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Electron Microscope Study of Gall Extract from *Quercus infectoria* in Combination with Vancomycin against MRSA Using Post-Antibiotic Effect Determination

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Abstract: The galls from *Quercus infectoria* have been considered as an alternative phytotherapeutic treatment against Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection. The aim of the study was to examine the mode of action of the combined effect of an extract of *Quercus infectoria* with vancomycin against MRSA. The acetone extract from the *Q. infectoria* was prepared from dried gall powder and the Post-Antibiotic Effect (PAE) time of acetone extract was determined in combination with vancomycin and singly, against American Type Culture Collection (ATCC) 43300 strain using the checkerboard assay. The morphology and ultrastructural changes of MRSA using scanning and transmission electron microscope following 6 h treatments, was also observed. The mean difference of PAE time between the combination and vancomycin was insignificant ($p > 0.05$). This indicated that the acetone extract from *Q. infectoria* did not significantly prolong the PAE time of vancomycin. Morphology changes in the combination-treated cells showed no evidence of bacterial lysis although cytoplasmic damage was visible from ultrastructural studies. The antagonistic activity of the combined antibacterials shown by electron microscopic study did not correlate with PAE synergism. The acetone extract from *Q. infectoria* antagonized the bacteriolytic action of vancomycin by acting at the same site of action at different point in the peptidoglycan cycle. This finding indicated that tannin-based medicinal plant targets enzymes involved in the synthesis of MRSA cell wall.

Key words: *Quercus infectoria*, PAE, SEM, TEM, MRSA, antagonism

INTRODUCTION

Staphylococcus aureus is one of the major pathogen involved in nosocomial and community acquired infections. *S. aureus* causes serious infectious diseases with high rates in mortality and morbidity. Introduction to penicillin for the first time in 1928 helped to improve the prognosis of critical patients suffering from *S. aureus* infection (Ji, 2007). The issues of resistance in *S. aureus* against β -lactam antibiotics were reported with the emergence of methicillin-resistant *S. aureus* (Huang *et al.*, 2006). Nowadays, the issue of pathogenic bacterial resistance is a major global health problem (Nakano *et al.*, 2011). About 100,000 ton of antibiotics are manufactured each year and the usage had big impact on the survival of the infected population directly, creating bacterial multi-drug resistance phenomenon (Mamikandan *et al.*,

2011). Antibiotic resistance may be resolved by combination therapy of phytochemical extract and commercial antibiotics. Combination therapy might increase the clinical efficacy by reducing the possibility of resistance or delaying the development of mutation towards resistance. Furthermore, combination widens the spectrum of antibacterial activity compared to monotherapy application. In addition to this, combination therapy may reduce the regimen dose and toxicological effects of the drugs. Combination of antimicrobial compounds contain lower effective dose compared to a single antimicrobial agent. Therefore, the undesirable effects of the vancomycin such as ototoxicity and nephrotoxicity may be reduced.

Plant extract was chosen to act in combination with the standard antibiotic instead of pure phytochemical. A pure active compound does not necessarily exhibit the

same level of bioactivity as the crude extract at the same dose (Wagner and Ulrich-Merzenich, 2009) because the interacting components are not present in the unrefined plant extracts (Rasoanaivo *et al.*, 2011). In relation to this, the main constituents of the galls from *Quercus infectoria* Olivier are gallotannic acid (50-70%), gallic acid (2-4%), ellagic acid, starch and sugar (Bruneton, 1999) which may contribute to the positive interactions between the various components of the whole plant extracts. The high tannin content in the galls of *Q. infectoria* accounts for its astringent property and its topical use in the treatment of infectious skin conditions. Previous research have showed that *Q. infectoria* galls in combination with vancomycin displayed synergistic effect based on microdilution checkerboard assay (Amman *et al.*, 2011) despite the additive interaction from time-kill kinetics (Basri and Khairon, 2012).

The present study was planned to confirm the type of interaction by the combined effect of acetone extract from *Q. infectoria* gall with vancomycin using Post-Antibiotic Effect (PAE) determination and Electron Microscopic (EM) studies. Following this assumption, this may be the first study of its kind which employed EM analysis of MRSA treated with acetone extract from *Q. infectoria* gall in combination with vancomycin. Hence, the present study focused on their mechanism of anti-MRSA action with emphasis on the morphological and ultrastructural changes in the MRSA after subjected to antibacterial combination treatment. The results may be beneficial for other researchers to identify the site of action of tannin-based medicinal plant against MRSA.

MATERIALS AND METHODS

Preparation of acetone extract: The acetone extract was prepared by immersing 100 g of dried material of galls in 500 mL acetone for 24 h at room temperature. The mixture was then filtered and process was repeated using the remaining residue with 300 mL acetone. The two filtrates were combined and concentrated under reduced pressure using a rotary evaporator. The resulting pellet was finally pounded to dryness under hot air-dryer to produce a powdery crude acetone extract (Basri and Fan, 2005).

Bacterial strains: The bacterial strains used in this study used were Methicillin-Resistant *Staphylococcus aureus* (MRSA) ATCC 43300. All the bacterial strains were grown and maintained on nutrient agar slants. The inoculum size of each strain was standardized to 10^6 bacteria mL^{-1} for each test by adjusting the optical density of the bacterial suspension to a turbidity corresponding to spectrophotometric absorbance corresponding to 0.08 at 620 nm.

Determination of minimal inhibitory concentration

(MIC): The MIC values for acetone extract and vancomycin were determined using 96-well plate and Mueller Hinton broth enriched with 2% sodium chloride (CLSI, 2006). The range of final concentration for acetone extract was within $4.882-10\ 000\ \mu\text{g}\ \text{mL}^{-1}$ and from $0.024-50\ \mu\text{g}\ \text{mL}^{-1}$ for vancomycin. The size of bacterial inoculum used was 10^8 CFU mL^{-1} . The MIC values was evaluated following 24 h incubation at 37°C and the results obtained were used in the determination of Post-Antibiotic Effect (PAE) of the extracts either in combination or singly as well as in electron microscope studies.

Determination of post-antibiotic effect (PAE):

The PAE time for MRSA ATCC 43300 was determined with synergistic combination of acetone extract and vancomycin using the viable plate count method. The treatment group was prepared with extract or vancomycin at concentration 10X MIC and diluted bacterial suspension (final inoculums of 10^6 bacterial mL^{-1}). On the other hand, the control group was prepared using Mueller Hinton broth and diluted bacterial suspension (final inoculums of 10^6 bacterial mL^{-1}). Dilution at 1:1000 was performed using Mueller Hinton broth after incubating both the treatment and control group for 1 h at 37°C . Afterwards, 2 μL 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 presented after 24 h of incubation at 37°C and it was performed in triplicate. Graph \log_{10} CFU mL^{-1} against time was plotted, where the duration of PAE were obtained from the graphs. The formula used to determine the duration of PAE was based on (Craig and Gudmundsson, 1996):

$$\text{PAE} = \text{T} - \text{C}$$

Where:

T = Time required for the treated organism to increase $1\ \log_{10}$ CFU mL^{-1} following dilution at 1:1000

C = Time required for the control organism to increase $1\ \log_{10}$ CFU mL^{-1} following dilution at 1:1000

Electron microscopic (EM) analysis: In order to investigate the effect of acetone extract of *Q. infectoria*, alone and in combination with vancomycin on morphological and ultrastructural changes of MRSA, treated and control cells were examined by Scanning Electron Microscopy (SEM) after 6 h treatment. The bacterial cells treated with 10% dimethyl sulfoxide (DMSO) were used as control whereas the bacterial cells treated with (1X MIC) vancomycin alone were used as positive control. The cells were collected by

centrifugation and washed with distilled water. The cells were fixed with 2% glutaraldehyde in 0.1 M Phosphate Buffer Solution (PBS) and pH 7.4 for 15 min subsequently; the cells were washed with distilled water three times and fixed with 1% osmium tetroxide in distilled water for 5 min at room temperature. The samples were dehydrated with a series of graded ethanol (70, 90% and absolute ethanol), respectively for 5 min each, then coated with 42 nm thickness gold and examined in a Philips XL30 ESEM (FEI Company, Oregon, USA) at 28-30 kV. Cells were also prepared for transmission electron microscopy by fixation with 2% glutaraldehyde in 0.1 M PBS and washed with distilled water (Santhana Raj *et al.*, 2007). Following staining with 2% uranyl acetate for 5 minutes, the bacteria were exposed to 1% osmium tetraoxide for 5 min to dehydrate with a series of acetone (70, 90% and absolute acetone), respectively for 5 min each. Polymerization was done with pure epoxy resin in an embedding oven at 90°C for 2 h after the bacteria have been infiltrated by a mixture of acetone and epoxy resin (1:1) for 5 min. The blocks were trimmed and cut to 90 nm ultrathin sections and stained with Reynold's stain for 1 min. Each specimen was examined using Tecnai G2 TEM at an accelerating voltage of 100 kV.

RESULTS

Figure 1 illustrates the result in the determination of PAE time for the combination study. The MIC values for acetone extract and vancomycin against ATCC 43300 was 5 and 0.0016 mg mL⁻¹, respectively. Regarding the PAE

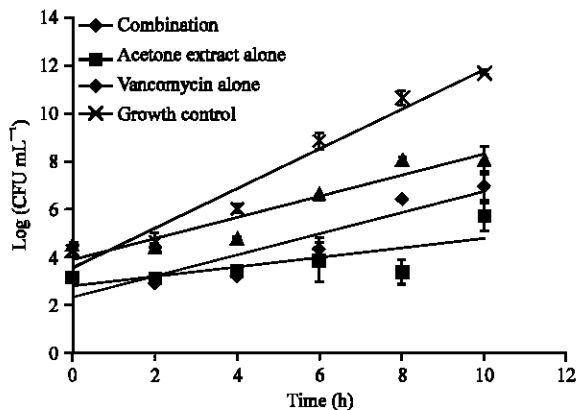


Fig. 1: Graph showing post-antibiotic effect (PAE) curves of combination of acetone extract with vancomycin, acetone extract alone and vancomycin alone against methicillin-resistant *Staphylococcus aureus* American Type Culture Collection MRSA ATCC 43300 at 10 times minimum inhibitory concentration (XMIC)

time for the combination study, it was shown that the PAE duration time for the acetone extract alone was the longest, followed by combination and vancomycin alone. Following 1 h exposure at 10X MIC, the PAE value for the acetone extract was 2.08 h. Acetone extract slightly increased the PAE time of vancomycin from 0.54-0.55 h (in combination) which indicated insignificant ($p > 0.05$) degree of prolonging the PAE time of vancomycin.

Morphological observation on the acetone extract-treated MRSA at 1X MIC using EM analysis showed that the bacteria became more irregular and inhomogeneous in shape (Fig. 2b) in contrast to control cells which showed a regular, smooth surface with spherical grape-like clusters (Fig. 2a). Most of the cells treated with acetone alone, were destroyed as indicated by the abnormal, distorted shape although some of the acetone extract-treated cells developed the formation of bleb-like structures on the surface. On the other hand, vancomycin at 1X MIC was used as a positive control and as expected, lysis was observed in most of the vancomycin-treated cells (Fig. 2c) as shown by the abnormal and elongated shape resulting from loss of normal spherical structure. Those cells that retained their coccoid shape had appearance of blebs on their surface. MRSA cells which were treated with acetone extract in combination with vancomycin were bigger in size (Fig. 2d) compared to other treatment. Interestingly, these acetone extract-treated MRSA maintained the spherical shape without separation of the central septa. However, no bacterial lysis was observed and the formation of blebbing structures was notably visible in all the combined-treated cells.

The findings from SEM study were confirmed by Transmission Electron Microscope (TEM) images (Fig. 3a-d). Ultrastructural analysis of combination-treated MRSA confirmed that the presence of blebbing on the surface of the cells which possibly account for the morphologically larger size of the combination-treated cells compared to control. There was a highly apparent septal line across cell wall and cytoplasmic white spots were clearly visible (Fig. 3d). Control cells displayed their structural integrity with a thick cell wall, dense cytoplasm and septal cross wall at the midline (Fig. 3a). TEM analyses of MRSA treated with acetone extract showed thickening of the cell wall and confirmed the distorted shape observed in SEM image. Additionally, cell division was impaired as demonstrated by the absence of septum formation in the non-separated cell (Fig. 3b), indicating that the acetone-extract from *Quercus infectoria* might affect the cell wall. The most pronounced ultrastructural

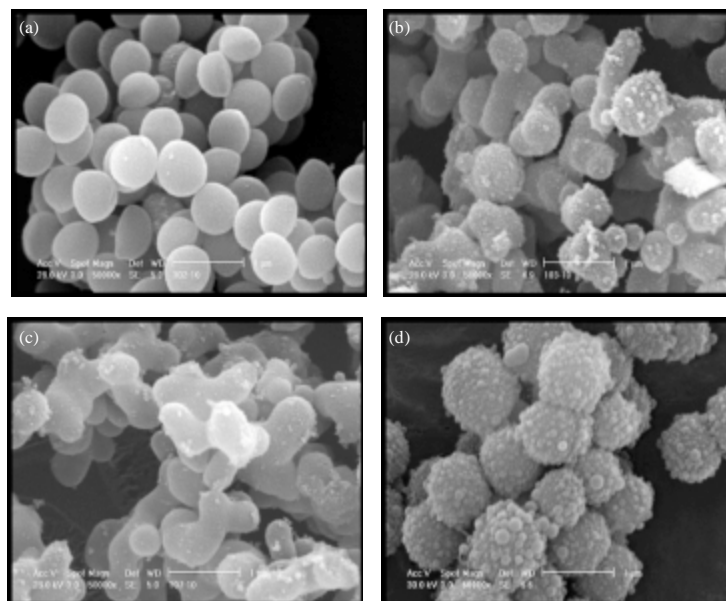


Fig. 2(a-d): Scanning electron microscope images of MRSA ATCC 43300 treated with acetone extract (AE) alone and in combination with vancomycin (VAN), all at 1XMIC after 6 h (a) Control, (b) Acetone extract (AE) (c) Vancomycin (VAN) and (d) AE (acetone extract) and vancomycin (VAN)

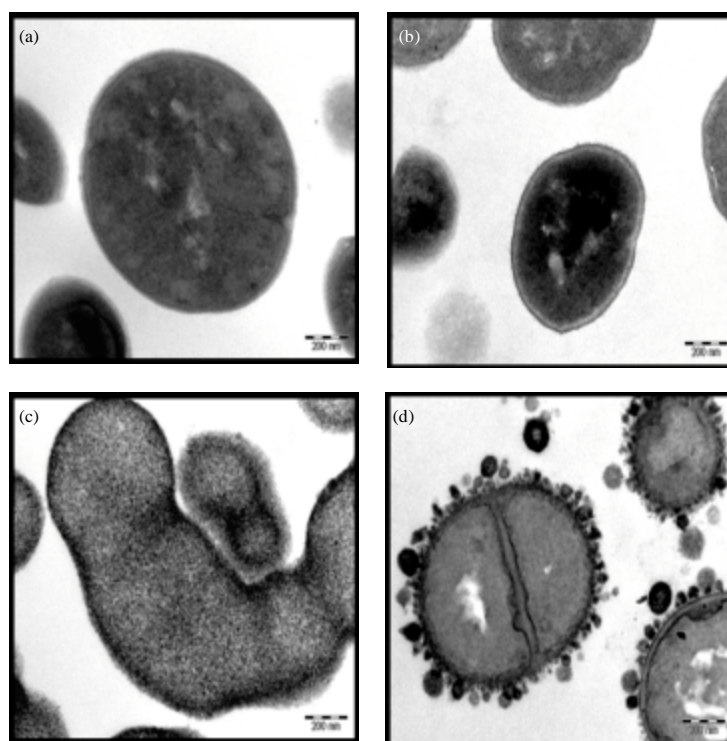


Fig. 3(a-d): Transmission electron microscope images MRSA ATCC 43300 treated with acetone extract (AE) alone and in combination with vancomycin (VAN), all at 1XMIC after 6 h (a) Control, (b) AE (c) VAN, (d) AE and VAN

changes following MRSA cells exposed to vancomycin after 6 h was the irregular thickness of the cell wall without any indication of septal aberration (Fig. 3c).

DISCUSSION

Post-Antibiotic Effect (PAE) analysis of acetone extract of *Q. infectoria* in this study was not in agreement with the present Electron Microscopic (EM) study. The presence of acetone in the combination form had no significant effect on the PAE time of vancomycin. In general, when the antibiotics are used in combination, the PAE time would be expected to be prolonged (Bozkurt-Guzel and Gerceker, 2012). However, the results of the present study showed that the acetone extract from *Q. infectoria* did not significantly increase the PAE time of vancomycin. This is probably attributed to the PAE time (0.54 h) of vancomycin contradictory to a previous research report (Totsuka *et al.*, 1999) that PAE time of vancomycin against MRSA strain (1.9-2.6 h). This may explain why acetone did not antagonize the persistent bactericidal effect of vancomycin. In fact the reverse is true i.e., vancomycin antagonized the prolonged persistent suppression of bacterial growth effect of acetone extract by almost 4-fold. In this study, result of PAE determination appeared to support the Fractional Inhibitory Concentration (FIC) synergistic interaction as previously reported (Basri and Khairon, 2012). However, there was an insignificant difference between the PAE time of combination and vancomycin alone. It is pertinent to mention here that if the PAE time of vancomycin is within the documented range (Totsuka *et al.*, 1999), then it may support the present EM findings. PAE study also did not show the significant synergism interaction between the acetone extract and the vancomycin. Hence, vancomycin appeared to antagonize the effect of acetone in sustaining its antimicrobial effect as indicated by PAE time study. However, the longest PAE time was shown by acetone extract alone whereas in the combination treatment, the acetone appeared to sustain the bactericidal effect of vancomycin.

The antagonistic interaction was confirmed by EM analysis. The marked irregular shape of most of the MRSA cells exposed to acetone may be due to the loss of cellular contents from the cells as a result of bacterial lysis. Hence, it may cause the acetone-treated cells to lose their coccal-shape. This clearly demonstrates that acetone-extract from *Q. infectoria* resembles the bactericidal action of vancomycin against MRSA. However, the irregular thickness of MRSA cell wall exposed to vancomycin without any indication of septal aberration as revealed by EM micrograph could be an evidence of reduced glycopeptide susceptibility.

It has been proposed that the latter is a multifactorial event due to several biological factors including thickened cell wall, reduced cell wall turnover and autolysis and increased cell-wall synthesis that eventually leads to reduced access of vancomycin to its active site, the division septum (Howden *et al.*, 2010). The most striking morphological changes observed in MRSA ATCC 43300 treated with the combined antimicrobials was the formation of vesicles blebbing out from the outer membrane similar to Al-Habib *et al.* (2010) for MRSA treated with grape seed extract. TEM analysis also appeared to correlate well with this result in which the intracytoplasmic black spots were observed, similar to those reported by Martins *et al.* (2004) in MRSA thioridazine-treated cells after 18 h of culture. This phenomena may be explained by the possibility that these structures arise so as to resist to cell lysis exerted by bactericidal effect of vancomycin. This is supported by ultrastructural study which showed either absence or lack of these structures in vancomycin-treated and acetone-treated MRSA cells.

Babiychuk *et al.* (2011) confirmed that the presence of blebbing actually confers resistance against cell lysis. The increased abundance of bleb-like structures in the combination treatment is probably due to bacterial mechanism to resist plasmalemmal injury. Hence, this further supports our finding that the acetone extract could well antagonize the bacteriolytic effect of vancomycin. As such, no such synergistic effect was observed between acetone extract and vancomycin from both the EM images.

This finding revealed that the EM analysis did not correlate to PAE synergistic effect. It has been suggested that electron microscopy observations seemed to be well correlated with the interactions observed in time-kill experiments (Jacqueline *et al.*, 2003). Hence, Time-Kill Analysis (TKA) is a more relevant test to evaluate the type of interaction between antimicrobial agents, compared to MDC technique and this is supported by an observation that TKA was favoured over the MDC assay (Basri and Khairon, 2012). In conclusion, acetone extract from *Q. infectoria* antagonized the bactericidal effect of vancomycin at early stage possibly by modification of the pathogen upon exposure to the extract which renders the pathogen less susceptible to the effect of vancomycin. This is supported by Tahany *et al.* (2002) on the antagonistic effect of lupeol acetate from *Moringa peregrina* and ampicillin against *E. coli* where the antibacterial activity decreased in the individually tested compound. Vancomycin is known to exhibit time-dependent bactericidal activity which retards the synthesis of bacterial cell wall and cause cell lysis (Boneca and Chiosis, 2003). Gall from *Q. infectoria*

contains high level of tannin, making it possible to possess similar antibacterial effect in this study (Evans, 1996). In general, the acetone extract from *Q. infectoria* gall may have the same mechanism of action with vancomycin against ATCC 43300 but at different steps in the biosynthesis of bacterial cell walls. Vancomycin has been proposed to attack a certain reaction step which is different from that inhibited by β -lactam antibiotic against MRSA (Aritaka *et al.*, 2001).

While EM provides useful information into the mechanism of action of antagonistic effect of antimicrobial agents, the resulting images are merely observational. Atomic focal microscopy and UV/VIS spectrophotometer are therefore recommended to confirm the type of interaction studies from the observations generated from the EM images. Moreover, the EM images obtained from this study were captured for 6 h which is undoubtedly, is not enough to give a more accurate and clearer picture in detail of the MRSA-treated cells. Longer time duration for the analysis of SEM and TEM are in progress to establish a rational combined therapy with *Q. infectoria* gall extract and vancomycin against MRSA. This finding would be useful as to gain further insight of the mechanism of the combined antibacterials against the MRSA cell wall at different time intervals.

CONCLUSION

It is hereby concluded that the acetone extract from *Q. infectoria* antagonized the bacteriolytic action of vancomycin by targeting different point in the peptidoglycan cycle but within the same site of action.

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