different pathways according to the antibacterial agent used against pathogenic infections [28]. The most common combination strategy is to use drugs, each of which inhibits a different bacterial pathway. In the present study, we chose ampicillin, oxacillin, and vancomycin as the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. Vancomycin has used treatment of serious infections caused by susceptible organisms resistant to penicillins (methicillin-resistant S. aureus) and multi resistant Staphylococcus epidermidis (MRSE) or in individuals with serious allergy to penicillins [4,5,27,29]. The combination of oxacillin and baicalein resulted in a reduction in the MICs/MBCs for isolates VRSA 1-2 by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 and MRSA 1-16 reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 7 of additive (FICI ≥ 0.625) and MRSA 10, 12, 13, and 15 of additive (FBCI ≥ 0.75). The combination of ampicillin and baicalein resulted in a reduction in the MICs/MBCs for isolates VRSA 1-2 by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 and MRSA 1-16 reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except and in most of MRSA tested were reduced by ≥4-fold evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 10, 13, and 16 (FBCI ≥ 0.75), respectively. The combination of vancomycin and baicalein resulted in a reduction against isolates MRSA 1-16 reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except MRSA 8 and 15 of additive (FICI ≥ 0.75) and MRSA 4, 10, 12, 13, and 15 of additive (FBCI ≥ 0.625) and VRSA 1-2 reduced by ≥4-fold, evidencing a synergistic effect as defined by a FICI and FBCI of ≤ 0.5 except VRSA3A066 of additive (FBCI ≥ 0.75). Synergy of baicalein associated with gentamicin against vancomycin-resistant Enterococcus has also been reported [25]. The synergistic actions of baicalein on MRSA may therefore involve other mechanisms of action such bacterial efflux pumps inhibition different from TetK, penicillin-binding proteins or interfering with the integrity of the cell wall [21,22].

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve a defence mechanism against prediction by many microorganisms, insects and other herbivores [11-13,20,24]. The compounds in the flavonol, flavan-3-ol

and flavone classes have been shown to inhibit energy metabolism (through ATP synthase inhibition) [30-32]. It has been reported that some plant derived compounds can improve the in vitro activity of some cell-wall inhibiting antibiotics by directly attacking the same target site, that is, peptidoglycan [33-35]. Some studies have shown that flavone derivatives are inhibitors of the NorA multidrug resistance pump in Staphylococcus aureus [22]. Baicalein acts as inhibitors of other bacterial resistant related enzymes such as methyl transferases associated to aminoglycosides resistance [36]. Flavonoid complexes attach with extra cellular soluble protein and with bacterial cell wall [37].

The effects of baicalein administered in combination with oxacillin and/or ampicillin and/or vancomycin against clinical isolates of VRSA and MRSA were confirmed by time-kill curve experiments. The time-kill curve of baicalein showed completely attenuated after 2-5 h of treatment and an increased rate of killing as compared to that observed with baicalein alone. A profound bactericidal effect was exerted when a combination of drugs was utilized.

In conclusion, our results of the antibacterial activity showed that baicalein exhibited strong inhibitory activities against isolates MRSA and VRSA. The combination effects of baicalein with antibiotics were synergistic effect by FIC/FBC index <0.5 against most of tested clinic isolated MRSA and VRSA. The more the antibacterial action and cell wall synthesis inhibition increased when used in combination baicalein with oxacillin and/or vancomycin. Baicalein is expected to be recognized as natural sources for the development of new functional drugs against multi-resistant S. aureus, MRSA and VRSA.

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