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Antagonistic Effect of Tannin on Oxacillin Efficacy Against Methicillin-Resistant *Staphylococcus aureus* (MRSA) by Time-Kill Assay

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Abstract: Tannin has been considered as an alternative phytotherapeutic treatment for controlling methicillin-resistant *Staphylococcus aureus* (MRSA) infection. The present study aimed to investigate the type of interaction between tannin with selected antibiotics, oxacillin, vancomycin and linezolid against MRSA ATCC 33591 in order to identify the possible site of action of tannin with regards to the known mechanism of action of the antibiotics studied. Tannin was bacteriostatic with the MBC value of four times greater than its MIC value of 31.25 µg/ml. As expected, oxacillin and vancomycin were bactericidal with the same MIC/MBC value of 31.25 µg/ml and 0.98 µg/ml, respectively. On the other hand, linezolid exhibited bacteriostatic action with MBC of 32XMIC value of 1.56 µg/ml. Microdilution checkerboard (MDC) finding showed an FIC index value of 0.625 for tannin-oxacillin combination which indicated partial synergism. Tannin exhibited indifference effect (FIC=1.06) in combination with vancomycin where as tannin-linezolid combination displayed an additive (FIC=1) interaction. Despite the partial synergism observed with tannin and oxacillin from FIC study, time-kill assay (TKA) revealed antagonistic effect of the combined treatment at 1/2XMIC of tannin and 1/8XMIC of oxacillin against ATCC 33591. As such, these findings provide an evidence that tannin acts at a same site as that of linezolid but on site different from oxacillin and in doing so, tannin antagonized the bactericidal efficacy of oxacillin. It can be suggested that consuming food or beverages that is rich in tannin such as tea and coffee should be avoided especially when β-lactam antibiotics are applied.

Key words: Methicillin-resistant *Staphylococcus aureus* · Tannin · Partial synergism · Antagonistic · Time-kill assay · Microdilution checkerboard · Bacteriostatic

INTRODUCTION

Antimicrobial resistance is an immense and serious global challenge. MRSA is a human pathogen that causes nosocomial, health care-associated and community-acquired infections [1]. Strain of this species is widely known for causing a minor skin infections to many of the life threatening diseases such as endocarditis, meningitis, bacteremia, osteomyelitis, toxic shock syndrome, food poisoning and scalded skin syndrome [2,3]. Increased emergence of multidrug-resistance (MDR) *Staphylococcus aureus* merits special attention in hospital environment, as it is associated with significant morbidity and mortality due to hard-to-treat systemic infections [4], thus made the therapy of staphylococcal disease a global challenge [5]. Other than that, the impact of MRSA infection also resulted in a more adverse outcome and higher cost due to the prolong length of hospital stay, additional diagnostic or therapeutic procedures and antibiotic use [6].

Currently, infections caused by MRSA are treated with groups of antibiotic such as vancomycin, linezolid, daptomycin and etc. However, frequent reports of the toxic effects, distressing adverse reaction and recent failure of antibiotic due to the dramatic emergence of multidrug resistant pathogen eventually place a significant burden on the economy with loss of productivity [7].

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Natural products and their derivatives have been recognized for many years as a significant source of new lead in the development of new pharmaceutical agents [8]. On top of that, the plant kingdom represents an enormous reservoir of the most structurally diverse compounds [9]. The phytochemicals are antibiotic principles of plant, many of which are proven to possess anti-bacterial, anti-inflammatory and anti-fungal activities [10]. They act as drug modulating or modifying agent when they are given together with other drug [11].Tannin or commercially known as tannic acid is a water-soluble phenolic compound with astringent property is particularly interesting since it is well known for its antibacterial activity especially against *Staphylococcus aureus* [12]. Additionally, tannin has shown potential as anti-oxidant [13], anti-viral [14], anti-malarial [15], anti-mutagenic and anti-carcinogenic properties [16].

An approach using combination therapy between standard antibiotic and phytochemical is the best alternative therapeutic tool to combat superbug pathogen [17,18]. Moreover, the treatment combination using the natural-plant derived compound source has received a great deal of attention in recent years owing to their versatile application [19] and as remedies for many infectious diseases [20] especially with the use of plant traditional medicine. Antibiotic with different mechanism of action which are active against MRSA were selected for this study. Linezolid (oxazolidinone, protein synthesis inhibitor), vancomycin (glycopeptide, inhibition of cell wall biosynthesis) and oxacillin (penicillin, cell wall inhibitor) were used in combination with tannic acid (tannin, found in tree bark and leaves) against MRSA ATCC 33591.

The present study, therefore intended to evaluate the anti-MRSA action of tannin in combination with selected antibiotics which may lead to the discovery of a novel, safe and effective therapeutic agent, thus hamper the development of microbial resistance towards antibiotic. Consequently, it can be used as an alternative treatment against MRSA infection hence decrease the total intake of antibiotics and accordingly, reduce their undesirable toxicological effects as well as reduce treatment cost, morbidity and mortality rate. The effectiveness of phytochemical-antibiotic combination was assessed by checkerboard assay and the bactericidal synergistic or partial synergistic pair was subjected to time-kill assay to confirm its interaction effect and to determine its rate of killing time.

MATERIALS AND METHODS

Bacterial Strains, Antibiotics, Chemicals and Reagents: The tested pathogenic bacteria MRSA ATCC 33591 strain was obtained from the American Type Culture Collection (ATCC). Tannin and the three standard antibiotics; oxacillin, vancomycin and linezolid, used in this study were obtained from Sigma-Aldrich, UK. Mueller-Hinton broth and agar were purchased from Oxoid (Basingstoke, UK).

Antimicrobial Agent Preparation: Stock solution of antibiotic and phytochemical was prepared according to the Clinical and Laboratory Standards Institute protocols [21] or manufacturer's recommendations. By using auto vortex mixer, the solution was mixed with their respective solvents until the powder was dissolved completely. The stock solution of those agents was filter sterilized through 0.45 µM filter membrane (CNW, Jiuding, China) and was freshly prepared in order to avoid any contamination.

Bacterial Inoculum Preparation: The obtained bacterial strain was grown and maintained on nutrient agar slant. The inoculum size of test strain was standardized by using cuvette and spectrophotometer at wavelength of 625 nm. The turbidity of the bacterial suspension was adjusted to the required bacterial density equivalent to 0.5 McFarland's standard or 10^6 colony-forming unit (CFU/ml).

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determinations: In the current work, the antimicrobial susceptibility of MRSA ATCC 33591 strain towards tannin and standard antibiotics (oxacillin, vancomycin and linezolid) were determined via standard broth microdilution method in accordance with guidelines [21]. For consistency, the antimicrobial susceptibility testing was performed in triplicates in a final volume of 0.1 ml per test well. The MICs value of tannin, oxacillin, vancomycin and linezolid against MRSA was accessed in a 96-well microtiter plate at final concentration ranging from 125 µg/ml to 0.24 µg/ml for vancomycin and 100 µg/ml to 0.19 µg/ml for linezolid, whereas the starting concentration of tannin and oxacillin was 2, 000 µg/ml to 3.91 µg/ml.

The tested agents were pipetted onto the sterile Mueller-Hinton broth (MHB) enriched with 2% NaCl before the bacterial suspension at final inoculum of 10^6CFUl/ml was added. The tested compound in MHB was used as negative control to ensure medium sterility

while the inoculum in MHB served as positive control to control the adequacy of the broth for bacterial growth. The MIC values were taken as the lowest concentration of the assayed antibacterial agents in the wells of the microtiter plate that inhibits the visible growth (inhibitory phase) of the microorganisms after 24 hr. of inoculation [22].

The MBC value was determined by subculture the well showing no apparent growth on a sterile Mueller-Hinton agar (MHA) free of antibacterial agent. The plate was reincubated for an additional 18 to 24 hr. The least concentration of an antibacterial agent showing no visible growth (either totally prevents growth or killing 99.9% of the bacterial inoculum) on agar plate after overnight incubation was considered as MBC value [23].

Fractional Inhibitory Concentration (FIC) Determination: The combined effect of tannin with oxacilin, vancomycin and linezolid against MRSA ATCC 33591 strain was assessed by microdilution checkerboard (MDC) method to obtain the FIC index value based on Basri *et al*. [24]. The study was performed in order to screen the potential activity of tannin treatment combining with individual antibiotics, which would exhibit synergistic outcome. The concentration of individual compound in the combination of tannin and selected antibiotic which prevented visible bacterial growth was recorded as the MIC of the individual compound in the respective combination. The effects of each combination were evaluated by calculating the FIC index to characterize interactions between the tested agents using the following formula:

$$FIC_{index} (\sum FIC) = FIC_A + FIC_B = A/MIC_A + B/MIC_B$$

Where,

A	MIC of drug A in combination
B	MIC of drug B in combination
MIC_A	MIC of drug A alone
MIC_B	MIC of drug B alone

FIC index by checkerboard method is interpreted as follows: synergistic effect if FIC index is ≤ 0.5; partial synergism as FIC$> 0.5 < 1$; additive effect as FIC index = 1; indifference as FIC index $> 1 \leq 4$; and antagonism as FICindex is of more than 4 [25].

Time-Kill Assay: The time-kill study of tannin in combination with antibiotics against a single bacterial isolate, MRSA ATCC 33591 was performed in sterile microtiter plate containing 96 wells contained 0.04 ml MHB [26]. The combined tested agents (tannin-oxacillin) were inoculated with 0.05 ml test organism to a density of $1x10^8$ CFU/ml in a final volume of 0.1 ml. Then, the plates were incubated at 37°C and viable count was calculated at 0, 4, 8, 12 and 24 hr. after addition of treatment agent.

At each hour, 0.01 ml of the sample was drawn from the well to be serially diluted in normal saline (0.9% NaCl) solution prior to being plated in triplicates on MHA plate using sterile wire loopfor determination of viable count. Total bacterial CFU/ml was counted after 24 hr. of incubation at 37°C. A well of inoculated MHB with antibiotic free serve as growth control. For each plate, a colony count of surviving bacteria between 30 to 300 were taken in to plot the time-mortality curve with $\log_{10}$ CFU/ml on the y-axis and time (hour) on the x-axis.

The interaction was interpreted as synergistic if there is a decrease of ≥ 2 $\log_{10}$ CFU/ml in colony counts between the combinations and the most active single drug after 24 hr. [27]. Additive or indifference effect was described as a < 2 $\log_{10}$ CFU/ml reduction in viable count after 24 hr. for the combination, in comparison to the most active single agent [28]. Antagonism was defined as an increase in the colony count of ≥ 2 $\log_{10}$ CFU/ml by the combination compared to the count obtained by the most active single agent alone after 24 hr. [29]. The combination was defined as a bactericidal if it produced a ≥ 3 $\log_{10}$ CFU/ml reduction in colony counts during incubation period denoting > 99.9% killing compared to the size of the starting inoculum [30, 31].

RESULTS

MIC and MBC Determinations: The MIC and MBC values of tannin and oxacillin against MRSA ATCC 33591 were shown in Table 1. The MIC value of tannin and oxacillin was the same i.e. 31.25 µg/ml whereas the MBC values of tannin and oxacillin were respectively, 125 µg/ml and 31.25 µg/ml. Therefore, tannin demonstrated equal antimicrobial activity with oxacillin but because MBC value of tannin was 4 times its MIC value, tannin was bacteriostatic against MRSA ATCC 33591.From Table 2 and 3, it was shown that MIC values of linezolid and vancomycin were 1.56 µg/ml and 0.98 µg/ml. From these MIC values, it can be deduced that tannin was 20-30 times less potent than linezolid and vancomycin. The MBC value of linezolid was 50 µg/ml (Table 2) whereas that of vancomycin was 0.98 µg/ml (Table 3). This means that oxacillin and vancomycin were expectedly bactericidal against MRSA ATCC 33591 because their MBC value equal to their MICs. On the other hand, the MBC value of linezolid against MRSA ATCC 33591 was 32 times higher than its MIC value which showed the same bacteriostatic action as tannin.

Table 1: Determination of MIC and MBC values of tannin and oxacillin against MRSA ATCC 33591

	MIC		MBC		Control	
Concentration (µg/ml)	Tannin	Oxacillin	Tannin	Oxacillin	Positive	Negative
2000	-	-	-	-	+	-
1000	-	-	-	-	+	-
500	-	-	-	-	+	-
250	-	-	-	-	+	-
125	-	-	-	-	+	-
62.50	-	-	+	-	+	-
31.25	-	-	+	-	+	-
15.63	+	+	+	+	+	-
7.81	+	+	+	+	+	-
3.91	+	+	+	+	+	-

+represents presence of growth (turbid well), - represents absence of growth (clear well),
positive control: bacterial suspension and Mueller-Hinton broth, negative control: tannin/oxacillin and Mueller-Hinton broth

Table 2: Determination of MIC and MBC values of linezolid against MRSA ATCC 33591

	Linezolid		Control	
Concentration (µg/ml)	MIC	MBC	Positive	Negative
100	-	-	+	-
50.0	-	-	+	-
25.0	-	+	+	-
12.5	-	+	+	-
6.25	-	+	+	-
3.125	-	+	+	-
1.56	-	+	+	-
0.78	+	+	+	-
0.39	+	+	+	-
0.19	+	+	+	-

+ represents presence of growth (turbid well), -represents absence of growth (clear well)
positive control: bacterial suspension and Mueller-Hinton broth, negative control: linezolid and Mueller-Hinton broth

Table 3: Determination of MIC and MBC values of vancomycin against MRSA ATCC 33591

	Vancomycin		Control	
Concentration (µg/ml)	MIC	MBC	Positive	Negative
125	-	-	+	-
62.5	-	-	+	-
31.25	-	-	+	-
15.63	-	-	+	-
7.81	-	-	+	-
3.91	-	-	+	-
1.95	-	-	+	-
0.98	-	-	+	-
0.49	+	+	+	-
0.24	+	+	+	-

+ represents presence of growth (turbid well), - represents absence of growth (clear well)
positive control: bacterial suspension and Mueller-Hinton broth, negative control: vancomycin and Mueller-Hinton broth

Table 4: Determination of FIC index values and interaction effect of tannin with three antibiotics against MRSA ATCC 33591

MIC (µg/ml) in combination				Individual FIC		
Antibiotics (A)	MIC (A)	Phytochemical (B)	MIC (B)	FIC A	FIC B	FIC Index
Oxacillin	3.91	Tannin	15.63	0.125	0.50	0.625 (PS)
Vancomycin	0.061	Tannin	31.25	0.06	1.00	1.063 (IN)
Linezolid	0.78	Tannin	15.63	0.50	0.50	1.000 (AD)

PS denotes partial synergism (FIC> 0.5 < 1), IN denotes indifference (FIC> 1 = 4), AD denotes additive (FIC = 1)

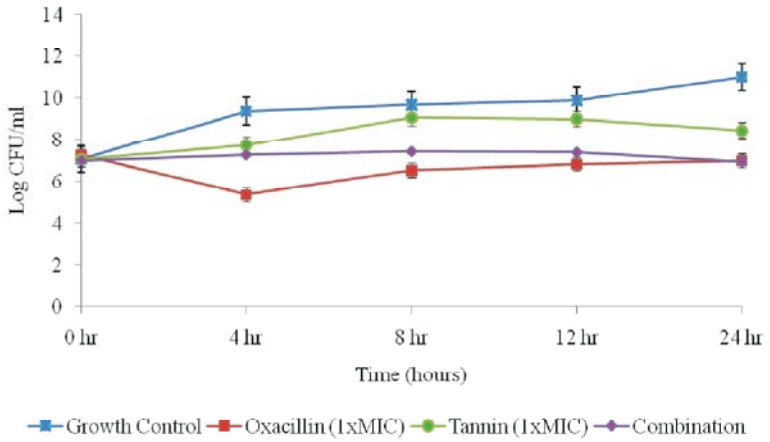

Fig. 1: Graph showing time-kill growth curves of combination of tannin with oxacillin, tannin alone and oxacillin alone against MRSA ATCC 33591. The data are presented as a mean of triplicates (n=3).

FIC Combination Test: Table 4 represents the FIC index values of the combination studies between tannin and three standard antibiotics. Combination of tannin and oxacillin displayed FIC index value of 0.625 which indicated partial synergism against MRSA ATCC 33591 being greater than 0.5 but less than 1. It was noted that tannin markedly reduced the MIC of oxacillin by eightfold from 31.25 µg/ml to 3.91 µg/ml. On the other hand, tannin showed indifference effect with vancomycin at FIC index values of 1.063 which was more than 1 but less than 4. The combination between tannin and linezolid was additive with FIC index value of 1.00. Since combination between tannin and oxacillin demonstrated the lowest FIC index value therefore, they were chosen for time-kill study to confirm such interaction effect.

Time-Kill Analysis: As shown in Figure 1, only oxacillin singly showed 2 $\log_{10}$ decrease in CFU/ml which occurred at the 4th hr. whereas tannin singly and the combination treatment did not exhibit a decrease in colony count over time. Hence, it was clearly shown that bacteriostatic action was observed for all treatments against ATCC 33591. In other words, the single antimicrobial agent as well as combined treatment did not demonstrate 3 $\log_{10}$ CFU/ml reduction in bacterial count, which was then followed by no change in the inhibitory effect against MRSA strain after 4 hours incubation. Thereby, this indicated that combination treatment exhibited antagonistic interaction between tannin and oxacillin against ATCC 33591 strain as the rate-killing effect of the combination treatment was greater than tannin but lower than oxacillin. This can be observed from the combination curve in Figure 1 which landed between the time-kill curve for tannin and oxacillin. Therefore, time-kill analysis finding clearly disputed the partial synergistic outcome of tannin in combination with oxacillin from the result of checkerboard technique.

DISCUSSION

The discovery of antibiotics was once thought to successfully control the diseases and agents of disease. However, the long history and widespread use of antibiotic have consequently led to the pathogen developing resistance to antibiotic therapy [32]. The antimicrobial property of *Quercus infectoria* galls extract have been found to be a result of the presence of high amount of hydrolysable tannin [33]. Tannin has been reported to be bacteriostatic and bactericidal against *S. aureus* [34].

The current study demonstrated that tannin showed equal anti-MRSA potency as oxacillin but exhibited lower antibacterial activity compared to vancomycin and linezolid. One of the possible factors that favour the activity of tannin against MRSA is that it is Gram-positive bacteria, which typically lack an outer membrane envelope [35]. It has been suggested that the level of component or active compound in the pure state is different from plant extract even at the same dose [36]. This is because crude extract is well-known to contain mixtures of bioactive compound while pure phytochemical such as tannin as being the only targeted compound [37]. On the other hand, tannin exhibited higher MIC value in comparison with linezolid and vancomycin. This result is in line with previous study which stated that generally phytochemical demonstrated lower antibacterial activity potency compared to standard antibiotics[18].

In this study, three standard antibiotics were chosen based on their different mechanism of action against MRSA. A combination therapy between phytochemical and commercial antibiotics with different target sites of action and mechanisms can be beneficial in the treatment of bacterial infections. This rely on the basic principle that in combination antimicrobial therapy, the formulation might increase the clinical efficacy, reduce regimen dose, thereby consequently lead to reduce toxicity and undesirable side effect as it contain lower effective dose or MIC value, increase bioavailability and importantly reduce the development of resistant mutant [38,39].

Synergistic interaction indicates that their mechanisms of antibacterial action are different or one compound enhanced the individual activity of another compound and vice versa [40-42]. Indifference, on the other hand, indicates that the combined action is the same as with either component alone [43]. Additionally, previous research has hypothesized that the additive activity was observed in combination of ellagic acid and gallic acid with β-lactam antibiotic, possibly due to their action at the same target site in the cytoplasmic membrane [44].

Therefore, it can bepostulated that tannin acts at the same target site with linezolid which correlated with the partial synergism with oxacillin indicating that tannin acts at different site with cell wall antibiotic. With vancomycin, it showed indifference; as such we can postulate that tannin could act by inhibiting protein synthesis, but at a cell wall where vancomycin acts. The MDC analysis in the current work seemed to demonstrate that the interaction with tannin markedly reduced the MIC value of vancomycin by sixteenfold eventhough the combination test showed indifference activity. It was also noted from FIC results that tannin improved the bactericidal activity of oxacillin by significantly reduced the MIC value of oxacillin by eightfold from 31.25 µg/ml to 3.91 µg/ml. With linezolid, tannin lowered its MIC value by twofold (1.56 to 0.78 µg/ml). As reported by Akiyama *et al*. [12], tannin has been suggested as a potential adjuvant compound for the therapy of *S. aureus* skin infection in the presence of β-lactam agent. In this case, as a pure compound, tannin possibly potentiates the effect of drug partner in combination by acting at an alternative target which consequently lead to the lysis of bacteria or inhibiting the microbial growth through interference with the bacterial cell wall.

Time-kill curve assay was utilized so as to verify the interaction from the MDC method and to evaluate the bactericidal activity and killing rate of combined antibacterial agents, namely between tannin and oxacillin since it exhibited lowest FIC index value. Time-kill analysis, however, did not correlate partial synergism interaction outcome of tannin in combination with oxacillin from the MDC study where a clear indication of antagonistic activity can be observed in the time mortality curve. Antagonism refers to a reduction in the activity of one component in the presence of the other [43]. Therefore, it was confirmed that tannin is a bacteriostatic anti-MRSA agent as it has been suggested that antagonism activity often occurs when a bacteriostatic agent is combined with bactericidal agent. The bactericidal drugs are more effective attack the multiplying bacteria. However, the inhibition of bacterial multiplication by bacteriostatic drugs may reduce the efficacy of the bactericidal agent when they were used together [45].

TKA result did not confirm MDC finding that tannin acts at different sites from oxacillin at the bacterial cell wall. Thus, we can postulate that tannin inhibited MRSA mainly by targeting on bacterial cell wall by complexing with cell wall protein as supported by Basri *et al*. [46] that in an action against MRSA infection, vancomycin attack at a certain reaction step which is different from that inhibited by β-lactam antibiotic. The hydroxyl groups of tannin play an important role in the molecule's ability to permeate the cell wall of bacteria [47]. From the TKA plotted graph, we could see that tannin, as a bacteriostatic agent antagonized the supposedly bactericidal activity of oxacillin.Oxacillin was shown to be bactericidal in the MIC/MBC finding despite no bactericidal rate-killing fall in 3 $\log_{10}$ CFU/ml was observed in TKA analysis. After 4 hr. incubation, tannin was not able to inhibit the bacterial growth and the initial bactericidal action of oxacillin effect was reversed. This is in agreement with Basri *et al*. [48] where the combination of epsilon-viniferin (ε-viniferin) with vancomycin against MRSA ATCC 33591 demonstrated antagonistic effect from time-kill assay but the synergistic effect was based on microdilution checkerboard technique.

Similar observation was also revealed in study of *Q. infectoria* galls extract in combination with vancomycin against MRSA 43300 [46] which reported that the electron microscope (EM) analysis of acetone extract of *Q. infectoria* galls did not correlate with the synergistic interaction as revealed by the post antibiotic effect study and it seemed that EM, was well correlated with the interaction observed in time-kill study. Hence, the result of the current study further supported previously reported literature, where TKA was favored over the MDC assay as a more relevant test in evaluating the type of interaction between antimicrobial agents[48].

Furthermore, the assay has been regarded as the highest accepted standard for synergy evaluation even though the checkerboard method has been widely used [49,50]. Therefore, the antagonistic effect displayed by time-kill assay in this study was also favored over the partial synergism effect of the checkerboard MIC test. Hence, we can conclude that tannin may have a similar mechanism of action with vancomycin but at different step in the biosynthesis of bacterial cell wall.

CONCLUSION

This findings provided evidence that tannin acts at a same site as that of linezolid but on site different from oxacillin and in doing so, tannin antagonized the bactericidal efficacy of oxacillin.

As such, precautions should be addressed when consuming tannin-based medicinal plant together with certain antibiotic since tannin has the ability to decrease the effectiveness of β-lactam antibiotic. However, further work should be carried out to investigate the detail information regarding the mechanism of action of tannin which displayed antagonistic effect with oxacillin.

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