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BACTERICIDAL ANTIBIOTIC-PHYTOCHEMICAL COMBINATIONS AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) infection is a global concern nowadays. Due to its multi-drug resistant nature, treatment with conventional antibiotics does not assure desired clinical outcomes. Therefore, there is a need to find new compounds and/or alternative methods to get arsenal against the pathogen. Combination therapies using conventional antibiotics and phytochemicals fulfill both requirements. In this study, the efficacy of different phytochemicals in combination with selected antibiotics was tested against 12 strains of *S. aureus* (ATCC MRSA 43300, ATCC methicillin sensitive *S. aureus* or MSSA 29213 and 10 MRSA clinical strains collected from National University Hospital, Singapore). Out of the six phytochemicals used, tannic acid was synergistic with fusidic acid, minocycline, cefotaxime and rifampicin against most of strains tested and additive with ofloxacin and vancomycin. Quercetin showed synergism with minocycline, fusidic acid and rifampicin against most of the strains. Gallic acid ethyl ester showed additivity against all strains in combination with all antibiotics under investigation except with vancomycin where it showed indifference effect. Eugenol, menthone and caffeic acid showed indifference results against all strains in combination with all antibiotics. Interestingly, no antagonism was observed within these interactions. Based on the fractional inhibitory concentration indices, synergistic pairs were further examined by time-kill assays to confirm the accuracy and killing rate of the combinations over time. The two methods concurred with each other with 92% accuracy and the combinatory pairs were effective throughout the 24 hours of assay. The study suggests a possible incorporation of effective phytochemicals in combination therapies for MRSA infections.

Key words: phytochemicals, antibiotic combinations, synergism, antibiotic resistance, methicillin-resistance *Staphylococcus aureus* (MRSA)

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of infection in hospitals (11). It has been nicknamed 'superbug' due to its multi-drug resistance to most of the contemporary antibiotics (8).

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Recently, it has also shown resistance to glycopeptide, vancomycin, which is known to be the last defense antibiotic against the pathogen. Due to its multi-drug resistance patterns and rapid adaptive resistance to various antibiotics, critical attention is necessary to find new ways to combat infections caused by MRSA. At this point, the use of drug combinations rather than single drugs provide better clinical outcomes, as the use of single agent is highly associated with occurrence of resistance (23). Many reports suggest that the use of drug combinations against multi-drug resistant bacterial pathogens have better efficacy compared to monotherapy (5). The use of western antibiotics, however, has encountered adaptive resistance over time, even in combinations (7, 12). This further limits the use of antibiotics in combinations, especially to overcome concerns of resistance. Identifying methods and strategies to prevent or delay the development of resistance in MRSA has therefore, become the cornerstone of antimicrobial drug research against resistant strains of *S. aureus*. Alternative compounds and secondary metabolites derived from plants or insects offer a rich source as antimicrobial agents (6).

Plants are a rich source of useful secondary metabolites that forms the plant defense mechanism against pathogenic invaders (6, 13). These include tannins, flavonoids, alkaloids, terpenoids and polyphenols. They have effective antibacterial properties against both Gram positive and Gram negative bacteria (6, 17, 18, 20). Since, phytochemicals have higher minimum inhibitory concentrations (MIC) (100-5000 µg/ml) than antibiotics (0.031-512 µg/ml), they oftentimes cannot be used in monotherapy as soul agents. On the other hand, phytochemicals are known to modulate or modify resistance mechanisms in bacteria (16, 20). Therefore, their potential use in combinations with antibiotics can help to potentiate the activity of the western drugs, resulting in increased efficacy.

Antibiotics with different mechanism of actions and that are active against *S. aureus* were chosen for this study. Fusidic acid and minocycline (protein synthesis inhibitor), rifampicin (inhibitor of DNA dependent RNA-polymerase), cefotaxime (third generation cephalosporin, disruption of cell wall), vancomycin (glycopeptides, inhibition of cell wall biosynthesis) and ofloxacin (quinolone, DNA-gyrase inhibitor) were used in combination with six phytochemicals against twelve *S .aureus* strains. The phytochemicals used were, tannic acid (tannins, found in tree bark and leaves), quercetin (flavanoid, found in colored fruits and vegetables), gallic acid ethyl ester (tea catechin, found in most teas), caffeic acid (plant phenol, found in leaves and stems), eugenol and menthone (essential oils). The combinations were assessed by checkerboard assay and the bactericidal synergistic pairs were assessed by time-kill assays.

MATERIALS AND METHODS

Bacterial strains, media and inoculums preparation

S. aureus MRSA 43300, MSSA 29213 and 10 MRSA clinical strains acquired from National University Hospital (NUH) were used in this study. Iso-Sensitest (IS) broth and agar powdered mixtures were used to prepare liquid and solid media, respectively, acquired from Oxoid, Singapore. Strains were stored in aliquots at -80 ˚C, suspended in IS broth containing 30% glycerol (v/v). For experiments, bacterial suspensions were spread onto IS agar plates and incubated at 37 ˚C for 24 hours. Inoculums were prepared in IS broth using 3 to 5 well formed colonies from the 24 hours culture of *S. aureus* to a concentration of 10^8 CFU (colony forming units)/ml as per 0.5 McFarland standards (1). It was further diluted into 1:100 dilutions to get concentration of 10^6 CFU/ml for further experiments.

Antibiotics and phytochemicals

All antibiotics, phytochemical and chemicals were obtained from Sigma-Aldrich, Inc. (Singapore). Purified powders of tannic acid (purity 98%), gallic acid ethyl ester (purity 99%), quercetin (purity ≥98%), Caffeic acid (purity ≥98%), menthone (purity 90%), eugenol (purity 99%), fusidic

acid (purity ≥98%), minocycline (purity 98%), cefotaxime (purity 91-96%), rifampicin (purity ≥97%), vancomycin (purity 80%) and ofloxacin (purity 98%) were used. Stock antibiotic solutions were prepared and dilutions were made according to the CLSI protocols (19) or manufacturer's recommendations. Tannic acid, quercetin and gallic acid ethyl ester were dissolved in ethanol (99% molecular grade, Sigma Aldrich). Cefotaxime sodium, vancomycin, ofloxacin and minocycline were dissolved in NaOH (sodium hydroxide, 1M, Sigma) and fusidic acid and rifampicin were dissolved in sterile distilled water. The stock solution concentration for all antibiotics and phytochemicals was 10 mg/ml and stored at -20 ˚C for subsequent use for up to 6 weeks.

Determination of minimum and fractional inhibitory concentrations (MIC and FIC)

The minimum inhibitory concentrations (MIC) were determined in triplicates by the broth microdilution method as described by Andrew (1). The antibiotic concentrations ranged from 0.0078-1024 µg/ml for antibiotics and 8-8192 µg/ml for the phytochemicals. The titer plates were inoculated with bacteria having 0.5 Macfarland turbidity (1), and incubated aerobically at 37°C for 24 hours.

The FIC (fractional inhibitory concentration) was established to understand the effect of the combination of two drugs under investigation. This was determined by checkerboard broth microdilution method explained elsewhere (14). The starting concentration of the phytochemicals and antibiotics for the checkerboard assay was 16 × MIC, which was determined earlier.

The FIC indices for the all combinations were calculated using the formula below:

(i) The FIC of drug 'A', given by

$$FIC_A = MIC_A \text{ combination} / MIC_A \text{ alone}$$

(ii) The FIC of drug 'B', given by

$$FIC_B = MIC_B \text{ combination} / MIC_B \text{ alone}$$

(iii) The FIC index the combination in each well is given by the sum of the FICs for each of the drugs present in the well:

$$FIC_{index} = FIC_A + FIC_B$$

Time-kill curves

Bactericidal activity of each antimicrobial agent and their respective combinations were determined by performing time-kill assays, according to the CLSI protocols (19). Viable colony forming units (CFUs) were counted by performing serial dilutions of the aliquoted sample at different time intervals. Antibiotics and phytochemicals were tested at ¼, ½, 1 and 2 MIC for each isolate. The combination pairs of antibiotics and phytochemicals were also assayed at ¼-¼, ½-½, 1-1 MICs. Aliquots were removed from each test sample at 0, 4, 8, 12, and 24 hours after inoculation and incubation at 37 ˚C aerobically. All readings were taken in triplicates.

Time-kill curves were plotted as Log_{10} CFU/ml versus time functions. Synergism was defined as more than $3Log_{10}$ CFU/ml decrease after 24 hours for the combination compared with that for the most active single agent, in this case the antibiotic. Antagonism was defined as more than $3Log_{10}$ CFU/ml increased in colony count after 24 hours (4, 13, 14, 21).

RESULTS

MIC and FIC Index

MIC values phytochemicals ranged from 128-4096 µg/ml, and for the antibiotics from 0.031-512 µg/ml (see Suppl. Table). In general, antibiotics against the clinical strains had higher MIC values compared with the ATCC strains (1024 folds higher than MIC for ATCC strains). Interestingly, phytochemicals showed uniform MICs against all strains tested with variation of one or two dilutions only.

The combination of phytochemicals with the antibiotics was assessed by calculating FICI index for each combinatory pair. The results are tabulated in Table 1. Tannic acid was

synergistic with fusidic acid, cefotaxime, minocycline and rifampicin (FICI ≤ 0.5), while it showed additivity with vancomycin and ofloxacin (FICI ≤ 1). Quercetin was synergistic with fusidic acid, minocycline and rifampicin. It showed indifference when combined with vancomycin, cefotaxime and ofloxacin. Gallic acid ethyl ester was additive with all the antibiotics tested (FICI ≤ 1) except with vancomycin whereas it showed indifference (FICI > 1 or ≤ 2). Caffeic acid, eugenol and menthone were also indifferent in action in combination with antibiotics.

Table 1. Fractional Inhibitory Concentration (FIC) indices of combinatory pairs of phytochemicals and antibiotics against MRSA strains

Antimicrobial Agents*	∑ FICI																			
	MRSA 43300		MSSA 29312		MRSA C1		MRSA C2		MRSA C3		MRSA C4		MRSA C5		MRSA C6		MRSA C7		MRSA C8		MRSA C9		MRSA C10	
TA + FA	0.5	(S)	0.375	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)
TA + Cefo	0.375	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.75	(A)	0.75	(A)	0.5	(S)	0.5	(S)	0.75	(A)	0.5	(S)	0.5	(S)
TA + Mino	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.75	(A)	0.5	(S)	0.5	(S)
TA + Van	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)
TA + Oflox	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)
TA + Rifam	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.75	(A)	0.5	(S)	0.5	(S)	0.5	(S)
Quer + FA	0.5	(S)	0.5	(S)	0.5	(S)	0.375	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.375	(S)
Quer + Cefo	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)
Quer + Mino	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.75	(A)	0.5	(S)	0.5	(S)	0.5	(S)	0.5	(S)
Quer + Van	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)
Quer + Oflox	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)
Quer + Rifam	0.375	(S)	0.375	(S)	0.5	(S)	0.5	(S)	0.5	(S)	0.75	(A)	0.5	(S)	0.5	(S)	0.375	(S)	1	(A)	0.5	(S)	0.5	(S)
GA + FA	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)
GA + Cefo	1	(A)	0.75	(A)	1	(A)	0.75	(A)	0.75	(A)	1	(A)	1	(A)	1	(A)	0.75	(A)	1	(A)	0.75	(A)	1	(A)
GA + Mino	0.75	(A)	0.75	(A)	1	(A)	1	(A)	1	(A)	0.75	(A)	0.75	(A)	1	(A)	1	(A)	0.75	(A)	1	(A)	0.75	(A)
GA + Van	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)
GA + Oflox	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	1	(A)	0.75	(A)	0.75	(A)	1	(A)	0.75	(A)	1	(A)	1	(A)
GA + Rifam	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	0.75	(A)	1.5	(I)	1	(A)	0.75	(A)	0.75	(A)
CA + FA	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)
CA + Cefo	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)	2	(I)	2	(I)
CA + Mino	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)
CA + Van	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)
CA + Oflox	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)
CA + Rifam	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)
Mth + FA	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)
Mth + Cefo	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)
Mth + Mino	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)
Mth + Van	2	(I)	2	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)	2	(I)	2	(I)
Mth + Oflox	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)
Mth + Rifam	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)
Eug + FA	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)
Eug + Cefo	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)
Eug + Mino	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	2	(I)
Eug + Van	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)
Eug + Oflox	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)
Eug + Rifam	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)

Interpretations: S - Synergism, A – Additive and I - Indifference.

*Abbreviations; TA - Tannic acid, Quer - Quercetin, GA - Gallic acid ethyl ester, CA - Caffeic acid, Eug - Eugenol, Mth - Menthone, FA - Fusidic acid, Cefo - Cefotaxime sodium, Mino - Minocycline, Van - Vancomycin, Oflox - Ofloxacin, Rifam - Rifampicin

Time Kill Assay

Time kill assays were performed with the synergistic pairs based on their FIC indices to assess bactericidal effects and killing rates over time. In the checkerboard assay, the addition of 0.25 MIC of tannic acid could reduce the MIC of fusidic acid, cefotaxime, minocycline and rifampicin by 4 to 8 folds in most of *S. aureus* strains under investigation. Similar observations were made in case of quercetin in combination with minocycline, fusidic acid and rifampicin.

Table 2 summarizes the time kill assays as Log_{10} difference between the combination curve and that of most active single agent in the combination, in this case was the

antibiotics. Based on the difference values, the synergistic effects (difference ≥ 3 Log_{10}) could be observed starting from 4 hours of incubation and continued up to 24 hours, with a few exceptions (marked italic in the table).

Table 2. Log_{10} (CFU/ml) difference between time-kill curve of combination (phytochemical + antibiotic) and most active single agent (antibiotic) used alone against MRSA strains.

Strains/ TA (0.25 MIC)	Fusidic Acid (0.25 MIC)				Cefotaxime (0.25 MIC)				Minocycline (0.25 MIC)				Rifampicin (0.25 MIC)			
	4H	**8H**	**12H**	**24H**	**4H**	**8H**	**12H**	**24H**	**4H**	**8H**	**12H**	**24H**	**4H**	**8H**	**12H**	**24H**
MRSA 43300	4	5.9	7.5	7.6	4.1	5	5.5	5.7	4	5.9	7.3	7.3	3.4	4.6	5.8	7
MSSA 29213	3.2	5.5	6.5	5.7	4	5	6.4	6	3.9	4	4.3	5.1	3.7	5.5	6.2	6.5
MRSA C_1	4.1	4.2	4.5	4.8	3.3	3.9	4.4	5.1	5	5.2	5.1	4.5	3.6	5.3	6.1	5.6
MRSA C_2	4.4	4.7	4.9	4.6	3.3	4.4	4.3	4.5	5	5	5.3	5.05	3.6	4.7	5.5	5.3
MRSA C_3	3.9	4.2	4.4	4.5	4.3	5.1	5.4	5.2	4.6	4.5	4.3	4.8	3.9	5.5	5.9	7.1
MRSA C_4	3.2	3.3	3.5	*1.9*	NT	NT	NT	NT	4.6	4.2	5	4.9	4.5	5.3	5.7	6.2
MRSA C_5	3.7	3.6	3.7	3.9	NT	NT	NT	NT	4.8	5.1	5.6	5.5	3.9	4.6	5.5	5.2
MRSA C_6	4.2	5.2	5.7	7.3	4.4	3.9	3.1	*1.7*	5.2	5.6	5.7	5.5	4	5.6	6.3	7.1
MRSA C_7	4.8	3.9	3.8	4.2	5.1	5.6	5.8	5.9	5.6	5.8	5.7	6.1	NT	NT	NT	NT
MRSA C_8	3.7	3.8	3.7	*2.2*	NT	NT	NT	NT	5.2	5.5	5.5	5.4	4.1	4.3	5.4	5.9
MRSA C_9	4.1	4.4	4.5	5	4.3	5.0	5.3	5.2	5.2	5.5	5.8	5.5	*2.5*	*2*	*2.2*	*1.9*
MRSA C_{10}	5.1	7.1	7.7	7.5	5.1	5.2	5.6	5.3	5.3	5.1	5.8	5.4	4.8	5.6	6.6	6.2

Strains/Quer (0.25 MIC)	Fusidic Acid (0.25 MIC)				Minocycline (0.25 MIC)				Rifampicin (0.25 MIC)			
	4H	**8H**	**12H**	**24H**	**4H**	**8H**	**12H**	**24H**	**4H**	**8H**	**12H**	**24H**
MRSA 43300	4.1	4.5	4.6	4.6	4.6	5.1	5.1	4.4	3.3	5.9	6	5.8
MSSA 29213	4.5	5.4	6.4	6.5	3.8	3.8	4.3	4.5	4.0	4.6	4.9	5.9
MRSA C_1	5.4	6.1	6.3	6.5	4.5	5.0	4.9	5.4	3.6	4.7	5.9	7
MRSA C_2	5.9	6.1	7.3	7.4	4.5	4.7	4.5	5.3	4.8	5.3	5.6	5.6
MRSA C_3	4.7	5.2	6.1	6.3	4.1	5.2	5.4	5.7	*2.2*	4.8	4.6	4.2
MRSA C_4	4.3	4.6	5.1	4.9	*2.8*	*2.7*	*2*	*2*	3.8	5.9	4.6	5
MRSA C_5	5	5.3	6.5	6.4	4.7	5.2	5.5	5.2	4.4	5.2	5.7	4
MRSA C_6	5.95	5.9	6.5	6.8	NT	NT	NT	NT	5.1	4.5	6.7	5.5
MRSA C_7	5.5	5.9	6.2	6.2	4.1	4.9	5.1	4.7	3.8	5.1	6.5	5.5
MRSA C_8	4.8	5.2	5.1	4.6	5	5.2	5.2	5.2	NT	NT	NT	NT
MRSA C_9	4.8	4.6	5.4	5.3	4.7	5.4	5.7	5.6	3.7	4.1	*2*	*0.8*
MRSA C_{10}	5.2	5.3	5.2	5.6	5.1	5.1	5.2	5.1	3.2	3.9	6.0	5.2

TA – Tannic acid, Quer – Quercetin
NT – Not Tested (not synergistic pair, Table 1)
Italic text – < 3 log_{10} Difference (Interpretation: Not Synergistic)

DISCUSSION

Combination therapies have been used with an aim of better efficacy and improved treatment options (7). Combinations of conventional antibiotics and phytochemicals are more recent alternative methods for the treatment of multi-drug resistant bacteria like MRSA. Khan *et al*., (9) reported the potentiating effect of phytochemical piperine on ciprofloxacin activity against *S. aureus* strains. Similarly, Soe *et al*. (18), found that ethyl gallate addition to fusidic acid and tetracycline in combinations could overcome the resistance in MRSA. These reports illustrate the potentiating effect of phytochemicals on the mechanism of antibiotics.

In this study, tannic acid and quercetin showed synergy with most of the antibiotics tested. Both phytochemicals were able to reduce the MIC of the antibiotics up to 4 to 8 folds. In the time-kill assay, these synergistic pairs were able to show high killing rates starting from 4 hours up to 24 hours. The Log_{10} difference between the most active drug of the combination, in this case, (antibiotics) and the combination with phytochemical, clearly suggested suppression of populations that otherwise had high growth rate with single

agent (see Table 2). The high Log_{10} difference was also observed until 24 hours, which demonstrated the potentiating effect of tannic acid and quercetin on the selected antibiotics. However, for some pairs where the FICI suggested synergism, the time-kill assay did not concurred with the synergism definition ($\geq 3Log_{10}$ difference). In total, interpretations of the FICI calculated for all combinations against all strains, the time-kill assay was 92% in concurrence with the checkerboard assay.

Phytochemicals have been shown to have antimicrobial activity against broad spectrum of microbes (6). Their multi-targeted approach suggested by many, plays a role in reducing the probability of development of resistance (15, 16, 20). In appropriate combinations and doses they also play a role in increasing the susceptibility of the pathogen to various drugs (3). In the present study, antibiotics with different mechanism of action were used with broad spectrum phytochemicals. Most of the combinations tested showed positive interaction (synergy and additivity) with none antagonistic. This clearly suggested that phytochemicals are able to potentiate various antibiotics in suitable combinations. Therefore, they can be considered as potential additives for resistance modulation when used with a suitable antibiotic in combination. In addition to lowering the dose of the antibiotic in combination (to up to 8 folds), the overall efficacy of the treatment is improved. However, detailed analysis of the resistance patterns of the pathogen under consideration is important for incorporation into clinical practice.

In general, phytochemicals are less potent than antibiotics. This was also evident in this study, as the MICs of the antibiotics were much lower than those of the phytochemicals (see supplementary table). Incorporation of these antibacterial compounds as single agents would require enormously high concentrations for sufficient bioavailability *in vivo*. Therefore, their combinations at sub-MIC levels with more active antibiotics, also at sub-MIC, would be more suitable and realistic in a clinical setting. This would even bring down the side effects caused by each of these antibacterial drugs. Although, for clinical applications, the total toxicity levels of the phytochemicals must be taken into account, including the pharmacokinetics and pharmacodynamics (PK/PD) models of the drug. The acute toxicity levels of tannic acid and quercetin were, $LD_{50} > 120$ mg/kg and $LD_{50} > 159$ mg/kg, respectively, obtained from the manufacturer. These levels are much higher than the MIC obtained for the phytochemicals alone as well as in combination (FIC), suggesting their therapeutic significance.

Supplementary Table. Minimal Inhibitory Concentrations of Antibiotics and Phytochemicals

Strains*	FA (µg/ml)	Cefo (µg/ml)	Mino (µg/ml)	Van (µg/ml)	Oflox (µg/ml)	Rifam (µg/ml)	TA (µg/ml)	Quer (µg/ml)	GA (µg/ml)	CA (µg/ml)	Eug (µg/ml)	Mth (µg/ml)
MRSA 43300	0.031	8	0.25	1	0.5	0.016	256	512	1024	4096	4096	4096
MSSA 29213 (Control)	0.031	2	0.25	1	1	0.004	256	1024	1024	1024	2048	4096
MRSA C1	0.25	16	0.5	1	1	0.008	256	1024	1024	1024	2048	4096
MRSA C2	0.25	4	4	1	1	0.016	256	1024	1024	512	512	4096
MRSA C3	0.25	512	4	1	256	0.016	256	512	1024	512	2048	4096
MRSA C4	0.25	512	64	2	256	0.032	128	512	1024	1024	2048	4096
MRSA C5	0.25	512	8	4	512	0.032	128	512	1024	1024	2048	4096
MRSA C6	0.25	512	4	1	128	0.016	128	1024	1024	1024	2048	4096
MRSA C7	0.5	128	16	2	128	0.5	128	1024	1024	1024	2048	4096
MRSA C8	0.25	512	128	1	128	0.5	128	1024	1024	1024	2048	4096
MRSA C9	0.25	128	64	1	64	0.25	256	512	1024	1024	2048	4096
MRSA C10	0.25	512	2	4	256	0.5	512	512	1024	1024	2048	4096

*MRSA - Methicillin resistant *S. aureus*, MSSA - Methicillin sensitive *S. aureus*, MRSA C1 to C10 - MRSA clinical strains collected from National University Hospital, Singapore.
Abbreviations; TA - Tannic acid, Quer - Quercetin, GA - Gallic acid ethyl ester, CA - Caffeic acid, Eug - Eugenol, Mth - Menthone, FA - Fusidic acid, Cefo - Cefotaxime sodium, Mino - Minocycline, Van - Vancomycin, Oflox - Ofloxacin, Rifam - Rifampicin

MRSA has been reported resistant to many antibiotics (8). Recently, combination therapy has been identified as a rational approach to tackle concerns of resistance in MRSA. This is because of its several advantages over monotherapy, including

reduction of resistance and total drug intake, thereby reducing side effects (5). In addition to antibiotic combinations, antibiotics with complementary and alternative medicines such as phytochemicals and insect extracts have also been shown to be effective *in vitro* (2, 13, 20, 22). The present study also illustrates the use of phytochemicals in useful combination with antibiotics that easily subjects *S. aureus* to adaptive resistance (10).

CONCLUSION

Both checkerboard and time kill assay results demonstrated that tannic acid was able to prolong and potentiate the bactericidal activity of fusidic acid, cefotaxime, minocycline and rifampicin. Similar effect was observed with quercetin in combination with fusidic acid, minocycline and rifampicin. The synergistic effects could be observed in as early as 4 hours post inoculation, with maximum effects observed at 24 hours of incubation. Therefore, phytochemicals (tannic acid and quercetin) in combination with antibiotics were able to provide stable therapeutic outcomes with higher efficacy in terms of killing rate throughout 24 hours. These synergistic therapeutic pairs could be useful in combating MRSA infections in a hospital or community setting.

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