

# Pterostilbene Reduced Post-antibiotic Effect Time of Linezolid against Methicillin-resistant *Staphylococcus aureus*

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## Basri, *et al.*: Reduction of PAE Time of Linezolid against MRSA by Pterostilbene

Pterostilbene is biologically classified as a phytoalexin, which is part of the plant's defence system in response to pathogen infection. The present study is to identify the type of interaction and post-antibiotic effect time of pterostilbene in combination with linezolid against methicillin-resistant *Staphylococcus aureus* American Type Culture Collection 43300 and 33591. The minimum inhibitory concentration values of individual antimicrobial agents were determined using microbroth dilution technique whereas the microdilution checkerboard assay was employed to verify the type of interaction of the combined agents from the fractional inhibitory concentration index values. The post-antibiotic effect time of pterostilbene and linezolid and in combination treatment was evaluated using viable plate count method. The minimum inhibitory concentration value of pterostilbene against both strains were the same (31.25 µg/ml) compared to linezolid at 0.78 µg/ml and 1.56 µg/ml, against 43300 and 33591, respectively. Microdilution checker analysis of pterostilbene in combination with linezolid against both methicillin-resistant *S. aureus* strains displayed fractional inhibitory concentration value of 1.25. Despite the indifference interaction between pterostilbene and linezolid as shown by microdilution checker study, post-antibiotic effect analysis demonstrated antagonism as depicted by a decrease of post-antibiotic effect time of linezolid by pterostilbene against both methicillin-resistant *S. aureus* strains. The duration of post-antibiotic effect time obtained for pterostilbene were twice (3.6±0.15 h) that of linezolid (1.8±0.31 h) against methicillin-resistant *S. aureus* American type culture collection 43300. However, pterostilbene exhibited shorter post-antibiotic effect time of 0.3±0.10 h compared to linezolid (0.9±0.06 h) against 33591 strain. In conclusion, pterostilbene was not able to prolong the post-antibiotic effect duration of linezolid in both strains and each of the antimicrobials demonstrated a more favourable timing of doses when these agents were used singly.

**Key words:** Pterostilbene, indifference, methicillin-resistant *Staphylococcus aureus*, minimum inhibitory concentration, fractional inhibitory concentration, post-antibiotic effect

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as an important cause of hospital-acquired and community-acquired infection worldwide. The extensively increased incidence of MRSA in the past few years merits special attention as these strains are not only resistant to methicillin but also to most clinically available antibiotics, including towards the newest antibacterial drug currently in the market<sup>[1,2]</sup>. The emergence of MRSA causes a wide range of illnesses, from skin and wound infections to pneumonia and blood stream infection that lead to significant morbidity and increased mortality<sup>[3]</sup>. Prolonged hospitalization of MRSA patient caused financial burden to government as well as to the patient<sup>[4,5]</sup>.

Vancomycin and linezolid are the major antibiotics currently used against MRSA infection<sup>[6]</sup>. However, the emergence of vancomycin-resistant *S. aureus* (VRSA) in 2002 in the United States<sup>[7]</sup> and the first outbreak of linezolid-resistant MRSA reported in 2008<sup>[8]</sup> limits the use of these antibiotics. The resistance to these antibiotics is constantly increasing<sup>[9,10]</sup> hence, there is an urgent need for a new antimicrobial agent which will be effective to combat these multidrug-resistant bacteria.

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One approach is treatment with antibacterial combination using two or more antibacterial to combat these multidrug-resistant bacteria<sup>[11]</sup>. In search of more effective chemotherapeutic agents for treating microbial infection, combination therapy becomes an important strategy as synergistic interaction can potentially increase efficacy, reduce toxicity, cure faster, prevent the emergence of resistance and provide broad-spectrum of activity than monotherapy regimen<sup>[12]</sup>. Phytochemicals have been considered as promising source of novel antibacterial<sup>[13,14]</sup> due to the successful defence mechanism developed by plants.

Pterostilbene is a phytochemical classified as a stilbene compound. A high content of these phenylpropanoid compounds, notably resveratrol and its analogue pterostilbene, identified in *Vitis vinifera* leaves<sup>[15]</sup>, heartwood of sandalwood *Pterocarpus marsupium*<sup>[16]</sup> and in some species of *Vaccinium* berries<sup>[17]</sup> are known to have diverse pharmacological activities<sup>[18]</sup>. Pterostilbene possesses potent antioxidant activity<sup>[19]</sup>, anticancer property<sup>[20]</sup>, anti-inflammatory<sup>[21]</sup>, antifungal<sup>[22]</sup> and to date, possess antibacterial activity against MRSA<sup>[23]</sup>.

Thus, the present study is to identify the interaction effect and post-antibiotic effect (PAE) of pterostilbene in combination with linezolid against MRSA. Linezolid was chosen in combination with pterostilbene as it has broad *in vitro* activity against antibiotic-susceptible and antibiotic-resistant Gram-positive bacteria including MRSA. This approach is hope to come up new antimicrobial agent for the battle against MRSA, hence, decrease the total intake of antibiotic as well as reducing treatment cost, morbidity and mortality rate.

## MATERIALS AND METHODS

Pterostilbene was purchased commercially from EMD Biosciences/Calbiochem (USA) and both pterostilbene and linezolid were prepared according to the manufacturer's recommendation which stated that dilution of these antimicrobial agents was performed depending on their weight with respect to their minimum inhibitory concentration (MIC) values. The antimicrobial solutions were mixed with 100% dimethylsulfoxide (DMSO) solvent using autovortex mixer until the powder dissolved completely prior to the experiments.

### Preparation of bacterial inoculum:

In the present study, the bacteria strains used were MRSA ATCC 33591 and 43300 obtained from the American Type Culture Collection (ATCC). The

stock cultures were grown on nutrient agar (Merck, Germany) slant at 37° for 24 h to obtain isolated colony. One or two single colony that grew on Mueller-Hinton agar (MHA) was cultured and transferred into the sterile Mueller-Hinton broth (MHB) and incubated at 37° for 24 h. The inoculum size of test strain was standardized to absorbance reading ranging from 0.08-0.13 equivalent to 0.5 McFarland's standard using spectrophotometer at a wavelength of 625 nm. The 0.5 McFarland suspension was then diluted to 1:100 in MHB resulting to a final bacterial inoculum size of approximately  $1 \times 10^6$  colony forming units (CFU)/ml.

### Determination of MIC:

MIC values of pterostilbene and linezolid against both MRSA strains were determined by microdilution technique using 96-well microtiter plate at final concentration ranging from 0.195 to 100 µg/ml and 0.98 to 500 µg/ml, respectively. The tested compound in MHB was used as negative control to ensure medium sterility while inoculum in MHB was used to control the adequacy of the broth for bacterial growth in positive control well. For confirmation, 40 µl of triphenyl tetrazolium chloride (TTC) at 2 mg/ml was added into each well to allow detection of viability. The MIC values were taken as the lowest concentration of the tested agents in the well of the microtiter plate that inhibited the visible growth of organism after 24 h of incubation at 37°. This assay was done in triplicate for consistency.

### Determination of fractional inhibitory concentration (FIC):

Interaction study between pterostilbene and linezolid against MRSA ATCC 43300 and 33591 were determined from the FIC index using microdilution checkerboard (MDC) method. The tested antimicrobials were prepared in the well of the microtiter plate at six different concentrations namely, 1MIC, 1/2×MIC, 1/4×MIC, 1/8×MIC, 1/16×MIC and 0×MIC in MHB before the diluted bacterial suspension at a final inoculum of  $10^6$  CFU/ml was added. FIC values for pterostilbene or linezolid were determined by dividing the concentration of the antimicrobial agents necessary to inhibit growth in a given row or column by the MIC value of the test organism for that individual agents alone. To characterize the interaction between each combination, FIC index was calculated using the formula<sup>[24]</sup>,  $FIC_{index} (\sum FIC) = FIC_A + FIC_B = A/MIC_A + B/MIC_B$ , where, A is the MIC of drug A in combination,

B is the MIC of drug B in combination,  $MIC_A$  is the MIC of drug A alone,  $MIC_B$  is the MIC of drug B alone.

The FIC index was then used to determine if synergy, antagonism, or indifference occurred as a result of interaction between the combinations. The FIC index range of between 0.5 and 4.0 is commonly used to define the type of combination whether they are synergistic (<0.5), additive (0.5-1.0), indifference (1.0-4.0) and antagonism (>4.0)<sup>[25]</sup>.

#### Determination PAE time:

PAE time of pterostilbene, linezolid and pterostilbene in combination with linezolid were determined using the viable plate count method in both the MRSA ATCC 43300 and 33591. The treatment groups at concentration  $10 \times MIC$  in the bacterial suspension at  $10^6$  bacteria/ml were incubated for 1 h at  $37^\circ$ . The control group comprised MHB and the bacterial suspension. After 1 h incubation, dilution at 1:1000 was performed in MHB. A volume of 2  $\mu$ l of the diluted sample was streaked onto MHA at 0, 2, 4, 6, 8, 10 and 24 h in order to count the number of colonies presented after 24 h of incubation at  $37^\circ$ . This was performed in triplicate aseptically. Graph of  $\log_{10}$  CFU/ml against time was plotted where the duration of PAE time was calculated from the graph using the equation  $PAE = T - C$ <sup>[26]</sup>, where T is the time required for the treated organism to increase  $1 \log_{10}$  CFU/ml after removal of treatment agents and C is the time required for the control organism to increase  $1 \log_{10}$  CFU/ml after completion of the same procedure.

## RESULTS AND DISCUSSION

The results of the microbroth dilution assay are shown in Tables 1 and 2. It can be seen from Table 1 that the

MIC values of pterostilbene against both MRSA strains were the same i.e. 31.25  $\mu$ g/ml. On the other hand, the MIC of linezolid (Table 2) against MRSA ATCC 43300 and 33591 were 0.78 and 1.56  $\mu$ g/ml, respectively. The antimicrobial activity of pterostilbene was 40 and 20 times less potent than that of linezolid, against MRSA ATCC 43300 and 33591, respectively. The MIC values of the combination of antimicrobials were equal to the MIC values of the single substances as can be concluded from Table 3 which showed the results of microdilution checkerboard assay. The present finding demonstrated that the FIC value between pterostilbene and linezolid was the same against MRSA ATCC 43300 and 33591 at FIC index value of 1.25. This indicated that the interaction between the two antimicrobials displayed indifference against both MRSA strains.

Figs. 1 and 2 illustrated the comparison of PAE time of pterostilbene alone, linezolid alone and their combination against MRSA ATCC 43300 and 33591, respectively. The duration of PAE obtained for pterostilbene, linezolid and pterostilbene-linezolid combination against MRSA ATCC 43300 were  $3.6 \pm 0.15$  h,  $1.8 \pm 0.31$  h and  $0.9 \pm 0.00$  h, respectively (fig. 1). It was observed that PAE duration time for pterostilbene alone was the longest, followed by linezolid alone whereas the combination treatment has the shortest suppression time of bacterial regrowth. However, pterostilbene exhibited shorter PAE time ( $0.3 \pm 0.1$  h) when exposed singly to MRSA ATCC 33591 compared to linezolid ( $0.9 \pm 0.06$  h) as shown in fig. 2.

The present finding demonstrated that pterostilbene showed equal antiMRSA potency against MRSA ATCC 43300 and 33591, which is in accordance with<sup>[22]</sup> both

**TABLE 1: DETERMINATION OF MIC VALUE OF PTEROSTILBENE AGAINST MRSA ATCC 43300 AND 33591**

Concentration ( $\mu$ g/ml)	ATCC 43300			ATCC 33591		
	Pterostilbene	Positive control	Negative control	Pterostilbene	Positive control	Negative control
500	-	+	-	-	+	-
250	-	+	-	-	+	-
125	-	+	-	-	+	-
62.50	-	+	-	-	+	-
31.25	-	+	-	-	+	-
15.63	+	+	-	+	+	-
7.81	+	+	-	+	+	-
3.91	+	+	-	+	+	-
1.95	+	+	-	+	+	-
0.98	+	+	-	+	+	-

(+) Presence of bacterial growth, (-) absence of bacterial growth. Positive control comprised bacterial suspension and MHB; negative control comprised pterostilbene and MHB

**TABLE 2: DETERMINATION OF MIC VALUE OF LINEZOLID AGAINST MRSA ATCC 43300 AND MRSA ATCC 33591**

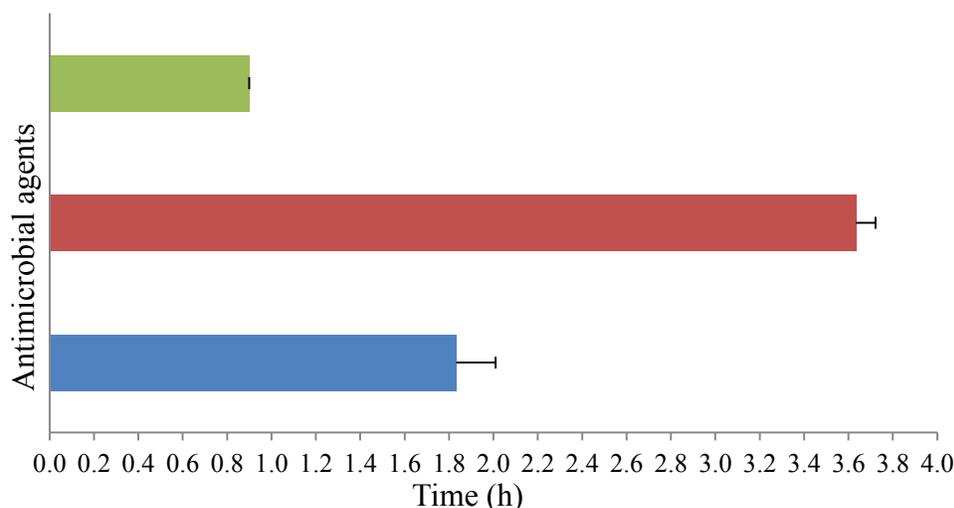
Concentration ( $\mu\text{g/ml}$ )	ATCC 43300			ATCC 33591		
	Linezolid	Positive control	Negative control	Linezolid	Positive control	Negative control
100	-	+	-	-	+	-
50	-	+	-	-	+	-
25	-	+	-	-	+	-
12.50	-	+	-	-	+	-
6.25	-	+	-	-	+	-
3.13	-	+	-	-	+	-
1.56	-	+	-	-	+	-
0.78	-	+	-	+	+	-
0.39	+	+	-	+	+	-
0.195	+	+	-	+	+	-

(+) Presence of bacterial growth; (-) absence of bacterial growth. Positive control comprised bacterial suspension and Mueller-Hinton broth; negative control comprised linezolid and Mueller-Hinton broth

**TABLE 3: FIC INDEX VALUES FOR PTEROSTILBENE IN COMBINATION WITH LINEZOLID AGAINST TWO MRSA STRAINS**

Strains	MIC ( $\mu\text{g/ml}$ ) in combination				FIC index		
	Antibiotic (A)	MIC (A)	Phytochemical (B)	MIC (B)	FIC A	FIC B	Outcome
ATCC 43300	Linezolid	0.78	Pterostilbene	31.25	0.195	31.25	1.25 (IN)
ATCC 33591	Linezolid	1.56	Pterostilbene	31.25	0.39	31.25	1.25 (IN)

PS denotes partial synergism ( $\text{FIC} > 0.5 < 1$ ); IN denotes indifference ( $\text{FIC} > 1 \leq 4$ ); AD denotes additive ( $\text{FIC} = 1$ )

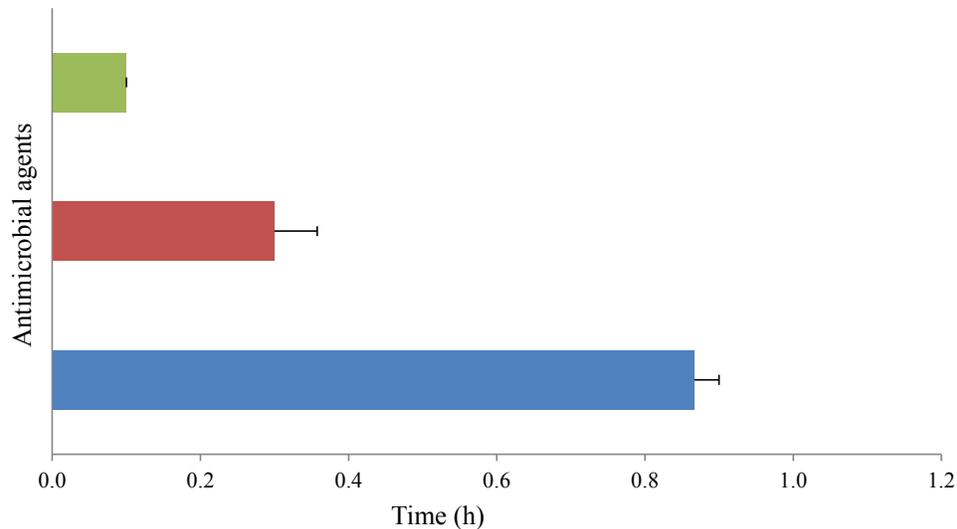


**Fig. 1: PAE time graph of pterostilbene, linezolid and pterostilbene in combination with linezolid against MRSA ATCC 43300.** Values are expressed as mean $\pm$ SEM; n=3. Comparison between PAE time combination of pterostilbene and linezolid, PAE time of linezolid alone, and PAE time of pterostilbene alone against MRSA ATCC 43300. ■ Combination (0.9 $\pm$ 0.0 h); ■ pterostilbene (3.6 $\pm$ 0.15 h); ■ linezolid (1.8 $\pm$ 0.31 h).

MRSA strains were equally susceptible towards the effect of pterostilbene. However, this is in contrast with previous study on other stilbenoids that showed less potency against MRSA ATCC 33591 with higher MIC of  $\epsilon$ -viniferin and johorenol A against this strain<sup>[27]</sup>. Pterostilbene, resveratrol,  $\epsilon$ -viniferin and johorenol A are formed in the flavonoid biosynthesis pathway belonging to the phenylpropanoid family<sup>[28]</sup>. Resveratrol, on the other hand was reported to possess antimicrobial activity against grapevine pathogens<sup>[29]</sup>.

Thus, pterostilbene could be an alternative treatment to overcome the problem of bacterial infection as it was known that selected phytochemicals have the ability to combat *Escherichia coli* and *S. aureus* and their biofilms<sup>[30]</sup>.

The FIC index value of  $>1$  but  $<4$  between pterostilbene and linezolid indicated that this interaction was indifference despite a marked reduction of MIC value of linezolid by fourfold against MRSA strains. This is also in line with the results reported by Zhang *et al*



**Fig. 2:** PAE time graph of pterostilbene, linezolid and pterostilbene in combination with linezolid against MRSA ATCC 33591. Values are expressed as mean±SEM; n=3. Comparison between PAE time combination of pterostilbene and linezolid, PAE time of linezolid alone, and PAE time of pterostilbene alone against MRSA ATCC 33591, ■ combination (0.1±0.0 h); ■ pterostilbene (0.3±0.10 h); ■ linezolid (0.9±0.06 h)

*al.*<sup>[31]</sup> that there was a significant reduction in the MIC value of antibiotic which showed both indifference and additive effects, when used in combination. According to Sopirala *et al.*<sup>[32]</sup> if the MIC of an antibiotic changed within a one-fold dilution, the result was considered as insignificant but because the decreased in the MIC of linezolid in this study was four-fold, the interaction could not possibly be indifference. However, the current FIC data was supported by our previous literature<sup>[23]</sup> that pterostilbene lacked interaction with linezolid despite the change in the MIC value of linezolid against both MRSA strains.

The PAE time of linezolid at 10×MIC in this study was within acceptable range of PAE parameters of between 0.7 h and 1.3 h after exposure of clinical strains of MRSA to linezolid at 1×MIC and 4×MIC<sup>[33]</sup>. Pterostilbene appeared to reduce the PAE time of linezolid by half suggesting that it antagonized the persistent post-antimicrobial effect of linezolid against 43300 strain. Linezolid, on the other hand, reduced the time taken for bacterial regrowth after removal of pterostilbene by 4 times which indicates that the interaction between the 2 agents was antagonism. As far as ATCC 43300 was concerned, pterostilbene demonstrated a prolonged PAE time after exposure to this MRSA strain as singly treatment. This is supported by a study on *Quercus infectoria* gall extract which showed longer PAE value compared to vancomycin as a standard antibiotic<sup>[34]</sup> against MRSA ATCC 33591. However, pterostilbene exhibited shorter PAE time when exposed singly to MRSA ATCC 33591 compared to linezolid despite the same pattern of combinational

PAE observed. In other words, pterostilbene still seemed to reduce the PAE time of linezolid meaning that it antagonized the effect of linezolid in sustaining its PAE. Linezolid, on the other hand, also reduced the time taken for bacterial regrowth after removal of pterostilbene by three-fold which confirms that the interaction between these 2 agents was antagonism. In short, pterostilbene was not capable of prolonging the duration of PAE time of linezolid in both MRSA ATCC strains. This defined pattern of antagonism in PAE study did not correlate with indifference effect from the FIC result. This contradicts with<sup>[35]</sup> that the antimicrobial combination used affected the PAE either as additive or an indifferent manner against MRSA. However, there is no report on the PAE of pterostilbene to date. Enhancement of prolongation of PAE against *S. aureus* was primarily dependent on the ability of each individual drug to induce a PAE since the final PAE was a rough mathematical sum of the individual PAE of both agents<sup>[35]</sup>.

In the current study, it was noted that when only one of the agents induced a PAE, the final result was similar to the PAE of that particular drug depicted as indifference. Pterostilbene did not increase the PAE of a standard antibiotic, despite exhibiting a PAE itself. Pterostilbene was unique in that it showed lasting suppression of bacterial regrowth, but when employed with linezolid, the combination effect reduced its PAE. Based on the data in this study, we can postulate that pterostilbene could possibly have the same mechanism of action as that of linezolid as proposed in our previous finding<sup>[23]</sup> that pterostilbene could target the cell wall different

from where oxacillin acts. Further studies to confirm that pterostilbene targets the bacterial protein different from the penicillin-binding protein where oxacillin acts are clearly needed to validate this hypothesis.

As conclusion, in spite of the indifference effect portrayed between pterostilbene and linezolid from FIC index value, PAE analysis confirmed antagonism interaction as pterostilbene was shown to decrease the duration of PAE time of linezolid against both the MRSA ATCC 43300 and 33591. This finding concluded that as a phytoalexin stilbenoid, pterostilbene was not capable of prolonging the duration of PAE time of linezolid in order to produce advantageous optimal antimicrobial dosing intervals in combination therapy against MRSA infection. The significance of the present study highlighted that pterostilbene and linezolid demonstrated a more favourable timing of dosage when these agents were used singly rather than in combination.

### Acknowledgements:

This project is funded by the Malaysian Government under the Fundamental Research Grant Scheme FRGS /2/2014/SG05/UKM/02/3.

### Conflict of interest:

The authors have declared that they have no competing interests.

### Financial support and sponsorship:

Nil.

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