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Synergistic Effects of Phytochemicals and Oxacillin on Laboratory Passage-Derived Vancomycin-Intermediate *Staphylococcus aureus* Strain

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The synergistic effects of 23 selected phytochemicals (10 phenolic acids, 8 flavonoids and 5 alkaloids) in combination with oxacillin against laboratory passage-derived Vancomycin-intermediate *Staphylococcus aureus* (VISA) strain were investigated. Minimum Inhibitory Concentration (MIC) values of each of the phytochemicals and oxacillin were determined. Fractional Inhibitory Concentration (FIC) index of each combinations were evaluated using the checkerboard broth microdilution assay. Out of 23 phyto-oxacillin combinations, 14 (60.87%) were synergistic and 9 (39.13%) were additive. Flavonoid-oxacillin combination showed 87.5% synergism whereas alkaloid-oxacillin and phenolic acid-oxacillin, respectively exhibited 60 and 40% synergism. The range of FIC index values for synergistic interaction was between 0.0417-0.1333. The synergistic interaction between all the phytochemicals and oxacillin combinations demonstrated reduction in MIC value of oxacillin from 30 to 1.5 μg μL^{-1} except for quercetin, which markedly reduced the MIC value of oxacillin to 0.5 μg μL^{-1}. Quercetin-oxacillin combination also displayed the lowest FIC index value of 0.0417. In conclusion, these findings suggested that flavonoid-based combination regimens may be useful candidate for treatment options of VISA infections.

Key words: Phytochemicals, synergism, MIC, FIC, flavonoid, VISA

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INTRODUCTION

Staphylococcus aureus continues to be one of the most common causes of nosocomial and community-acquired infections in the world (Centers for Disease Control and Prevention, 1996; Waldvogel, 1995). Widespread emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) that demonstrated reduced susceptibility to vancomycin has created alarming concern, especially in the hospital setting. Since the first report of the first clinical isolate of vancomycin-intermediate *S. aureus* (VISA) in Japan (Hiramatsu *et al*., 1997), widespread cases of VISA infections in other countries such as United States (Rotun *et al*., 1999), France (Ploy *et al*., 1998), United Kingdom (Howe *et al*., 1998) and Germany (Bierbaum *et al*., 1999) have been reported. The Minimum Inhibitory Concentration (MIC) of vancomycin for the Japanese VISA isolate was 8 μg mL^{-1}, which correlated with intermediate range using interpretive criteria defined by the National Committee for Clinical Laboratory Standards (2000). However, three isolates of vancomycin-resistant *S. aureus* (VRSA) with MIC≥32 μg mL^{-1}, which had been reported since 2002 (Chang *et al*., 2003; Weigel *et al*., 2003; Centers for Disease Control and Prevention, 2004) has created a more serious concern. Despite the fact that there are no reported cases of VISA in Malaysia as yet, nevertheless an alternative treatment need be explored for combating this new emergence strains. One way to overcome antibiotic-resistant bacteria is through the use of new antimicrobial compounds and/or combination therapy. This study was carried out to seek an approach considered as an alternative treatment in terms of combination therapy between phytochemical and an available antibiotic against laboratory passage-derived strains of VISA.

MATERIALS AND METHODS

Bacterial strain: The strain (*S. aureus* N1441b) used in this study was prepared as a laboratory- passage derived strain with decreased susceptibility to vancomycin (vancomycin MIC = 15 mg mL^{-1}) throughout the period when the present study was conducted from July 2006 until June 2007 in our laboratory in Forest Research Institute of Malaysia.

Antimicrobial agents: A total of 23 selected phytochemical powders from phenolic acid (caffeic acid, chlorogenic acid, cinnamic acid, ferulic acid, ellagic acid, gallic acid, p-anisic acid, salicylic acid, tannic acid and vanillin), flavonoid (catechin, chrysin, epicatechin, hesperidin, hesperetin, naringenin, quercetin, rutin) and alkaloid (berberine, colchicine, piperine, quinine anhydrous and aesculetin) were examined for their combined effect with oxacillin against selected bacteria. All agents were obtained from Sigma (St. Louis, Minneapolis, USA). Stock solutions of these agents were prepared in sterile dimethyl-sulphoxide (DMSO) solvent to concentration, which depend on their respective MIC values.

Susceptibility testing: Tryptic Soy broth (Difco Laboratories, Detroit, Michigan, USA) was used for susceptibility testing. The MICs of each of the pyhtochemicals and oxacillin were determined by broth microdilution according to the standards of the National Committee for Clinical Laboratory Standards (2000).

Combination assay: The combination effects of the phytochemicals and oxacillin on the growth of VISA were investigated using the checkerboard dilution method (Sato *et al*., 2004a). 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 h at 37°C to confirm sterility. After incubation, the combinations that inhibited visible bacterial growth were confirmed and recorded to be the MIC value of the individual and combined antibacterial agents. The FIC values were then calculated from the MIC of antibacterial alone and in combination in accordance with Isenberg (1992) in which the criteria for synergy, additive and antagonism when the FIC index were ≤0.5, 05 to 2.0 and ≥2.0, respectively.

RESULTS AND DISCUSSION

Three groups of phytochemicals, from the phenolic groups, alkaloids and flavonoids were studied. The FIC indices of the combination of oxacillin and the phenolic acids against VISA were found to be between 0.1125 and 2.000; i.e., the effects are either synergistic or additive (Table 1). Similarly, the flavonoids also showed both synergistic and additive interaction with oxacillin (Table 2). However, all the synergistic effect of combining oxacillin with flavonoids produced FICs between 0.0417 to 0.1333 whereas the only additive effect was observed with naringenin with FIC index of 1.0333. All of synergistic interactions between the phytochemicals (except for quercetin) and oxacillin showed a twenty-fold decreased in MIC of oxacillin from 30 to 1.5 μg μL^{-1} whereas,

Table 1: Antibacterial activity of phenolic acids alone and in combination with oxacillin against vancomycin-intermediate *S. aureus* (VISA)

	MIC (μg μL^{-1})			
		Combination[a]		
Phytochemicals	Alone	Phytochemical	Oxacillin[b]	FIC index
Caffeic acid	5.0	0.3125	1.5	0.1125 (S)[c]
Chlorogenic acid	15.0	0.6250	30.0	1.0417 (A)
Cinnamic acid	2.5	2.5000	1.0	1.0333 (A)
Ferulic acid	2.5	2.5000	1.0	1.0333 (A)
Ellagic acid	5.0	0.3125	1.5	0.1125 (S)
Gallic acid	7.5	7.5000	1.5	1.0500 (A)
P-anisic acid	15.0	0.9375	1.5	0.1125 (S)
Salicylic acid	5.0	0.3125	1.5	0.1125 (S)
Tannic acid	7.5	7.5000	1.5	1.0500 (A)
Vanillin	7.5	7.5000	30.0	2.0000 (A)

Table 2: Antibacterial activity of flavonoids alone and in combination with oxacillin against vancomycin-intermediate *S. aureus* (VISA)

	MIC (μg μL^{-1})			
		Combination[a]		
Phytochemicals	Alone	Phytochemical	Oxacillin[b]	FIC index
Catechin	10.00	0.6250	1.5	0.1125 (S)
Chrysin	30.00	0.6250	1.5	0.0708 (S)
Epicatechin	10.00	0.8333	1.5	0.1333 (S)
Hesperidin	30.00	0.6250	1.5	0.0708 (S)
Hesperetin	15.00	0.9375	1.5	0.1125 (S)
Naringenin	2.50	2.5000	1.0	1.0333 (A)
Quercetin	1.25	0.0313	0.5	0.0417 (S)
Rutin	40.00	2.5000	1.5	0.1125 (S)

Table 3: Antibacterial activity of alkaloids alone and in combination with oxacillin against vancomycin-intermediate *S. aureus*, (VISA)

	MIC (μg μL^{-1})			
		Combination[a]		
Phytochemicals	Alone	Phytochemical	Oxacillin[b]	FIC index
Berberine	5.0	0.3125	1.5	0.1125 (S)
Colchicine	20.0	20.0000	1.5	1.0500 (A)
Piperine	40.0	2.5000	1.5	0.1125 (S)
Quinine anhydrous	5.0	0.3125	30.0	1.0625 (A)
Aesculetin	7.5	0.6250	1.5	0.1333 (S)

[a]This MIC by combination study expresses the concentration at FIC index obtained. [b]The MIC of oxacillin alone was 30 μg μL^{-1}. [c]The combination was interpreted as synergistic (S) or additive (A)

quercetin remarkably reduced MIC of oxacillin by sixty-fold to 0.5 μg μL^{-1}. Besides quercetin with FIC index equal to 0.0417, chrysin and hesperidin also exhibited promising synergistic interactions with equal FIC index value of 0.0708. The interaction study of alkaloids in combination with oxacillin produced FICs values between 0.1125 to 1.0625, indicating either synergistic or additive activity against VISA (Table 3). Synergistic effects in combination with oxacillin were observed with berberine and piperine by twenty-fold reduction in MIC of oxacillin with the same FIC index of 0.1125.

The combination of phytochemicals with antibiotics would be useful for the therapy of bacterial infections with respect to the reduction of MIC values, which would possibly result in potentiating antibacterial potency, reducing side-effects and eliminating the development of resistant mutants. Although DMSO has antibacterial activity, it however, never inhibited the growth of the VISA strains at a concentration that corresponded to the MIC of the tested phytochemicals. Thus, the antibacterial activity observed in the present study is attributed to the action of phytochemicals. Lewis and Ausubel (2006) hypothesized that in general, plant antibacterials are individually relatively weak but function in synergy. This study evaluated that the combination of phytochemicals with oxacillin were mostly synergistic and to a limited extent, additive against laboratory passage VISA strains. Among the phenolic acid groups, ellagic acid showed synergistic effect whereas gallic acid and tannic acid were additive in combination with oxacillin. Akiyama *et al.* (2001) observed that the antistaphylococcal activity of β-lactam antibiotic had increased in the presence of tannic acid at 100 mg L^{-1} and ellagic acid at 5000 mg L^{-1}. In addition to this, Kwon *et al.* (2007) have recently shown that gallic acid, in pure form showed high anti-*S. aureus* activity although gallic acid does not appeared to be synergistic in combination with oxacillin against VISA strains studied. Nevertheless, Chung *et al.* (1998) concluded that the ester linkage between gallic acid and glucose to form tannic acid was important to the antimicrobial potential of these compounds. Tannic acid is an important gallotannin which are esters of gallic acid and glucose whereas ellagic acid was formed by lactonization of ellagitannin (Chung *et al.*, 1993). In addition to ellagic acid, other phenolic compounds that seemed to act in synergy with oxacillin were caffeic acid, p-anisic acid and salicylic acid against laboratory passage VISA strains. Phenolic acids have been shown to potentiate the activity of β-lactam antibiotics against various strains of *Staphylococcus aureus* (Kwon *et al.*, 2007; Muroi *et al.*, 2004; Shimizu *et al.*, 2001; Zhao *et al.*, 2001; Nascimento *et al.*, 2000).

As far as the polyphenols are concerned, it was the flavonoid groups that exhibited the lowest FIC indices, which correspond to the excellent synergistic interactions in this study. According to Pathak *et al.* (1991), flavonoids were among the many phytochemicals that appear to be one of the most potent candidates for treating bacterial infections because a number of flavonoids have been shown to possess unique and sufficient antibacterial potency. In the present study, quercetin showed the most potent anti-VISA activity and markedly reduced MICs of oxacillin by sixty-fold with the lowest FIC index value. This is in agreement with Alvarez *et al.* (2006) who recently observed synergism of bacteriostatic action using combination of quercetin with

other flavonoids against *Staphylococcus aureus* ATCC 25923; quercetin was found to reduce the MIC of morin from 157.4 to 29.9 μg mL^{-1} and responsible for inhibitory effect of rutin against *S. aureus* ATCC 25923, that is, from devoid of antibacterial activity to having MIC value of 40.2 μg mL^{-1}. It was postulated that binding of a flavonoid makes an easier passage of another flavonoid by diffusion across structural membrane proteins and as such, the binding of quercetin to porines change the tridimensional conformation thereby, exposuring the hydrophilic character of the pore (Alvarez *et al*., 2006) hence, possibly results in potentiating the binding of oxacillin to Penicillin-Binding Protein (PBP) in VISA strain. The activities of plant flavonoids against MRSA have instilled a great deal of interest (Tsuchiya *et al*., 1996; Xu and Lee, 2001; Nanayakkara *et al*., 2002; Sato *et al*., 2004a, b). The FIC indices of the combination of 6,7-dihydroxyflavone and oxacillin against MRSA isolates were found to be between 0.251 and 0.504 (Sato *et al*., 2004a) whereby synergistic effects of isoflavanone and mupirocin was also observed against MRSA (Sato *et al*., 2004b). The mechanism of antibacterial action of flavonoid might be due to the ability of the compound to reverse bacterial resistance to β-lactam antibiotics against MRSA (Eumkeb and Richards, 2005).

In addition to polypehenol as plant antibacterials, there is excellent rationale that plant alkaloids also possess antibacterial activity and in fact, the most active anti-staphylococcal compound from the goldenseal (*Hydrastis canadensis*) is the quarternary ammonium alkaloid berberine (Gibbons, 2004). In this study, berberine and piperine were the phytochemicals in the alkaloid groups that indicated synergism in combination with oxacillin against VISA. This is supported by Yu *et al*. (2005) that berberine markedly lowered the MICs of oxacillin against MRSA and a synergistic effect was found between berberine and oxacillin against MRSA. On the other hand, the combination of piperine with ciprofloxacin was also effective in reducing the MIC against the MRSA isolates (Khan *et al*., 2006). This article is in line with our finding that piperine alone was not potent as antibacterial agent but in combination with other antibiotic, there was a reduction in MIC of oxacillin.

One of the strategies in overcoming the problem of resistance in bacteria is combination therapy between the existing antibiotic with phytochemicals. This is because one of the major mechanisms contributing to the resistance of microorganisms to antibiotics was reported by Levy (2002) and Poole (2004) to be due to the multidrug resistance efflux pumps (MDREP). Therefore inhibition of this NorA eflux in resistant-strain of *Staphylococcus aureus* by potential phytochemical that can act as a potentiator of the existing antibiotic would offer an ideal solution to deal with threat associated with infections due to resistant strains of *Staphylococcus aureus* including VISA strains. Present study has shown that piperine and berberine act in synergy with oxacillin against VISA which is supported by Khan *et al*. (2006) suggesting the role of piperine as an efflux pump inhibitor. In addition to this, Lewis and Ausubel (2006) has reviewed synergism that involved multidrug resistance pump inhibitors in a combination of 5'-methoxyhydnocarpin and berberine. Tegos *et al*. (2002) suggested that use of Multidrug Resistance Pump (MDR) inhibitors may uncover mechanism of plant antimicrobial action. Based on the FIC indices in the present study, the combination of most of the phytochemicals tested and oxacillin exert synergistic effect on the growth of laboratory passage-derived *S. aureus* strains. However, there are not many reports concerning the use of phyto-antibiotic combinations against these strains, probably due to the minority cases for such pathogen in certain countries. Further studies to determine the mode of action of the antimicrobial combinations against VISA strains are currently in progress.

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