

Full Length Research Paper

# Determination of the post-antibiotic effect (PAE) of combinations of extracts from galls of *Quercus infectoria* with vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Post-antibiotic effect (PAE) is one of the pharmacodynamic parameters that can be defined as the time it takes for the microorganisms to regain its normal growth after the complete removal of the antimicrobial agent. PAE on 2 strains of methicillin-resistant *staphylococcus aureus* (MRSA)-*Staphylococcus* were induced by galls of *Quercus infectoria* in combination with vancomycin. The determination of minimum inhibitory concentration (MIC) and PAE were carried out on two strains of *S. aureus* with vancomycin, methanol and acetone extracts. The test for fractional inhibitory concentration (FIC) index was done to verify the type of interaction of the combinations using checkerboard assay. The FIC value obtained for methanol and acetone extract with vancomycin against both strains of MRSA indicated the interaction of these combinations as synergistic. The combination of methanol and acetone extract with vancomycin significantly enhanced the PAE for both MRSA strains compared to the PAE when these agents were used singly. Both combinations of methanol extract with vancomycin and acetone extract with vancomycin gave slightly higher PAE values for reference strain, MRSA ATCC 33591 compared to the passaged strain, Mu 9495. The longer PAE of extracts from galls of *Quercus infectoria* in combination with vancomycin in comparison to that of singly tested extracts and antibiotic could have some potential implications for the timing of doses during therapy with antimicrobial combinations against MRSA.

**Key words:** Post antibiotic effect, combination, *Quercus infectoria*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin.

## INTRODUCTION

*Staphylococcus aureus* is a major pathogen both within hospitals and in the community. *S. aureus* is an opportunistic pathogen and given the right circumstances

can cause serious infections (Stapleton and Taylor, 2002). Methicillin-resistant *S. aureus* (MRSA) was first isolated from a patient in United Kingdom in the year 1961, a year after the introduction of methicillin (Jevon, 1961; Chambers, 1997). Vancomycin has been the most reliable therapeutic agent against infections caused by MRSA. However, the first MRSA to acquire resistance to vancomycin, vancomycin-intermediate *S. aureus* (VISA) was isolated in Japan in the year 1996 (Hiramatsu, 2001). Fully vancomycin-resistant *S. aureus* (VRSA) were first reported from USA in 2002 (CDC, 2002; Chang et al., 2003). The resistance of *S. aureus* strains towards many

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**Abbreviations:** PAE, Post-antibiotic effect; MRSA, methicillin-resistant *staphylococcus aureus*; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration.

antibiotics has triggered a need to develop alternative antimicrobial agents especially of plant origin.

*Quercus infectoria* Olivier (Fagaceae) is one of the medicinal plants proved to have reliable antimicrobial properties. *Q. infectoria* is a small tree native of Greece, Asia Minor and Iran. The galls arise on young branches of this tree due to the attack by the gall-wasp *Adleria gallaetictoria* (Dar et al., 1976; Samuelsson, 1999). The galls are locally known as *manjakani* in Malaysia (Muhamad and Mustafa, 1994) and have been shown to have many medicinal properties such as astringent, antibacterial (Fatima et al., 2001), antifungal (Yamunarani et al., 2005), antiviral (Hussein et al., 2000), antidiabetic (Dar et al., 1976; Hwang et al., 2000), local anaesthetic (Dar et al., 1976; Hussein et al., 2000), larvicidal (Redwane et al., 2002) and anti-inflammatory (Kaur et al., 2004) activities.

The secondary metabolites from plants are good sources for combination therapy (*Hemaiswarya* et al., 2008). Tannin, one of the main constituents in the galls of *Q. infectoria* (Dar and Ikram, 1979; Wiart and Kumar, 2000) inhibits bacterial growth by interacting with the enzymes and proteins or acting indirectly on the bacterial membrane (Scalbert, 1991); whereas vancomycin inhibits bacterial growth by interfering with the protein synthesis of the bacterial cell wall (Hiramatsu, 2001). Therefore, the combination of extract from galls of *Q. infectoria* with vancomycin could provide a potential synergistic effect towards MRSA. Effective regimen dosing of this combination therapy can be obtained by determining the post-antibiotic effect (PAE) a well established pharmacodynamic parameter. Duration of PAE is the time it takes for the microorganisms to regain its normal growth following the complete removal of the antimicrobial agent (Craig and Gudmundsson, 1996). Determination of PAE helps to reduce or prevent problems such as toxicity effects of and resistance towards the many antibacterial agents. Here we report studies on post-antibiotic effect combination of extracts from *Q. infectoria* with vancomycin against MRSA.

## MATERIALS AND METHODS

### Bacterial strains

The bacterial strains used in this study were reference strain, MRSA ATCC 33591 and passaged strain, MRSA Mu 9495.

### Plant materials

The galls of *Q. infectoria* used in this study were purchased from the local market and were identified based on its physical characteristics. The voucher number obtained from Forest Research Institute Malaysia (FRIM) was EZ186/93. The galls were crushed to small pieces with a pestle and mortar before powdered using an electric grinder.

### Preparation of methanol and acetone extract

The extract was prepared by immersing 100 g of dried material in

500 ml solvent (methanol/acetone) for 24 h at room temperature. The mixture was then filtered and the process was repeated by immersing the remaining residue again into 300 ml of solvent (methanol/acetone). Both the filtrates were added and concentrated under reduced pressure using rotary evaporator at 45°C. The resulting pellet was pounded dry under hot air-dryer and finally a powdery crude (methanol/acetone) extract was produced.

### Preparation of extract solution

The extracts were dissolved in sterile distilled water to a final concentration of 20 mg/ml. Extracts were sterilized by passing through a 0.45 µm membrane filter.

### Preparation of vancomycin solution

The vancomycin (Sigma-Aldrich, USA) was prepared in accordance with the instructions of the manufacturers; dilutions were made in appropriate medium on the day on which the experiments were performed.

### Determination of minimum inhibitory concentration (MIC) value

The (MIC) were determined for both MRSA ATCC 33591 and MRSA Mu 9495 in Mueller Hinton broth by a microtiter dilution techniques, using final inoculums of approximately 10<sup>6</sup> colony-forming units (CFU)/ml. The MIC values were taken as the lowest concentration of the extracts or vancomycin in the wells of the microtiter plate that inhibited the visible growth of organisms after 24 h of incubation at 37°C.

### Determination of frictional inhibitory concentration (FIC)

The FIC for the combination of extracts (methanol/acetone) with vancomycin against both MRSA ATCC 33591 and MRSA Mu 9495 were determined using the checkerboard assay in order to indicate the types of interactions involved synergistic, additive or antagonistic. Both the extract and vancomycin were prepared in the wells of the microtiter plate at four different concentrations such as 1MIC, 1/8MIC, 1/16MIC and 0MIC (Mueller Hinton broth) before the diluted bacterial suspension (final inoculums of 10<sup>6</sup> bacterial/ml) were added. FIC values for each extract or vancomycin were derived by dividing the concentration of that extract or vancomycin necessary to inhibit growth in a given row or column by the MIC value of the test organism for that extract or vancomycin alone. The FIC index was then calculated by summing the separate FICs for each of the extract and vancomycin present in that particular well. Formula to determine FIC index (Bharadwaj et al., 2003)

$$FIC_A = \frac{MIC_A \text{ in combination}}{MIC_A} \quad (1)$$

$$FIC_B = \frac{MIC_B \text{ in combination}}{MIC_B} \quad (2)$$

Where A and B are the tested agents.

FIC index = (1) + (2)

**Table 1.** Determination of MIC values of extracts of galls of *Q. infectoria* against MRSA ATCC 33591 and MRSA Mu 9495.

Concentration (mg/ml)	MRSA ATCC 33591		MRSA Mu 9495	
	Methanol	Acetone	Methanol	Acetone
5.0000	-	-	-	-
2.5000	-	-	-	-
1.2500	-	-	-	-
0.6250	+	-	-	-
0.3125	+	+	+	+
0.1563	+	+	+	+
0.0781	+	+	+	+
0.0391	+	+	+	+
0.0195	+	+	+	+
0.0098	+	+	+	+
0.0049	+	+	+	+
0.0024	+	+	+	+

- = Absence of growth, positive control: Bacterial suspension and Mueller Hinton broth; + = presence of growth, negative control: Extracts and Mueller Hinton broth.

#### Interpretation

Synergistic	= $x \leq 0.5$
Additive	= $0.5 < x \leq 2.0$
Antagonistic	= $x > 2.0$

#### Determination of post-antibiotic effect (PAE)

PAE for both the MRSA ATCC 33591 and MRSA Mu 9495 were determined with methanol extract, acetone extract and vancomycin using the viable plate count method. Treatment group were prepared with extracts or vancomycin at concentration 10X MIC and diluted bacterial suspension (final inoculums of  $10^6$  bacterial/ml) whereas the control group were prepared using Mueller Hinton broth and diluted bacterial suspension (final inoculums of  $10^6$  bacterial/ml). Dilution at 1:1000 was done using Mueller Hinton broth after incubating both the treatment and control group for 1 h at 37°C. 2  $\mu$ l of the diluted sample was streaked on Mueller Hinton agar at 0,2,4,6,8,10 and 24 h in order to count the number of colonies present after 24 h of incubation at 37°C and it was performed in triplicate. Graph  $\log_{10}$  cfu/ml against time was plotted, where the duration of PAE were obtained from the graphs.

PAE Calculation (Craig and Gudmundsson, 1996)

$$PAE = T - C$$

Where, T is the time required for the treated organism to increase 1  $\log_{10}$  cfu/ml following dilution at 1:1000 and C is the time required for the control organism to increase 1  $\log_{10}$  cfu/ml following dilution at 1:1000

#### Determination of combinational PAE

Combinational PAE for both the MRSA ATCC 33591 and MRSA Mu 9495 were determined with combinations of methanol extract with vancomycin and acetone extract with vancomycin using the viable plate count method. This was performed after obtaining the FIC values for both the extracts and vancomycin. The method used was the same as for the determination of PAE for the methanol extract, acetone extract and vancomycin alone.

## RESULTS

#### Determination of MIC

The MIC values of the methanol and acetone extracts from the galls of *Q. infectoria* against both MRSA ATCC 33591 and MRSA Mu 9495 are shown in Table 1. The MIC values of methanol and acetone extract against MRSA ATCC 33591 were 6250 mg/ml and 0.3125 mg/ml respectively whereas, the MIC value of methanol and acetone extract against MRSA Mu 9495 was 0.3125 mg/ml.

Table 2 shows the MIC values of vancomycin against both MRSA ATCC 33591 and MRSA Mu 9495. The MIC of vancomycin against MRSA ATCC 33591 and MRSA Mu 9495 were 0.2500 mg/ml and 0.0039 mg/ml respectively. This suggested that MRSA Mu 9495 was less sensitive towards vancomycin.

#### Determination of FIC

The FIC index obtained for both combinations of methanol extract with vancomycin and acetone extract with vancomycin gave a value less than 0.5 for both MRSA ATCC 33591 and MRSA Mu 9495, thus indicating the interaction involved for both the strains was synergistic.

#### Determination of PAE

The duration of PAE obtained for methanol extract, acetone extract and vancomycin against MRSA ATCC 33591 were  $1.2 \pm 0.17$  h,  $1.0 \pm 0.21$  h and  $1.0 \pm 0.40$  h respectively whereas against MRSA Mu 9495 were  $1.0 \pm 0.16$  h,  $0.8 \pm 0.10$  h and  $1.2 \pm 0.07$  h respectively. It is shown that the duration of PAE were lower against MRSA Mu 9495 as it is a clinical isolate strain thus it may

**Table 2.** Determination of MIC values of vancomycin against MRSA ATCC 33591 and MRSA Mu 9495.

Concentration (mg/ml)	Vancomycin	
	MRSA ATCC 33591	MRSA Mu 9495
1.0000	-	-
0.5000	-	-
0.2500	-	+
0.1250	-	+
0.0625	-	+
0.0313	-	+
0.0156	-	+
0.0078	-	+
0.0039	+	+
0.0020	+	+
0.0010	+	+
0.0005	+	+

- = Absence of growth, positive control: bacterial suspension and Mueller Hinton broth; + = presence of growth, negative control: extracts and Mueller Hinton broth.

require a stronger antimicrobial effect compared to MRSA ATCC 33591.

#### Determination of combinational PAE

The duration of PAE obtained for combinations of methanol extract with vancomycin and acetone extract with vancomycin against MRSA ATCC 33591 were  $2.3 \pm 0.31$  h and  $1.9 \pm 0.07$  h respectively whereas against MRSA Mu 9495 were  $2.1 \pm 0.10$  h and  $1.6 \pm 0.55$  h respectively. The duration of the combinational PAE were longer compared to the duration of PAE of methanol extract, acetone extract and vancomycin alone.

#### Statistical analysis

Comparison between the combinational PAE and the PAE of the extracts and vancomycin alone using the paired T-test showed that both strains gave a persistent longer duration of PAE for the combinational therapy. The PAE of the combination of methanol extract with vancomycin were significantly ( $p < 0.05$ ) longer against both MRSA ATCC 33591 and MRSA Mu 9495; whereas the PAE of the combination of acetone extract with vancomycin were significantly ( $p < 0.05$ ) longer against MRSA ATCC 33591 but, unfortunately not significantly ( $p > 0.05$ ) longer against MRSA Mu 9495.

#### DISCUSSION

This study shows that the combinational therapy with extracts from the galls of *Q. infectoria* with vancomycin could provide a prolonged PAE against both the MRSA

strains compared to the PAE when the extracts and vancomycin were used alone. This was supported by other researches that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens including *S. aureus* (Adwan and Mhanna, 2008; Betoni et al., 2006).

The synergistic effect shown by the combination of extracts with vancomycin suggests that they could provide a potential effect against the pathogens. Both the extracts and vancomycin could benefit each other during the process of inhibiting the bacterial growth takes place. This was also proven in other researches that the plant extracts could increase the activity of antimicrobial drugs *in vitro* against bacteria (Chang et al., 2007; Horiuchi et al., 2007).

All the extracts from galls of *Q. infectoria* showed a decrease in MIC to vancomycin and this could referred to that these crude extracts have many different phytochemicals which might inhibit bacteria growth by different mechanisms. This double attack of both agents on different target sites of the bacteria could theoretically lead to either an additive or synergistic effect (Esimone et al., 2006). In this study, vancomycin inhibits the polymerization of glycopeptides by binding tightly to the D-alanyl-D-alanine precursor of the bacterial cell wall. Thus, interrupting the cross linking of the peptidoglycan, further causing the cells to lyse (Lacy et al., 2008); whereas, extracts from galls of *Q. infectoria* attacks the bacterial enzyme such as the autolysin and  $\beta$ -lactamase (Chusri and Voravuthikunchai, 2009).

This study also shows that the combination of methanol extract with vancomycin gave a significantly longer PAE against both the MRSA strains compared to the combination of acetone extract with vancomycin that gave a significantly longer PAE against MRSA ATCC 33591 but not significantly longer PAE against MRSA Mu 9495. This

can be due the different type of solvent used in the extract preparation as this can greatly influence the bioactive compound being extracted (Pinelo et al., 2005). Plant extraction using methanol solvent could provide a consistent antimicrobial activity compared to those extracted by other solvents (Lin et al., 1999). It is also proven in other research that alcohol is a suitable solvent for the extraction of the plant bioactive compounds (Ahmad and Beg, 2001).

In conclusion, the combination of extracts from galls of *Q. infectoria* with vancomycin could provide efficient synergistic effects against the MRSA strains. The combination of methanol extract with vancomycin is more potential to be further developed as a potent anti-MRSA agent in order to reduce the emergence of multi-drug resistance and toxicity due to the indiscriminate use of antibiotics.

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