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Synergistic Effects of the Combination of Galangin with Gentamicin against Methicillin-Resistant *Staphylococcus aureus*

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The antimicrobial killing activity toward methicillin-resistant *Staphylococcus aureus* (MRSA) has been a serious emerging global issue. New effective antimicrobials and/or new approaches to settle this issue are urgently needed. The oriental herb, *Alpinia officinarum*, has been used in Korea for several hundreds of years to treat various infectious diseases. As it is well known, one of the active constituents of *Alpinia officinarum* is galangin. Against the 17 strains, the minimum inhibitory concentrations (MICs) of galangin (GAL) were in the range of 62.5~125 μg/ml, and the MICs of gentamicin (GEN) ranged from 1.9 μg/ml to 2,000 μg/ml. The fractional inhibitory concentrations (FICs) of GAL, in combination with GEN, against 3 test strains were 0.4, 3.9, and 250 μg/ml, and were all 15.62 μg/ml in GEN. The FIC index showed marked synergism in the value range of 0.19 to 0.25. By determining time-kill curves, also confirmed the low synergism of the GAL and GEN combination against 4 h, 8 h, 12 h, and 24 h cultured MRSA. The time-kill study results indicated a low synergistic effect against 3 test strains. Thus, the mixture of GAL and GEN could lead to the development of new combination antibiotics against MRSA infection.

***Keywords*:** galangin, *Alpinia officinarum*, methicillin-resistant *Staphylococcus aureus* (MRSA), checkerboard test

Staphylococcus aureus is an important human pathogen, causing life-threatening systemic infections such as pneumonia, septicemia, endocarditis, and osteomyelitis. Furthermore, *S. aureus* bacteria can spread easily, and have been found in the noses of approximately 40~50% of healthy people, and are also commonly found on the skin (Marraro and Mitchell, 1975; Sanford *et al*., 1994; Lowy, 1998). MRSA is one of the most important nosocomial pathogens of the past two decades (Lowy, 1998). Today, the ongoing emergence of multi-drug resistant bacteria and the infectious diseases caused by them are serious global problems (Rountree, 1978; Linton *et al*., 1988). Thus, Novel antimicrobials and/or new approaches to combat these problems are urgently needed (Liu *et al*., 2000).

Therefore, combination therapy is often profitable for patients with serious infections caused by drug-resistant pathogens (Dawis *et al*., 2003). The use of combination therapy can broaden the spectrum, of antimicrobial activity, minimize the emergence of resistant microbial variants and can sometimes result in synergistic interaction, thereby exhibiting antimicrobial activity greater than would be expected from each antimicrobial drug individually (Eliopoulos and Eliopoulos, 1998).

Natural products have traditionally been a rich source of antimicrobial agents of many nations (Silver and Bostian, 1990). Flavonoids are increasingly becoming the many subjects of medical research (Cushnie and Lamb, 2006). The rhizome of *Alpinia officinarum* has been used as a traditional medicine in Orient for relieving stomach-ache, treating colds, invigorating the circulatory system, and reducing swelling (An *et al.,* 2008). The flavonol galangin (3,5,7-trihydroxyflavone) is present in numerous plants and is a major constituent of *Alpinia officinarum* (Cushnie and Lamb, 2006).

In the present study investigated antimicrobial activity of GAL and the synergistic effects of the mixture of GAL and GEN against MRSA strains. GAL is known as an antibacterial, antifungal, and antiviral agent (Shin *et al*., 2002; Kim *et al*., 2006). In the present study, performed MICs, the checkerboard test, and a time-kill assay to evaluate the susceptibility of GEN and the synergism of the mixture of GAL and GEN against 17 MRSA strains.

Materials and Methods

Bacterial strains and growth conditions

Among the 17 *S. aureus* strains used in this study, 15 clinical isolates (MRSA) were obtained from 15 unique patients at Wonkwang University Hospital (Iksan, Korea). The other 2 strains were *S. aureus* KCCM 40510 (methicillin-resistant strain) and *S. aureus* ATCC 25923 (methicillin-susceptible strain). ATCC 25923 (American Type Culture Collection, Manassas, USA) and KCCM 40510 (Korea Culture Center

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of Culture Collections) 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 Laboratories, USA)

Determination of the *mecA* gene

Detection of the *mecA* gene in the MRSA strains was performed by PCR (Polymerase Chain Reaction) amplication. Prior to the DNA extraction, frozen bacteria were subcultured twice onto 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 Co., Korea). The PCR amplification consisted of 30 cycles (94°C, 60 sec; 55°C, 60 sec; 72°C, 60 sec).

The primers used in this study were as follows: mecA-forward primer; 5'-ATGAGATTAGGCATCGTTTC-3', reverse primer; 5'-TGGATGACAGTACCTGAGCC-3' and femA-forward primer; 5'-CATGATGGCGAGATTACAGG-3', reverse primer; 5'-CGCTAAAGGTACTAACACACGG-3'. The final PCR products were separated on 2% agarose gel.

Antibiotics and chemicals

GAL and GEN were commercially purchased from Sigma Chemical Co. (USA).

Determination of MICs

The MIC was performed by the microdilution broth method (CLSI, 2006). Serial two fold dilutions of GAL and GEN was prepared in sterile 96-well micro plates and microtube with concentrations ranging between 1/2 by using MHB. The *S. aureus* 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 MHB was supplemented with serial GEN concentrations ranging from 0.19 to 5,000 µg/ml, and with GAL at concentrations from 31.25 to 1,000 µg/ml. The data were reported as MICs, the lowest concentration of GEN and GAL inhibiting visible growth after 24 h of incubation at 37°C (Shahverdi *et al*., 2007). The MIC of GEN was also determined, and similarly defined as the lowest antibiotic concentration at which no visible bacterial growth was observed.

The checkerboard dilution test and time-kill assay

The antibacterial effects that resulted from combining the two antimicrobial agents were assessed by the checkerboard test (Noble *et al*., 1992). The serial dilutions of the GAL and GEN were mixed in cation-supplemented MHB. The inocula were prepared from colonies that had been grown on MHA overnight. The final bacterial concentration after inoculation was 10^6 CFU/ml, and the MIC was determined after 24 h of incubation at 37°C.

The fractional inhibitory concentration (FIC) index was determined by the following formula: FIC index = $FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$, where [A] is the concentration

Table 1. The *S. aureus* strains used in the experiments

S. aureus strain	Class	*mecA* gene[a]	β-Lactamase activity	Antibiotic resistance pattern[b]
ATCC 25923	MSSA	-	-	-
KCCM 40510	MRSA	+	+	AM, OX
Clinical isolates[c]				
DPS-1	MRSA	+	+	AM, OX
DPS-2	MRSA	+	-	AM, OX
DPS-3	MRSA	+	+	AM, OX
DPS-4	MRSA	+	-	AM, OX
DPS-5	MRSA	+	-	AM, OX
DPS-6	MRSA	+	-	AM, OX
DPS-7	MRSA	+	-	AM, OX
DPS-8	MRSA	+	+	AM, OX
DPS-9	MRSA	+	+	AM, OX
DPS-10	MRSA	+	+	AM, OX
DPS-11	MRSA	+	+	AM, OX
DPS-12	MRSA	+	-	AM, OX
DPS-13	MRSA	+	+	AM, OX
DPS-14	MRSA	+	-	AM, OX
DPS-15	MRSA	+	-	AM, OX

[a] +, positive; -, negative.
[b] AM, ampicillin; OX, oxacillin.
[c] DPS indicates staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Table 2. Antimicrobial activity of galangin and gentamicin against 16 MRSA strains, a standard MRSA strain, and an MSSA strain

		MIC (μg/ml)	
S. aureus strain	Class	GEN[a]	GAL
ATCC 25923	MSSA	3.92	125
KCCM 40510	MRSA	62.5	125
Clinical isolates			
DPS-1[b]	MRSA	2000	125
DPS-2	MRSA	500	62.5
DPS-3	MRSA	2000	62.5
DPS-4	MRSA	1.9	125
DPS-5	MRSA	2000	62.5
DPS-6	MRSA	2000	125
DPS-7	MRSA	2000	125
DPS-8	MRSA	2000	62.5
DPS-9	MRSA	2000	125
DPS-10	MRSA	500	62.5
DPS-11	MRSA	2000	62.5
DPS-12	MRSA	2000	125
DPS-13	MRSA	2000	125
DPS-14	MRSA	2000	62.5
DPS-15	MRSA	1.9	125

[a] GEN, gentamicin; GAL, galangin
[b] DPS, 15 clinical isolates from Wonkwang University Hospital

Table 3. The interpreted FICI response for antimicrobial agent combinations against an MRSA isolate, a standard MRSA strain, and a standard MSSA strain

S. aureus strain	Class	FIC (μg/ml)			FICI
		GEN[a]	+	GAL	
ATCC 25923	MSSA	0.1		0.12	0.22 synergy
KCCM 40510	MRSA	0.06		0.12	0.18 synergy
Clinical isolates					
DPS-1[b]	MRSA	0.125		0.12	0.25 synergy

[a] GEN, gentamicin; GAL, galangin
[b] DPS, clinical isolates from Wonkwang University Hospital.

of drug A, MIC_A is its MIC, and FIC_A is the FIC of drug A for the organism; while [B], MIC_B, and FIC_B are defined in the same fashion for drug B. The FIC index thus obtained was interpreted as follows: <0.5, synergy; 0.5 to 0.75, partial synergy; 0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism. Finally, the varying rates of synergy between the two agents were determined (Mazumdar *et al*., 2005; Guadalupe *et al*., 2006).

The time-kill curve assay was performed according to the method described by synergistic bactericidal activity of Nascimento *et al.* (2007), in order to study the combined effects of time and antimicrobial agent concentration on the bacterial growth. For this assays, a standard inoculums of approximately 10^6 CFU/ml of an overnight culture was used. GAL (0.5 MIC) and GEN (0.5 MIC) were used. Combinations of GAL plus GEN were also evaluated. A test plate containing only MHB was inoculated and served as control. Counts of viable strains were carried out at different intervals up to 24 h at 37°C. The rate and extent of killing was determined by plotting viable colony counts (CFU/ml) against time in MHA. According to the method of synergistic bactericidal activity of Nascimento *et al.* (2007), if the final concentration of viable bacteria growing on media containing two antimicrobial agents is 100 fold less than the concentration of bacteria growing on media containing a single antimicrobial agent, then synergy is considered to be present If the final concentration of viable bacteria in media containing two antimicrobial agents is >100 fold higher than the concentration of bacteria growing on media containing a single antimicrobial agent, the result is regarded as antagonistic. Additivity or indifference to the treatment was defined as any other scenario not meeting the criteria for synergy or antagonism. In this paper, all experiments were independently repeated three times.

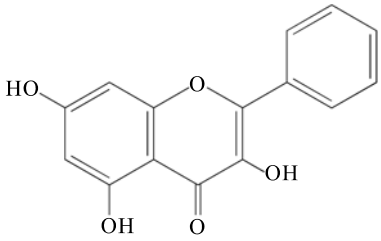

Fig. 1. Chemical structure of the flavonol galangin.

Results

Antimicrobial susceptibility

This study investigated the antimicrobial killing activity of select drugs against 16 MRSA strains and 1 MSSA strain. The checkerboard test results, GEN in combination with GAL, showed good antimicrobial activity against the MRSA strains. The mixture of GAL and GEN showed antibacterial activity not only against the single MSSA strain, but also against 3 MRSA strains tested. The MICs of GAL against the MRSA strains ranged from 62.5 to 125 μg/ml, and 66% of the tested MRSA strains had MICs of 125 μg/ml. The MICs of GEN against the MRSA strains ranged from 1.9 to 2,000 μg/ml, and 69% of the tested MRSA strains had MICs of 2,000 μg/ml. The MIC of GAL against a standard MSSA was 125 μg/ml, and that of GEN were 3.92 μg/ml. Eleven MRSA strains from the 16 tested strains (69%) showed high levels of GEN resistance.

Assessment of synergy

The GAL+GEN combinations exhibited against the 3 tested strains were markedly lowered the MICs. The fractional inhibitory concentrations (FICs) of GAL and GEN against the 3 tested strains were 0.4∼250 μg/ml and 15.62 μg/ml, respectively. The FIC index of GAL in combination with GEN ranged from 0.19 to 0.25, indicating remarkable synergism (Table 3).

Time-kill tests were performed to study the synergistic effects of GAL and GEN with time. Figures 2, 3, and 4 show the test results of the time-kill assay against a standard MRSA strain, a standard MSSA strain, and the DPS-1 strain.

On the tested MHA plates, remarkably lower numbers of colonies were detected as compared to the MHA plates treated with each GAL and GEN. The effect of GAL with GEN was synergistic in FICIs. But the time-kill tests showed different antibacterial activities against 3 strain. Each of these combination regimens were then tested together in a time kill at 4 times the respective isolate MIC. In this same time kill, GAL significantly enhanced GEN activity, but unfortunately, the combination was indifferent in DPS-1.

Discussion

Aminoglycosides, including gentamicin was commonly used antibiotics worldwide because of their low cost and rapid and potent bacteriocidal activity in life-threatening infections (Mascaretti, 2003). But unfortunately, aminoglycosides can induce ototoxicity and nephrotoxicity. Although the nephro-

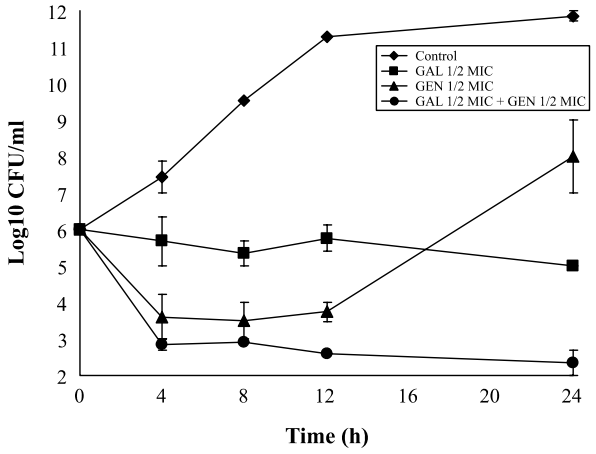

Fig. 2. The time-kill curves of galangin and gentamicin against the standard MSSA (ATCC 25923) strain.

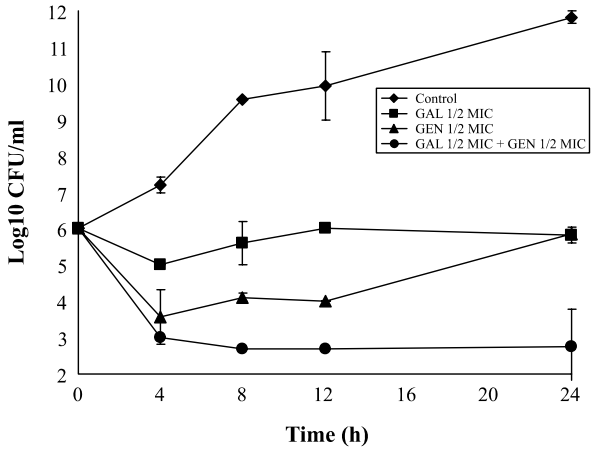

Fig. 3. The time-kill curves of galangin and gentamicin against the standard MRSA (KCCM 40510) strain.

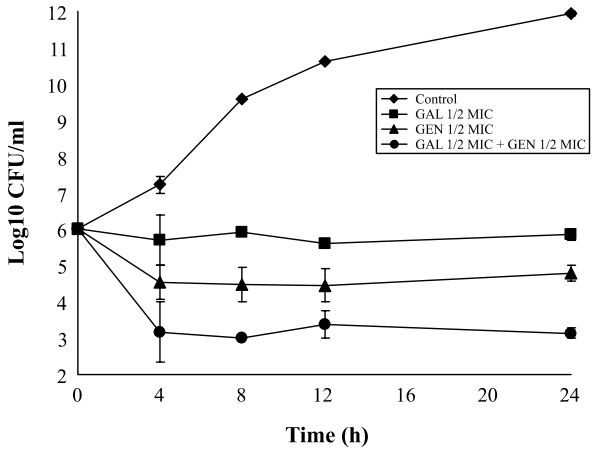

Fig. 4. The time-kill curves of galangin and gentamicin against the MRSA clinical isolate (DPS-1) strain.

toxicity is usually reversible, the ototoxicy is often permanent. Still and all, in developing countries, they are favored because they are relatively less expensive than newer of other antimicrobial drugs, nonototoxic agents (Drobbin *et al*., 2007).

Besides, one of the major problems in the use of topical antibiotics is the development of resistance. The widespread emergence of multi-drug resistant bacteria has made it more difficult to prevent and cure infectious diseases. Although the use of antibiotics, including misuse and overuse, has treated infectious diseases, it has also aided natural bacterial evolution and led to the appearance of drug resistant bacteria (Barrett *et al*., 1968; Parker and Hewitt, 1970; Lowy, 1998). MRSA is very dangerous, and produces serious medical problems because it causes the most common infectious diseases and often acquires multi-drug resistance.

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* (Tsuji and Rybak, 2005).

GAL, a member of the flavonol class of flavonoid, is present in high concentrations in honey and *Alpinia officinarum*, a plant which has been using as a spice and as a traditional herbal medicine for a variety of ailment in Orient for centuries. GAL is also present in higher concentrations in propolis, which is a resinous material made by bees, used in many countries for the medical treatment of numerous diseases, including airway affections, cutaneo-mucosal and viral infections (Capasso and Mascolo, 2003). Moreover, galangin has been recently proposed as a candidate for chemoprevention of cancer (Heo *et al.,* 2001). In the study by Denny *et al*. (2002), GAL was shown to inhibit the activity of β-lactamase from *Stenotrophomonas maltophilia* (Cushnie and Lamb, 2006).

In the present study, GAL plus GEN of combination showed synergism against MRSA. Further, the results of this in vitro trial provide evidence that GAL in combination with GEN could make clinically relevant synergy against MRSA. But this time-kill study showed low synergistic effect in DPS-1. The results obtained here cannot be applied currently in clinical treatment, but consider that the combination treatment of GAL and GEN will prove to be helpful to treat MRSA.

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