Synergy of flavone with vancomycin and oxacillin against vancomycin-intermediate *Staphylococcus aureus*

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Abstract: This study evaluated *in vitro* activity of 9 flavonoids in combination with vancomycin or oxacillin against vancomycin-intermediate *Staphylococcus aureus* (VISA) ATCC 700699 by employing the checkerboard method to obtain Minimal inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) index. Six flavonoids namely hesperitin, rutin, naringenin, flavones, naringin and 3, 7-dihyroxyflavone which exhibited notable inhibitory activity (MIC values ≤ 3200 µg/mL) were further evaluated for combination assay with antibiotics. The combinations of vancomycin+flavone and oxacillin+flavone were found synergistic with the FIC index value 0.094 and 0.126, respectively. Other combinations showed an additive interaction (FIC index = 1.063) but no antagonistic reaction (FIC index > 4) were observed. In time kill studies, oxacillin-flavone combination at synergistic concentration demonstrated bactericidal effect at 24 h period with concentration-dependent manner on the VISA strain. Following 1 h exposure, the combination also produced persistent effect on the bacteria growth for 2.9 hrs at 1x sub-MIC and more than 24 h at 5x of sub-MIC and there was a significant difference between both concentrations ($p<0.05$). Vancomycin-flavone combination, however, showed no concentration-dependant effect and lower PAE values (1.159 h and 2.322 h at 1x and 5x sub-MIC, respectively) on the VISA strain. In conclusion, flavone markedly intensifies the susceptibility of oxacillin against VISA and the combination can be implicated for further interaction studies at molecular level.

Keyword: Flavonoid, vancomycin, oxacillin, VISA.

INTRODUCTION

Staphylococcus aureus continues to be one of the most common causes of nosocomial and community-acquired infections in the world (CDC, 1996; Waldvogel, 1995). Widespread emergence of methicillin-resistant *S. aureus* (MRSA) that demonstrated multi-resistant to a wide variety of antibiotic (Lowy, 1998) has created alarming concern, especially in the hospital setting. Vancomycin has been used successfully for the treatment of serious MRSA infections for the past 30 years. However, since the first clinical isolate of vancomycin-intermediate *S. aureus* (VISA) was reported in Japan (Hiramatsu *et al.,* 1997), widespread cases of its infections in other countries such as United States of America (Rotun *et al.,* 1999), France (Ploy *et al.,* 1998), United Kingdom (Howe *et al.,* 1998) and Germany (Bierbaum *et al.,* 1999) have been reported. In view of this, the development of a new anti-VISA agent is urgently needed. One alternative treatment to overcome antibiotic-resistant bacteria is through the use of new antimicrobial compounds or combination therapy using different compounds.

This study was carried out to seek an alternative treatment approach using combination therapy between vancomycin oroxacillin with 9 flavonoids against VISA strain. Also evaluated were the *in vitro* activities by time-kill kinetics and post-antibiotic effect assays.

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MATERIALS AND METHODS

Antibiotic and phytochemical

Vancomycin, oxacllin (Fluka 46589) and a total of 9 selected phytochemical powders from flavonoid group i.e., hesperidin, hesperetin, naringenin, naringin, quercetin, rutin, colchicine, flavones and 3,7-dihydroxyflavone were commercially purchased from Sigma-Aldrich (St. Louis, Minneapolis, USA). Stock solutions of these agents were prepared in sterile dimethyl-sulphoxide (DMSO) solvent to various concentrations, which depend on their respective MIC values.

Bacterial strains

S. aureus ATCC 700699 was obtained from American Type Culture Collection (ATCC). The minimum inhibitory concentration (MIC) of vancomycin (8 µg/mL) was measured in duplicate using the E-test strip (AB BIODISK, Solna, Sweden).

Antibacterial activity

Tryptic soy broth (Difco Laboratories, Detroit, Michigan, USA) was used for susceptibility testing. The MIC of each of the flavonoids and vancomycin or oxacillin was determined by broth microdilution method according to the Clinical and Laboratory Standard Institute (CLSI), formerly known as a National Committee for Clinical Laboratory Standards (NCCLS 2004). Initial assessment of the interaction between vancomycin and flavonoid was

performed by traditional checkerboard experiment for each drug combination (Sato *et al.,* 2004). Fractional inhibitory concentration (FIC) indices of ≤ 0.5 were defined as synergy, whereas FIC indices of > 0.5 - 4 and > 4 were defined as addition and antagonism, respectively (LaPlante, 2007). Serial dilutions of two antimicrobial agents were mixed in a microtiter plate so that each row contained a fixed amount of one agent and increasing concentration of the second agent. The ranges of concentrations used were based on the MICs of each antimicrobial agent against the tested bacteria. The microtitre plates were then incubated for 24 hours at 37ºC to confirm sterility. After incubation, the concentrations from the combination that inhibited visible bacterial growth were taken to be the sub-MIC value of the individual and combined antibacterial agents.

Time-kill and post-antibiotic effect (PAE) assay

Further pharmacodynamic evaluation was done to confirm the synergistic combinations of the drugs. Time-kill studies were carried out with tryptic soy broth (TSB). All antimicrobial agents were tested at 4 and 8 times for their respective sub-MIC with a starting inoculum of 5 x 10^5 CFU/ml prepared by direct colony suspension method (LaPlante and Rybak, 2004). The following antibiotic concentration at sub-MIC were prepared; vancomycin at 0.1875, 0.75 and 1.5 µg/ml with or without flavones at sub-MIC values which were 312.5, 1250 and 2500 µg/ml. Sample aliquots (10 µl) were removed from cultures at 0, 2, 4, 8 and 24h. Antimicrobial activity was acertained by serial dilution (10- to 10,000-fold) of plated samples with TSB. Growth control wells for each organism were prepared without antimicrobial agent and performed in parallel to the antimicrobial test tubes. Synergy of a combination of antibiotics was defined as a decrease of at least 2 $\log_{10}$ CFU/ml at 24h compared to the single most active drug in combination (Domaracki *et al.,* 2000). An antibiotic or its combination was considered bactericidal when it produced a reduction of at least 3 $\log_{10}$ CFU/ml from starting inoculum at 4 or 24 h post incubation (Rand and Houck, 2004).

RESULTS

The results of the susceptibility testing and checkerboard studies of combinations of vancomycin or oxacillin and 9 selected flavonoids are shown in table 1. The MIC of all flavonoids alone were 3200 µg/ml or more with the exception for naringenin (400 µg/ml), flavone and 3, 7 dihyroxyflavone (both at 1600 µg/ml). Of all combinations, the combination of vancomycin+flavone and oxacillin+flavone were significant synergistic effect (FIC index = 0.094 and 0.126, respectively). The result also showed that there was no antagonistic interaction (FIC index > 4) noted between both antibiotics and flavonoids.

The antimicrobial activity of both antibiotic and flavone at synergistic concentration (sub-MIC level) were further evaluated by time-kill and post-antibiotic experiments during 24 h period. Time kill curves in fig. 1 showed that there were no bactericidal activity noted at 8 h of the treatment among three different synergistic concentrations (KS) used (1x-, 4x-, and 8x of the KS). In addition, the combinations at all these concentrations were considered active; with clear separation from the growth control curve at all the time points for the bacteria. Flavone-oxacillin combination at the concentration of 1xKS essentially retained the bacterial counts close to the initial inoculums after 24 h. Only at the concentration of 8xKS of the combination resulted in a bactericidal killing which was higher than 3 $\log_{10}$ CFU/ml of growth reduction (table 2). A repeated measure independent t-test was carried out to confirm hypotheses that there was a significant mean difference between the concentrations applied.

Table 1: Susceptibilties of VISA to vancomycin (MIC=8 µg/ml) or oxacillin (MIC=800 µg/ml) in combination with flavonoids

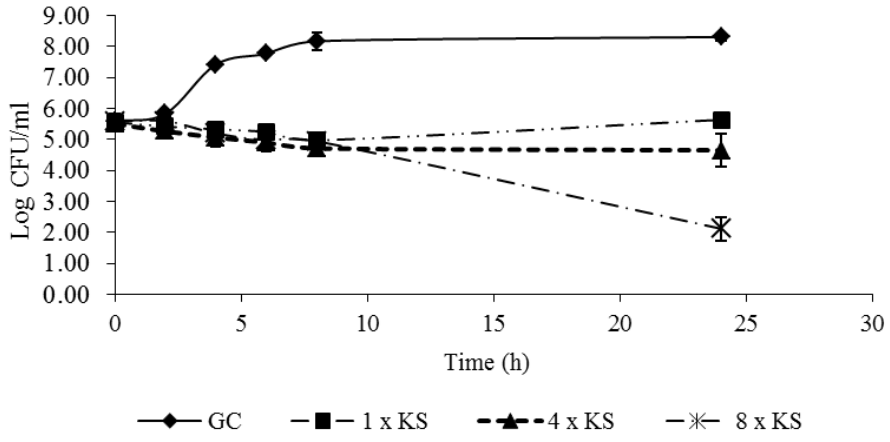
Flavonoid	MIC alone (µg/ml)	MIC in combination (µg/ml)		FIC index	MIC in combination (mg/ml)		FIC index
		Vancomycin	Flavonoid		Oxacillin	Flavonoid	
Hesperetin	3200	8	200	1.063	800	200	1.063
Hesperidin	>3200	ND	ND	ND	ND	ND	ND
Rutin	3200	8	200	1.063	800	200	1.063
Naringenin	400	0.25	400	1.031	50	400	1.063
Quercetin	>3200	ND	ND	ND	ND	ND	ND
Flavone	1600	0.25	100	0.094*	50	100	0.126*
Fisetin	>3200	ND	ND	ND	ND	ND	ND
Naringin	3200	0.25	3200	1.031	800	200	1.063
3,7- dihydroxyflavone	1600	8	100	1.063	2	200	1.063

FIC index < 0.5 , synergy effect; 0.5 < FIC index ≤ 4, additive effect and FIC >4, antagonistic effect.
*The combination was interpreted as synergistic; ND, not determined

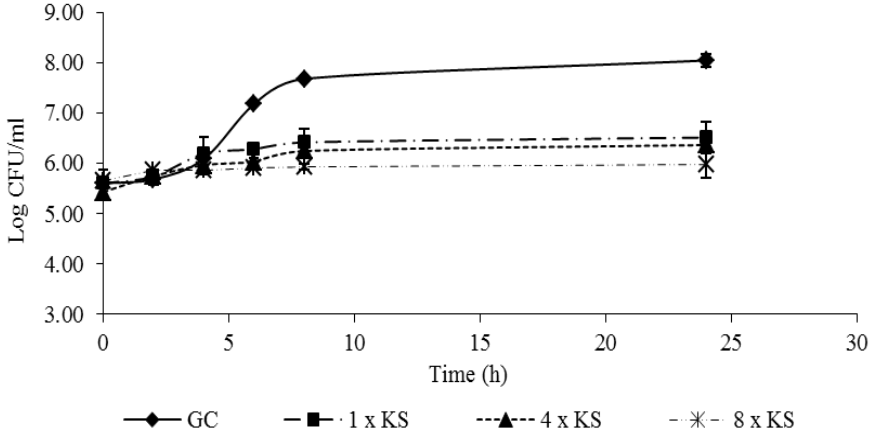
The mean PAE values with the treatment of antibiotic alone and in combination with flavone (at the concentration of 1x and 5x of the KS) are shown in table 3. After 1 hour of simultaneous exposure time with the treatment of flavone-oxacillin combination, the mean value of PAE at 1x KS showed a significant different ($p<0.05$) between the mean value produced by 5x. In contrast, the same strain treated with flavone-vancomycin combination exhibited much less PAE values which were 1.159 h and 2.322 h with the concentration of 1x and 5x of the KS, respectively. When tested alone, vancomycin at 24 h showed the most significant different between PAE value produced by 1x and 5 xs of the KS in which PAE were 0.491 h and > 24 h for both concentrations, respectively.

DISCUSSION

In the present work**,** we demonstrated that some flavonoids, on their own, by themselves had only exhibited weak antibacterial effects on VISA but the effect of combining two antibiotics, dramatically increased the bacteria susceptibility. Plant-derived compound alone may have low efficacy or are relatively weak, but antimicrobial activity may be enhanced synergistically when in combination with antibiotics (Lewis and Ausubel, 2006). Mono-therapy with antibiotics such as oxacillin (β-lactam) and vancomycin (Glycopeptide) have been included as the gold standard for the treatment of *S. aureus* infection. As far as treatment for VISA is concern, currently there is no guideline of standard treatment to combat the superbug.



(a)



(b)

Fig. 1: Time-kill curves for ATCC VISA with the treatment of flavone in its combination with (a) oxacillin and (b) vancomycin for 24 hours Bacteria were incubated without treatment (growth control (◆), 1xKS (■), flavone 1/16x-oxacillin 1/16xMIC; 4xKS (▲), flavone 4/16x-oxacillin 4/16xMIC; 8xKS **(×)**, flavone 8/16x-oxacillin 8/16xMIC. Data points are the mean values ± S.D of three experiments. CFU, $\log_{10}$ colony forming units; KS, synergistic concentration; GC, growth control.

Table 2: Mean of growth reduction (log_{10} CFU/ml) for ATCC VISA strain with the following treatments after 8 h and 24 h

Compound	Concentration	Mean of growth reduction (log_{10} CFU/mL)	
		8 h	24 h
Oxacillin	1 x MIC	3.449	3.449
	4 x MIC	3.677	3.677
	8 x MIC	3.367	3.367
Flavone+Oxacillin	1 x K	0.480	-0.044
	4 x KS	0.535	2.344
	8 x KS	0.598	3.454
Vancomycin	1 x MIC	3.645	3.645
	4 x MIC	3.431	3.431
	8 x MIC	3.438	3.438
Flavone+Vancomycin	1 x KS	-0.813	-0.911
	4 x KS	-0.814	-0.933
	8 x KS	-0.266	-0.319

MIC: Minimum inhibitory concentration; KS: Synergistic concentration from the checkerboard assay

At present years, more focus of antimicrobial combination studies against resistant strain of *S. aureus* are on the efficacy of combination among available antibiotics (da Silva *et al.,* 2011; Ermertcan *et al.,* 2010; Hadji Nejad *et al.,* 2010). In this study, we hypothesized that some flavonoids, although relatively weak antibacterial by themselves, may exhibit synergistic effects in combination with either oxacillin or vancomycin thereby suggesting an alternative therapy using phytocompounds for synergistic antimicrobial combination

Naringenin (flavonoid group, flavonone) has shown better antibacterial effect (with lower MIC value, i.e., 400-800 µg/mL) compared to flavone. However, there was no evidence of synergism when it was tested in combination with neither oxacillin nor vancomycin. This indicates that the lower MIC value of individual antimicrobial agent did not necessarily produce synergy when it has been tested in combination with other antimicrobial agent. On the other hand, oxacillin required an exceedingly high MIC value (800 µg/mL) to inhibit ATCC VISA, reduced to 50 µg/mL when tested in combination with flavones thus confirmed our hypothesis. This phenomenon is in agreement with the study by Sato and colleagues (2004) which found that 6, 7-dihydroxyflavone had a weak antibacterial effect on MRSA, but at sub-MIC concentration elevated the susceptibility to β-lactams and they named the compound as the 'intensifier to β-lactam-susceptibility in MRSA'. On the other hand, it was such a promising finding in effort of scavenging new alternative to combat the resistance by considering combination therapy with flavones as the VISA strain for this study showed completely resistance trait to oxacillin (while tested alone), reduced to over 15-fold for its susceptibility to the antibiotic by only concurrently administered with flavones.

Next, we tested the selected synergistic combination using time-kill assay to confirm the dynamic effect of sub-MICs of the synergistic combination at different time point for 24 h. The results, however, showed contradictory findings to that of observations in the checkerboard method. This finding was in agreement with Domaracki and co-workers (2000) which suggested that not all isolates for which synergy was detected by the checkerboard method showed synergy ($\geq$ 2 logs of killing) in time-kill studies. They added that the synergy was only produced when the antibiotics were re-administered after 6 h, however in this study we did not observe the effect of the antibiotics re-administration in order to produce synergism.

The PAEs value, defined as time for delayed bacterial growth after a short on-off exposure to an antibiotic for 1 or 2 h (Craig and Gudmundsson, 1986), produced by the flavone in combination with oxacillin and vancomycin have been found to be different against ATCC VISA (table 2). At 5x of the synergic concentration, the negative PAE value (PAE > 24 h) was shown by flavone-oxacillin combination compared to PAE = 2.322 h for flavone-vancomycin at the same concentration. This indicated that the combination of flavone with the ß-lactam produced persistent effect longer than 24 h after antibiotic removal, while flavone with the same concentration in combination with vancomycin yielded lower PAE value. We could not conclude whether the effect of the combination exposure during the delay re-growth of the VISA strain was either transient or lethal damage in spite of the concentration falls below MIC as no further microscopic confirmation have been done.

This study provides preliminary result of alternative anti-VISA treatment by applying potential phytocompound concurrently to the existing antibiotics in view that we

Table 3: PAE values for antibiotic treatment alone and in combinations against VISA strain for 24 h test period

Antimicrob	Concentration (MIC)	P_0	P_1	Time to reach P_1 (hours)		PAE (hours) = T - C	R^2
Oxa+Flav	1x	5.563	6.563	(6.563- 5.644)/0.151	6.086	2.935	0.988
Oxa+Flav	5x	5.484	6.484	(6.484- 5.511)/-0.480	-2.027	> 24*	0.902
Oxa	1x	5.559	6.559	(6.559- 5.649)/-0.165	-5.515	> 24*	0.923
Oxa	5x	5.332	6.332	(6.332- 5.501)/-0.251	-3.311	> 24*	0.965
Van+Flav	1x	5.552	6.552	(6.552- 5.453)/0.255	4.31	1.159	0.943
Van+Flav	5x	5.521	6.521	(6.521-5.421)/0.201	5.473	2.322	0.966
Van	1x	5.439	6.439	(6.439-5.128)/0.361	3.642	0.491	0.965
Van	5x	5.471	6.471	(6.471- 5.594)/-0.120	-7.308	>24*	0.942
Control	-	5.312	6.312	(6.312- 5.209)/0.350	3.151	0.000	0.995

P_0 . Mean value of colony counting after rapid removal of the antimicrobial by dilution method (t = 0)
P_1 - Mean value for the count of CFU in the test culture to increase 1 $\log_{10}$ above the count observed immediately after antimicrobial removal
T - The time required for P_1 for the treatment with antimicrobial
C - The time required for P_1 without treatment (control); R^2 - Coefficient of determination
Van – Vancomycin; Oxa – Oxacillin; Flav - Flavone
*The values of PAE >24 indicate the PAE produced after 24 hours

can exert the same efficacious antimicrobial effect, but with less administration dosage. However, further interaction study at molecular level is necessary to support the findings. This will eventually reduce the reported VISA prevalence in both hospital and community as one of the factor concerning antibiotic resistance is the widespread use of high dose antibiotics.

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