Pterostilbene Enhanced Anti-Methicillin Resistant Staphylococcus aureus (MRSA) Activity of Oxacillin

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Abstract: Methicillin-resistant Staphylococcus aureus (MRSA) is a deadly pathogen that initially was limited to hospital and healthcare facilities but has gradually become a growing problem in healthy children and adults. Pterostilbene belongs to the phenylpropanoid phytoalexin which is involved in plant response to various pathogen and herbivores attack. The aim of this study is to evaluate the anti-MRSA action of pterostilbene in combination with selected antibiotics; vancomycin, linezolid and oxacillin against ATCC 43300 and ATCC 33591. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and fractional inhibitory concentration (FIC) index values were determined. Microbroth dilution technique and microdilution checkerboard (MDC) assay were employed. The MIC and MBC of pterostilbene against ATCC 33591 was 31.25 and 62.50 µg mL\(^{-1}\), respectively. While for ATCC 43300, the MBC value was also twice (62.50 µg mL\(^{-1}\)) its MIC value of 31.25 µg mL\(^{-1}\). This indicated that pterostilbene was bacteriostatic against both MRSA strains. Our MIC/MBC study also showed that linezolid exhibited bacteriostatic action but, oxacillin and vancomycin were bactericidal. MDC study showed that pterostilbene-oxacillin combination exhibited lowest FIC value (0.56) against both MRSA strains which indicated partial synergistic interaction. On the other hand, pterostilbene was additive (FIC 1.00) in combination with vancomycin whereas pterostilbene-linezolid combination displayed indifference effect with FIC of 1.25 against both MRSA strains. Pterostilbene in combination with oxacillin partially enhanced anti-MRSA activity with twofold reduction in MIC of oxacillin by acting at different site at the bacterial cell wall from that of oxacillin but more specific to the site of action of vancomycin.

Keywords: Pterostilbene, MRSA, Synergism, MIC, FIC, Bacteriostatic

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a human pathogen that causes both nosocomial and community-acquired infection (Boucher et al., 2010; Grundmann et al., 2006; McCarthy et al., 2010). The emergence of MRSA which has caused an increase in incidence and prevalence rate in many country, is widely known for causing endocarditis, meningitis, bacteremia, osteomyelitis and toxic shock syndrome (Lowy, 2003). As resistance toward antibiotic arises, cure towards bacterial infection becomes more difficult (Alexander and Wang, 2015). The impact of MRSA infection will increase burden to patient due to longer duration of hospital stay and also higher cost (Cosgrove et al., 2005; Short et al., 2006).

Vancomycin is currently the last defense against MRSA infection due to the fact that MRSA has shown resistance to most β-lactam antibiotics including penicillin, cephalosporin and carbapenem. In addition to its parental indication, the side effects associated with vancomycin are nephrotoxicity, hearing impairment and ‘red man syndrome’. The latter is known to cause skin redness and itching resulting from histamine reaction (Alexander and Greenberger, 1996). To complicate the situation, uncontrolled spreading of MRSA and the increased usage of vancomycin make the drug more susceptible to failure due to emergence of vancomycin-resistant organism (Hiramatsu, 2001).
Thus, the problem of bacterial resistance has triggered many researchers to seek alternative treatment and overcome this phenomenon from becoming more serious. One approach using combination of antimicrobial therapy that consists of existing antibiotic and plant extract is the best alternative therapeutic weapon to combat superbug pathogen (Cowan, 1999; Kyaw and Lim, 2012). Pterostilbene is the component that belongs to a phenolic group known as stilbene (Pan et al, 2008). Stilbene is a secondary plant metabolite formed from the flavonoid biosynthesis pathway and belongs to the phenylpropanoid family (Chong et al, 2009). Pterostilbene is found in the heartwood of sandalwood Pterocarpus marsupium (Maurya et al., 1984), leaves of Vitis vinifera (Langcake et al., 1979) and in some species of Vaccinium berries (Pezet and Pont, 1988). Pterostilbene is the analog of resveratrol, found in grapes Vitis vinifera and blueberries (Rimando et al, 2004). This component is biologically classified as phytoalexin. Phytoalexin has antimicrobial property which serves as part of the plant defense system against pathogen and herbivore (Chong et al., 2009). Additionally, pterostilbene has demonstrated potential antioxidant (Rimando et al., 2002), anti-fungal (Li et al., 2014), anti-glycemic (Grover et al., 2005), anti-mutagenic and anti-carcinogenic properties (Chio et al., 2011; Ghazali et al., 2012; McCormack and McFadden, 2012) which indicated the potential of pterostilbene in the prevention of certain illnesses and infectious diseases. However, there is no report on the antimicrobial activity of pterostilbene to date, which triggers the present study to be carried out. As such, the present study could also be assumed as the first report for the antibacterial activity of pterostilbene and hence, evaluation of the anti-MRSA action of pterostilbene in combination with selected antibiotics may lead to discovery of a novel, more effective treatment for combating MRSA infection.

Materials and Methods

Preparation of Antimicrobial Agents

The phytochemical used in this study is pterostilbene purchased commercially from EMD Biosciences/Calbiochem (USA). Stock solutions of antibiotic and phytochemical were prepared according to the manufacturer’s recommendations. The chemicals were dissolved in their respective solvents by using auto vortex mixer prior to the experiments.

Preparation of Bacterial Inoculum

The bacteria used was the reference strains of Methicillin-Resistant Staphylococcus aureus (MRSA) ATCC 43300 and ATCC 33591 obtained from the American Type Culture Collection (ATCC), grown and maintained on nutrient agar slant. Three to five colonies that grew on Mueller Hinton Agar (MHA) was cultured and transferred into Mueller Hinton broth (MHB). After incubation for 24 h at 37°C, the turbidity of the bacterial inoculum was adjusted to optical density reading of between 0.08-0.1 using spectrophotometer at a wavelength of 625 nm.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was determined using microbroth serial dilution method in a sterile 96-well microtiter plate. This assay was performed in triplicate for consistency in accordance with guidelines provided by CLSI (2014). MIC value is the lowest concentration of antimicrobial agent in inhibiting the visible growth of bacteria after overnight incubation at 37°C. The concentration of the tested antimicrobial agents used in the study against MRSA ATCC 33591 and ATCC 43300 ranged from 500 to 0.98 µg mL$^{-1}$ for pterostilbene, 125 to 0.24 µg mL$^{-1}$ for vancomycin, 100 to 0.19 µg mL$^{-1}$ for linezolid whereas the concentration of oxacillin was from 2, 000 to 3.91 µg mL$^{-1}$. The sterile MHB was enriched with 2% NaCl before the tested antimicrobial agents were inserted into the well at concentration gradient in a serial dilution. Then the diluted bacterial suspension at final inoculum of 10$^7$CFU mL$^{-1}$ 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 ensure the adequacy of the broth for bacterial growth. To facilitate the observation of the growth of bacteria in each well, 20 µL of 2,3,5-triphenyltetrazolium chloride (TTC) at 2 mg mL$^{-1}$ was added into each well. The minimum bactericidal concentration (MBC) was determined by culturing the sample from each clear well from the 96-microtiter plate onto the MHA plate. Then, the plates were incubated at 37°C overnight. The MBC value was determined as the least concentration of an antibacterial agent showing no visible growth (either totally inhibiting growth or killing 99.9% of the bacterial inoculum) on agar plates after overnight incubation (Pankey and Sabath, 2004).

Determination of Fractional Inhibitory Concentration (FIC)

FIC index value of combination between pterostilbene with each of the respective standard antibiotics; oxacillin, vancomycin and linezolid against MRSA ATCC 33591 and 43300 were obtained using microdilution checkerboard (MDC) method. The concentration of individual compound in the combination of pterostilbene with the selected antibiotics which inhibited 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 using the following formula (Drago et al., 2007):

$$FIC_{index} = \frac{A}{MIC_A} + \frac{B}{MIC_B}$$

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

The FIC index by checkerboard method is interpreted as follows: Synergistic effect if FICI of ≤ 0.5; partial synergism as FICI > 0.5; additive effect as FICI = 1; indifference as FICI > 1 ≤ 4; and antagonism as FICI of more than 4 (Drago et al., 2007).

**Results**

The MIC and MBC values of pterostilbene against MRSA ATCC 33591 and ATCC 4330 were shown in Table 1. The MIC value of pterostilbene was 31.25 µg mL\(^{-1}\) whereas its MBC was twice the MIC value against both MRSA strains. As far as linezolid is concerned, the MIC value is lower than pterostilbene at 1.56 and 0.78 µg mL\(^{-1}\), respectively against ATCC 33591 and ATCC 4330 as shown in Table 2. The MBC value of linezolid indicated that it was bacteriostatic against MRSA strains as it was fourfold and sixteen fold higher than its MIC values against ATCC 33591 and ATCC 43300 strains. The standard antibiotics, vancomycin (Table 3) and oxacillin (Table 4) was shown to have MIC values of 0.98 and 62.5 µg mL\(^{-1}\) respectively against ATCC 33591 whereas ATCC 43300 strain was more susceptible to the effect of vancomycin and oxacillin at MIC values of respectively, 0.49 and 31.25 µg mL\(^{-1}\). From the MBC values of vancomycin and oxacillin, it can be seen from Table 3 and 4 that both antibiotics were bactericidal against MRSA strains with MBC values that equaled their MIC values. In other words, the MIC and MBC values of vancomycin were 0.98 µg mL\(^{-1}\) against ATCC 3591 whereas for ATCC 43300, both the values were 0.49 µg mL\(^{-1}\). Oxacillin against ATCC 33591 also showed the same MIC/MBC value which was 62.5 µg mL\(^{-1}\) whereas, it was 31.25 µg mL\(^{-1}\) against ATCC 43300 (Table 4).

**Table 1. Determination of MIC and MBC values of pterostilbene against MRSA ATCC 33591 and ATCC 43300**

| Concentration (µg/mL) | ATCC 33591 | ATCC 43300 | Control |
|-----------------------|------------|------------|---------|
|                       | MIC | MBC | MIC | MBC | Positive | Negative |
| 500                   | -   | -   | -   | -   | +        | -        |
| 250                   | -   | -   | -   | -   | +        | -        |
| 125                   | -   | -   | -   | -   | +        | -        |
| 62.5                  | -   | -   | -   | -   | +        | -        |
| 31.25                 | -   | +   | -   | +   | +        | -        |
| 15.63                 | +   | +   | +   | +   | +        | -        |
| 7.81                  | +   | +   | +   | +   | +        | -        |
| 3.91                  | +   | +   | +   | +   | +        | -        |
| 1.95                  | +   | +   | +   | +   | +        | -        |
| 0.98                  | +   | +   | +   | +   | +        | -        |

* represents presence of growth (turbid well)
- represents absence of growth (clear well)
Positive control: Bacterial suspension and Mueller-Hinton broth
Negative control: Pterostilbene/solvents used and Mueller-Hinton broth

**Table 2. Determination of MIC and MBC values of linezolid against MRSA ATCC 33591 and ATCC 43300**

| Concentration (µg/mL) | ATCC 33591 | ATCC 43300 | Control |
|-----------------------|------------|------------|---------|
|                       | MIC | MBC | 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/solvents used and Mueller-Hinton broth
### Table 3. Determination of MIC and MBC values of vancomycin against MRSA ATCC 33591 and ATCC 43300

| Concentration (µg/mL) | ATCC 33591 |          | ATCC 43300 |          | Control |          |
|-----------------------|------------|----------|------------|----------|---------|----------|
|                       | MIC        | MBC      | 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/solvents used and Mueller-Hinton broth

### Table 4. Determination of MIC and MBC values of oxacillin against MRSA ATCC 33591 and ATCC 43300

| Concentration (µg/mL) | ATCC 33591 |          | ATCC 43300 |          | Control |          |
|-----------------------|------------|----------|------------|----------|---------|----------|
|                       | MIC        | MBC      | MIC        | MBC      | Positive | Negative |
| 2000                  | -          | -        | -          | -        | +        | -        |
| 1000                  | -          | -        | -          | -        | +        | -        |
| 500                   | -          | -        | -          | -        | +        | -        |
| 250                   | -          | -        | -          | -        | +        | -        |
| 125                   | -          | -        | -          | -        | +        | -        |
| 62.50                 | -          | -        | -          | -        | +        | -        |
| 31.25                 | +          | +        | -          | -        | +        | -        |
| 15.63                 | +          | +        | +          | +        | +        | -        |
| 7.81                  | +          | +        | +          | +        | +        | -        |
| 3.91                  | +          | +        | +          | +        | +        | -        |

- Represents absence of growth (clear well)

Positive control: Bacterial suspension and Mueller-Hinton broth
Negative control: Oxacillin/solvents used and Mueller-Hinton broth

### Table 5. Determination of FIC values and interaction effects with three antibiotics against MRSA ATCC 33591

| Antimicrobial agents | MIC (µg/mL) | FIC (µg/mL) |
|----------------------|------------|------------|
|                      | Alone      | Combination| FICI       | FICI       | Outcome   |
| Oxacillin            | 62.50      | 31.25      | 0.500      | 0.56       | Partial Synergism |
| Pterostilbene        | 31.25      | 1.95       | 0.062      | 0.56       |          |
| Vancomycin           | 0.98       | 0.49       | 0.500      |            |          |
| Pterostilbene        | 31.25      | 15.62      | 0.500      | 1.00       | Additive |
| Linezolid            | 1.56       | 0.29       | 0.250      |            |          |
| Pterostilbene        | 31.25      | 31.25      | 1.000      | 1.25       | Indifference |

FICI > 0.5 < 1 denotes partial synergism; FICI > 1 ≤ 4 denotes indifference and FICI = 1 denotes additivity

### Table 6. Determination of FIC values and interaction effects with three antibiotics against MRSA ATCC 43300

| Agents              | MIC (µg/mL) | FIC (µg/mL) |
|---------------------|------------|------------|
|                      | Alone      | Combination| FICI       | FICI       | Outcome   |
| Oxacillin            | 31.25      | 15.620     | 0.500      | 0.56       | Partial Synergism |
| Pterostilbene        | 31.25      | 1.950      | 0.062      | 0.56       |          |
| Vancomycin           | 0.49       | 0.240      | 0.500      |            |          |
| Pterostilbene        | 31.25      | 15.620     | 0.500      | 1.00       | Additive |
| Linezolid            | 0.78       | 0.195      | 0.250      |            |          |
| Pterostilbene        | 31.25      | 31.250     | 1.000      | 1.25       | Indifference |

FICI > 0.5 < 1 denotes partial synergism; FICI > 1 ≤ 4 denotes indifference and FICI = 1 denotes additivity
The results for combination studies between pterostilbene and three selected antibiotics against ATCC 33591 and ATCC 43300 were presented in Table 5 and 6, respectively as well as in Figure 8. Out of the three standard antibiotics, oxacillin showed the lowest FIC index value of 0.56 in combination with pterostilbene against both MSRA strains. An FIC index value of greater than 0.5 but less than 1 denotes partial synergism hence, combination of pterostilbene and oxacillin exhibited partial synergism interaction against both ATCC strains. In order words, pterostilbene was capable of reducing the MIC value of oxacillin by twofold from 62.50 to 31.25 μg mL⁻¹ against ATCC 33591 while for ATCC 43300, the twofold reduction was from 31.25 to 15.62 μg mL⁻¹. Pterostilbene in combination with vancomycin exhibited additivity (FIC 1.00) against both ATCC strains indicating that pterostilbene could well act at the same site of antibacterial action as vancomycin. Linezolid, on the other hand, showed indifference against ATCC 33591 and ATCC 43300 at FIC value of 1.25. This indicates that the combined action of pterostilbene-linezolid was the same as with either pterostilbene or linezolid alone. There is lack of interaction between pterostilbene and linezolid because there is no change in the MIC value of the individual agents in the combination treatment. As such, we can postulate that pterostilbene could target the cell wall different from where oxacillin acts but more specific to the site of action of vancomycin.

Discussion

The current study provides lead that pterostilbene possessed stronger antibacterial property compared with oxacillin against MRSA ATCC 33591 and comparable anti-MRSA potency as oxacillin against ATCC 43300 strain. Pterostilbene is a phytoalexin which belongs to the phenylpropanoid family and is involved in plant response to various pathogens and herbivore attack (Chong et al., 2009). In comparison with antibiotics used as current therapy against MRSA infection, pterostilbene still showed 20 to 31 times lower antimicrobial activity than linezolid and vancomycin against ATCC 33591 and 40 to 60 times less inhibitory potency against ATCC 43300. Despite the observation that ATCC 33591 strain is less susceptible towards linezolid and vancomycin, it is interesting to note that both MRSA reference strains were equally susceptible towards the effect of pterostilbene. This mean to say that whilst ATCC 33591 has developed resistance towards the last line of defense antibiotics, pterostilbene has not induced resistance in this particular strain. The same phenomenon was seen in the anti-MRSA effect of oxacillin whereby the susceptibility of ATCC 33591 was less than that of ATCC 43300. Pterostilbene could possibly reduce the problem of microbial resistance as supported by (Monte et al., 2014) that phytochemical have the ability to demonstrate significant potential to reverse antibiotic resistance. Despite the comparable antimicrobial efficacy of pterostilbene with oxacillin, pterostilbene acts bacteriostatic in the same manner as linezolid with MBC exceeding their MIC values. This is in agreement with Basri et al. (2012) that stilbenoid compounds belonging to the phenylpropanoid family were reported to exhibit bacteriostatic action against MRSA strains. In general, phytochemicals or secondary metabolites from the plants are capable of inhibiting or slowing the growth of bacteria rather than killing the pathogen (Nazzaro et al., 2013).

Individually, pterostilbene and linezolid were bacteriostatic but pterostilbene in combination with linezolid actually produced indifference effect. This is in line with Kyaw and Lim (2012) which reported that eugenol which is a bacteriostatic phytochemical (Rastogi et al., 2008; Yadav et al., 2015) in combination with a bacteriostatic antibiotic minocycline produced indifference interaction. According to Qin et al. (2013), indifference is when the combined action is the same as with either component alone. Previous study (Sopirala et al., 2010) reported that, if the MIC of an antibiotic changed within onefold dilution, the resulting effect is indifference. Ironically, although the FIC index was interpreted as indifference, but it was conflicting to note that there was a marked reduction of MIC value of linezolid by fourfold by pterostilbene which indicated synergism. This is because synergy occurs when a combination of two drugs causes inhibition or killing by fourfold lower concentration than that of either component drug used separately (Qin et al., 2013).

As far as oxacillin is concerned, pterostilbene displayed partial synergism in combination with this bactericidal agent against MRSA in spite of their comparably similar anti-MRSA potency. When the interaction is synergistic, it indicates that their mechanisms of antibacterial action might be different (Bassolé and Juliani, 2012). The MDC analysis in this study demonstrated that the interaction between pterostilbene with oxacillin partially enhanced the activity of oxacillin by reducing the MIC of oxacillin by twofold. In other words, the significant finding in this study was that pterostilbene partially enhanced antimethicillin resistant Staphylococcus aureus (MRSA) activity of oxacillin by acting at partially different target as that of oxacillin action at the bacterial cell wall and at site that does not involved site of linezolid action at protein level. This is further supported by the present FIC study that pterostilbene with vancomycin showed additive effect against MRSA. Previous study (Jayaraman et al., 2010) reported that additivity was observed in combination of ellagic acid and gallic acid with β-lactam antibiotics possibly due to their action at the same target site in the cytoplasmic membrane. Hence, this could well indicated that pterostilbene acts at the
same target as vancomycin at the bacterial cell wall but different with that of oxacillin. This additivity in pterostilbene-vancomycin combination was disputed by Basri et al. (2014) as synergism was portrayed by the combination of ε-viniferin and vancomycin. This is probably because ε-viniferin and pterostilbene differs in their chemical structure (Fig. 1-7) whereby ε-viniferin lacks two methoxy groups on the benzene ring which could account for the same site of action of pterostilbene and vancomycin.

Fig. 1. Determination of MIC and MBC values of pterostilbene against MRSA ATCC 33591 and ATCC 43300

Fig. 2. Determination of MIC and MBC values of linezolid against MRSA ATCC 33591 and ATCC 43300

Fig. 3. Determination of MIC and MBC values of vancomycin against MRSA ATCC 33591 and ATCC 43300
Fig. 4. Determination of MIC and MBC values of oxacillin against MRSA ATCC 33591 and ATCC 43300

Fig. 5. Determination of FIC values and interaction effects with three antibiotics against MRSA ATCC 43300 and ATCC 33591

Fig. 6. Chemical structures of pterostilbene and ε-viniferin
Methicillin-resistant \textit{S. aureus} (MRSA)

Fig. 7. Gram stain of Methicillin-resistant \textit{S. aureus} (MRSA)

Fig. 8. Flowchart of combination drugs and the effect of their combination
Conclusion

In summary, it can be concluded that pterostilbene have an antibacterial activity against MRSA with partial synergistic interaction in combination with oxacillin by reduction of MIC value by twofold and reduction of MIC value of linezolid by fourfold despite indifference effect in FIC analysis. It can also be postulated that pterostilbene acts at different site of target as oxacillin at the bacterial cell wall but more specific to that of vancomycin. Therefore further studies involving time-kill assay and postantibiotic effect (PAE) are currently ongoing to validate these interactions. This is because time-kill assay can confirm the partial synergism between pterostilbene-oxacillin combination as well as indifference effect between pterostilbene-linezolid as displayed by the FIC index values. These combinations have huge potential to be further studied and can be developed as an alternative treatment in combating MRSA infection.

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Author’s Contributions

Siti Fairuz Ishak: Performed the experiments and drafting the article.

Ahmad Rohi Ghazali: Cosupervised the project and contributing to context.

Noraziah Mohamad Zin: Provide the laboratory facilities for the study.

Dayang Fredalina Basri: Design the experiments and edit the manuscript.

Conflicts of Interest

The authors wish to declare that there are no conflicts of interest. This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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