

Research Article



Mode of action of Endophytic *Streptomyces* sp., SUK 25 extracts Against MRSA; Microscopic, Biochemical and Time-Kill Analysis

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ABSTRACT

Bioactive compound from endophytic *Streptomyces* sp. has been claimed as a source of antibiotic. This study focused on the investigation of pharmacodynamic pattern and visualization of the mechanism of SUK 25 extracts against MRSA. The pharmacodynamic characteristic of the extract against MRSA 43300 was determined using time-kill assay. Then, the mode of action of the extracts was observed through biochemical assay and transmission electron microscopy. The SUK 25 extracts displayed bacteriostatic mode of action and concentration-dependent manner. The action of SUK 25 extracts against MRSA ATCC 43300 caused irregular shape of cells, which affected changes in Crystal Violet uptake by the cells. The release of UV absorbing materials and protein from the cells was caused by cell lysis. In conclusion, the action of the SUK 25 extracts against MRSA ATCC 43300 led to the internal change in cells, through which permeability of cells was altered by a decrease in the Crystal Violet uptake, shape of cell changes, and in turn brought about the lysis of cell.

Keywords: Mode of action, endophytic *Streptomyces*, MRSA

INTRODUCTION

Streptomyces sp. was classified as filamentous Gram-positive bacteria and naturally lives in plants, soils and marine environments. Its bioactive secondary metabolites have proved to exhibit antibiotic, anticancer, anti-inflammation and anti-viral activities.^{3,29} For example, peptide antibiotic isolated from *Streptomyces* sp. in Monstera tree, Peru displayed antifungal property against *Cryptococcus neoformans*, and anti-malarial activity against *Plasmodium falciparum*.¹²

Infectious disease caused by resistant bacteria, such as Methicillin Resistance *Staphylococcus aureus* (MRSA), has emerged as virulent pathogen in public and clinical settings. Vancomycin is the last resort of available antibiotic, but the current development of VRSA is making the treatment to become more difficult. It also has some side effects administered, such as nephrotoxicity.¹⁴ Nowadays, alternative medicine from natural sources is an important solution to overcome the side effects of synthetic drugs.

The purpose of this study is to determine the pharmacodynamic pattern of SUK 25 extract treatments against MRSA ATCC 43300, and to visualize the mechanism of action of SUK 25 extracts against MRSA.

MATERIALS AND METHODS

Culture Condition

MRSA ATCC 43300 and SUK (UKM Strain) 25 were obtained from Novel Antibiotic Laboratory, UKM. The SUK 25 was isolated from *Zingiber spectabile* root. The overnight culture of MRSA ATCC 43300 at 37 °C on Muller Hinton Agar (MHA), (Merck, USA) supplement with 2%

sodium chloride (Sigma-Aldrich, USA) was performed before testing. The mature spore of 14-day culture of SUK 25 at 28 °C on International *Streptomyces* Project (ISP) 2 Agar was used for extraction and testing. Both MRSA ATCC 43300 and SUK 25 were cultured in the 20% glycerol (Merck, USA) solution and placed at -80 °C for prolonged storage.^{8,33}

Secondary Metabolite Extraction

SUK 25 culture in Thornton media was extracted for exploitation of its secondary metabolites. Ethyl acetate (R & M Chemical, Malaysia) extraction was carried out by extracting the culture filtrates with three half-volumes of ethyl acetate. The extracts were collected (from upper layer) using a separating funnel (Pyrex, USA). The solvent phase was dried through the rotating evaporator (Buchi, Switzerland) at 40 °C with 240 mbar. The extracts (in solvent phase) were dried up, weighed and tested as anti-MRSA agents.³²

Anti-MRSA Activity

The MIC (Minimum Inhibitory Concentration) of the dried extracts of SUK 25 was determined against MRSA ATCC 43300⁸. The crude extracts of SUK 25 were dissolved in 10% methanol (R & M Chemical, Malaysia) and two-fold dilution technique was applied to prepare the concentration of 0.488 µg/mL to 1000 µg/mL in a 96-well microtiter plate (Thermo-Scientific, USA). Then, 50 µl of 1 x 10⁶ CFU/mL of bacterial inoculums was added to Mueller Hinton Broth (MHB) to make a total volume of 100 µl. Vancomycin (Sigma-Aldrich, USA) was used as positive control. The plate was incubated at 37 °C overnight. This assay was carried out three times to obtain consistent readings.



Time-Kill Assay

Time-kill studies were carried out in MHB at time intervals of 2 h until overnight. All antimicrobial agents were tested at 1X to 8X of their respective MIC with a starting inoculum of 5×10^5 CFU/mL prepared by direct colony suspension method based on modification and the procedure approved by CLSI.^{7,10}

The concentrations of SUK 25 extracts used in the study were 1.95 µg/mL, 3.9 µg/mL, 7.8 µg/mL, and 15.6 µg/mL whereas those of vancomycin were 1.5625 µg/mL, 3.125 µg/mL, 6.25 µg/mL and 12.5 µg/mL. The 50 µl aliquot of SUK 25 extracts at the corresponding concentration was added to 450 µl of MHB. Then, 500 µl of bacteria inoculum at concentration of 1×10^6 CFU/mL was added. The growth control comprised a well without the antimicrobial agent, performed in parallel to the treated wells whereas Vancomycin (Sigma-Aldrich, USA) was used as positive control.²²

Afterwards, the time kill assay was determined throughout the 24 h incubation at 37 °C. The viability of bacteria cells was done by withdrawing 10 µL aliquots at each time interval at every 2 h interval, and incubated at 37 °C. This test was done in triplicates.

Lysis Cell Assay

This autolysis assay was carried out according to modification of Mani and Hanaki^{14,20}. MRSA ATCC 43300 suspension with an inoculum size of 5×10^5 CFU/mL was incubated with extracts at 7.8 µg/mL of SUK 25 (4X MIC value) for 2 h at 37 °C. The untreated sample was used as negative control whereas MRSA ATCC 43300 treated with 6.25 µg/mL of Vancomycin (4X MIC value) was used as positive control. Then, 1 mL of each sample was centrifuged at 4500xg for 5 min at 4 °C. After that, the pellets were washed twice with 1 mL pH 7.4 PBS (Phosphate Buffer Saline) (First BASE, Singapore) solution, before dissolving them in 1 mL 0.05 M, pH7.2 Tris-HCl (Sigma Aldrich, USA) solution containing 0.05% (I/I) Triton X-100 (Sigma Aldrich, USA). The Absorbance reading (A_{580}) was taken at every one hour for 4 h of incubation period.

The percentage of A_{580} was determined as follows :

$$\frac{A_{580} \text{ at every 1 h}}{A_{580} \text{ at 0 h}} \times 100$$

Crystal Violet Assay

This assay was carried out according to modification of Devi¹¹ MRSA ATCC 43300 at 5×10^5 CFU/mL was centrifuged at 4500xg for 5 min at 4 °C.

Then, the cells pellet was washed twice and dissolved in pH 7.4 PBS solutions (First BASE, Singapore). For treatment reaction, about 7.8 µg/mL of SUK 25 extracts were added to the bacteria suspension and incubated for 2 h at 37 °C. While the suspension bacteria without extracts acted as negative control, the bacteria treated with 6.25 µg/mL of Vancomycin acted as an internal positive control. In addition, 0.25 M EDTA (Sigma Aldrich,

USA) was mixed with the suspension bacteria as external positive control. After 2 h of incubation, the samples were centrifuged at 9300xg speed for 5 min at 4 °C. Then, the pellet was dissolved in pH 7.4 PBS solution containing 5 µg/mL of Crystal Violet (Merck, USA). After that, the samples were incubated for 10 min at 37 °C before they were centrifuged at 13,400xg for 15 min. All the samples were recorded at 590nm using a spectrophotometer (SECOMAM, France). The A_{590} reading for Crystal Violet (Merck, USA) stock solution was assumed as 100% Crystal Violet uptake by the sample cells. The percentage of Crystal violet uptake was determined as the formula given below:

$$\frac{A_{590} \text{ treatment sample}}{A_{590} \text{ of } 5 \frac{\mu\text{g}}{\text{ml}} \text{ Crystal Violet Stock Solution}} \times 100\%$$

Release of UV Absorbing Materials 260nm and 280nm Assay

The release of UV absorbing materials was measured using UV spectrophotometer (SECOMAM, France), with several modifications.⁶ The MRSA ATCC 43300 with inoculum concentration of 1×10^6 CFU/mL was washed for three times and dissolved with pH 7.4 PBS. The extract of SUK 25 at 7.8 µg/mL concentration was added to 1 mL bacteria suspension, and incubated for 2 h at 37 °C. The bacteria suspension treated with 6.25 µg/mL Vancomycin was used as positive control, while bacteria suspension without any treatment served as negative control. After incubation, 10 µl of each sample was tenfold diluted and then cultured on MHA overnight at 37 °C. The remaining suspension cell was centrifuged at 13,400xg for 15 min, and the absorbance reading at 260 nm and 280 nm (A_{260} and A_{280}) from the supernatant was recorded.

Transmission Electron Microscopy

In order to observe the effect of SUK 25 extracts on ultra structural changes of MRSA ATCC 43300, the treated MRSA cells were examined under transmission electron microscope (FEI Tecnai™, USA) after 3 h treatments. This microscopy procedure was carried out according to Liu.¹⁹ The MRSA ATCC 43300 overnight culture in MHB was diluted to final absorbance (A_{630}) value of 0.4 at 630 nm. After that, 1 mL of the cell suspension was centrifuged at 11,000xg for 10 min. Then, cell pellet obtained was washed twice with pH 7.4 PBS. The washed cells were incubated with 1 mL of 7.8 µg/mL SUK 25 extracts for 3 h at 37 °C. For the positive control, the cell suspension was treated with Vancomycin at 6.25 µg/mL while the negative control was without any treatment. The cells were fixed with 3% glutaraldehyde (Sigma Aldrich, USA) in 0.1M PBS. The cells were post-fixed with 1% (w/v) Osmium tetroxide (Sigma Aldrich, USA) for 2 h at room temperature, washed once with the 0.1M PBS, dehydrated in a graded series of ethanol (Sigma Aldrich, USA) solution (70%, 90% and absolute alcohol) and the processes were followed by staining procedure, using 1% Toulidine blue (Sigma Aldrich, USA) and 2% Borate (Sigma Aldrich, USA) in distilled water for semi-thin procedure to



determine the area of interest. Then, polymerization was carried out by epoxy resin (Sigma Aldrich, USA) in an embedding medium at 90 °C for 2 h, after the bacteria had been infiltrated by a mixture of acetone (Fisher Scientific, Canada) and epoxy resin (Sigma Aldrich, USA) (1:1) for 5 min. The blocks were trimmed and cut into 90nm ultra section with a diamond knife on an Ultracut Ultramicrotome (Leica Microsystems Inc. USA), and the sections were double-stained with 3% uranyl acetate (Sigma Aldrich, USA) and 1% lead nitrate (Sigma Aldrich, USA). The specimens were examined with transmission electron microscope (FEI Tecnai™, USA) at an accelerating voltage of 120kV.

Statistical Analysis

One-way ANOVA and Post-hoc turkey analysis were done using IBM SPSS version 21. The p-value < 0.05 was considered significantly different.

RESULTS AND DISCUSSION

Bacteriostatic effect of MRSA ATCC 43300 on SUK 25 extract treatments on Figure 1 showed the growth of MRSA ATCC 43300 after treatment with increasing concentration of SUK 25 extracts at 1X, 2X, 4X and 8X MIC after 24 h incubation.

Results showed that the outcome of increasing concentration of SUK 25 extract towards MRSA ATCC 43300 exhibited bacteriostatic effect.

After 2 h incubation, 4X and 8X MIC treatment of SUK 25 extracts showed apparent bacteriostatic effect with a reduction in growth value, 2.71 log₁₀ CFU/mL and 1.85 log₁₀ CFU/mL, respectively. Meanwhile, 2X MIC and 1X MIC treatments caused bacteriostatic effects at 6 h and 4 h incubation, respectively.

The 2X MIC treatment against bacterial growth declined about 2.81 log₁₀ CFU/mL; whereas 1X MIC treatment caused the bacterial growth to be inhibited about 1.21 log₁₀ CFU/mL. The Vancomycin treatment at increasing concentration (1X MIC till 8X MIC) showed steady decline to more than 3 log₁₀ CFU/mL after 24 h incubation.

One-way ANOVA and Post-hoc turkey analysis, showed significant differences (p < 0.05) between the different concentrations of SUK 25 (1X MIC till 8X MIC) with the rate of bacterial growth reduction after 2 h treatment.

Therefore, the treatment of SUK 25 extracts against MRSA ATCC 43300 was concentration-dependent, whereas the treatment of Vancomycin against the bacteria was time-dependent. Bacteriostatic effect of the growth inhibition on MRSA ATCC 43300 is shown in Figure 1, with the increasing concentration of SUK 25 extracts treatment (1X, 2X, 4X and 8X MIC) in overnight culture. This effect was that of linear relationship with increasing concentration of the extracts.

The re-growth phase of MRSA was seen after 6 h reaction, excluding 8X MIC treatment of SUK 25 at 15.6 µg/mL.

In this study, the time-kill assay was based on post-MIC test and suspension method. It was conducted to determine the rate of MRSA killing by SUK 25 extracts. This assay also determined the time frame of treatment, and the concentration of exposed antibiotic.²⁴ Li and friends stated that there were three identical phases in time-kill curve, namely lag phase, exponential killing phase and re-growth phase.¹⁷ Therefore, the killing curve of pathogenic bacteria provides detailed information regarding time course of antibacterial effect. CLSI guidelines stated that bacteriostatic effect occurred as a result of bacterial growth inhibition, which led to inoculum suspension of less than 3 log₁₀ CFU/mL.⁷ On the other hand, the bactericidal effect of bacterial growth occurred when inoculum suspension value dropped to more than 3 log₁₀ CFU/mL.

The re-growth phase occurred possibly due to MRSA resistance that had developed towards the extracts. However, the resistance did not occur at high concentration of antibiotic treatment.¹⁸

In addition, this extract was not found to be toxic at IC₅₀ (50% inhibitory concentration) of 43.31 ± 1.24 µg/mL which was lower than 15.6 µg/mL.² Sudha & Masilamani stated the regulation of National Cancer Institute that the range of crude extracts is not toxic if the IC₅₀ value is more than 30 µg/mL.³⁰ Meanwhile, Malebo stated that the IC₅₀ value of more than 30 µg/mL is classified as non-toxic.²¹ Therefore, the extract of SUK 25 had a potential medicinal value to be developed as antibiotic candidate, despite being a concentration-dependent treatment. Ackermann & Rodloff stated that microlide antibiotic such as telithromycin, an antibiotic, which has interaction with microbial growth including MRSA, would have the concentration-dependent interaction pattern.¹

On the other hand, the MRSA ATCC 43300 treated with 7.8 µg/mL SUK 25 extracts showed 50% autolysis after 127.5 min incubation and exposure to lysis buffer (Figure 2). On the other hand, Vancomycin, at 6.25 µg/mL against MRSA ATCC 43300, underwent 50% autolysis after 105 min incubation when exposed to the lysis buffer.

For non-treated sample, the MRSA cells were subjected to 50% autolysis after 172.5 minutes incubation with the lysis buffer. Statistical analysis of one-way ANOVA and post-hoc Turkey test data indicated that the difference was significant (p-value < 0.05).

In this study, autolysis process of MRSA ATCC 43300 was found to be abnormal when treated with SUK 25 extracts (Figure 3), through which the lysis of cells dropped to 50% at shorter time (127.5 min) compared to untreated cells (172.5 min), and through which the fastest 50% lysis was demonstrated in Vancomycin treatment cells (105 min).

Figure 5 (b, e) showed that MRSA ATCC 43300 was totally lysed after 3 h treatment with 7.8 µg/mL of SUK 25 extracts, such as cells' treatment with 6.25 µg/mL of Vancomycin (Figure 5 (c, f)).



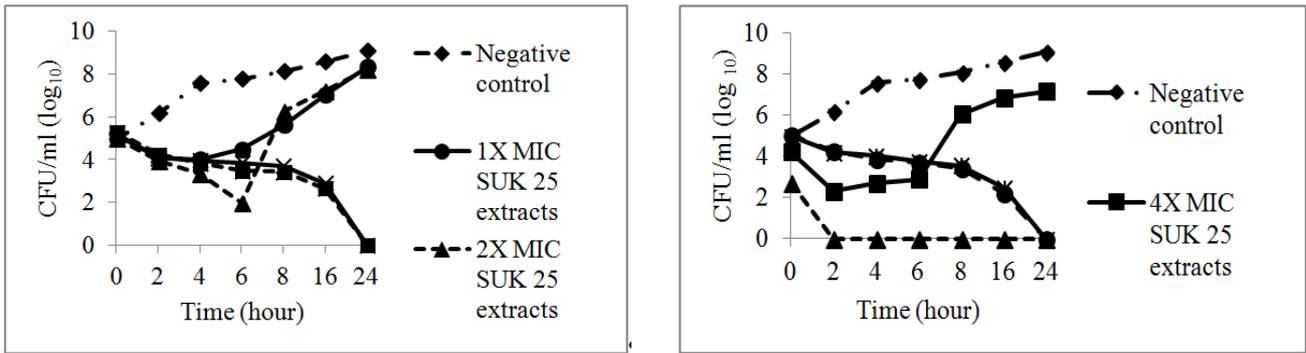


Figure 1: Growth curve of MRSA ATCC 43300 in time kill assay treated with increasing concentration of SUK 25 and Vancomycin (1X MIC and 2X MIC in Figure 1 a; 4X MIC and 8X MIC in Figure 1 b). The MRSA ATCC 43300 without any treatments used as negative control.

(Note the concentration of the following extracts: 1X MIC SUK 25 extracts: 1.95 µg/mL, 2X MIC SUK 25 extracts: 3.9 µg/mL, 4X MIC SUK 25 extracts: 7.8 µg/mL, 8X MIC SUK 25 extracts: 15.6 µg/mL. In contrast, the concentration of the following should also be noted: concentration of 1X MIC Vancomycin: 1.5625 µg/mL, 2X MIC Vancomycin: 3.125 µg/mL, 4X MIC Vancomycin; 6.25 µg/mL, 8X MIC Vancomycin; 12.5 µg/mL. Log CFU/mL = 10₁₀ colony forming unit).

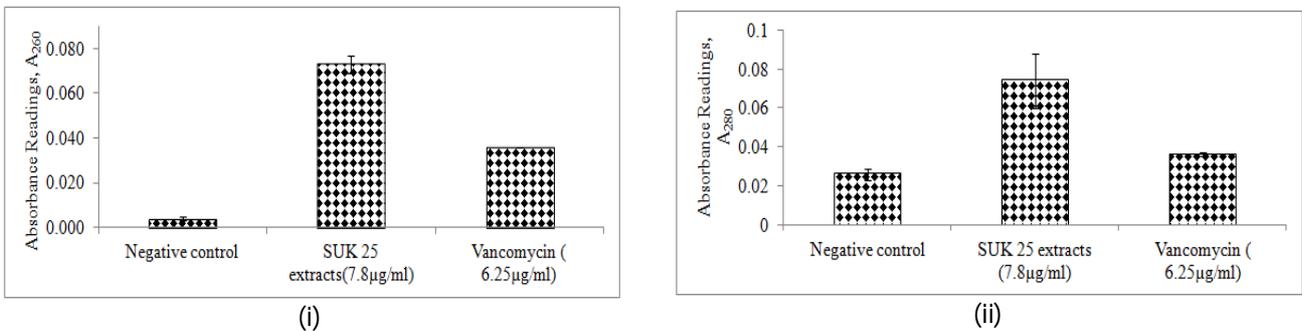


Figure 4: Release of UV absorbing materials at (i) 260 nm and (ii) 280nm from MRSA ATCC 43300 after treatment of 7.8 µg/mL SUK 25 extracts. The untreated cells were denoted as negative control and the cells treated with Vancomycin at 6.25 µg/mL were used as comparison.

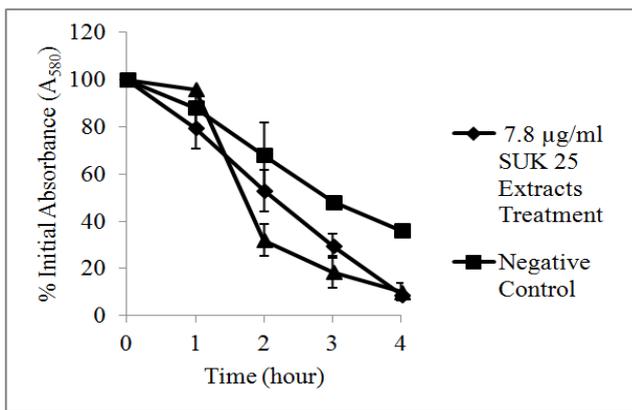


Figure 2: Autolysis MRSA ATCC 43300 after treatment with SUK 25 extracts at 7.8 µg/mL. It was compared with Vancomycin at 6.25 µg/mL as positive control and the MRSA cells without any treatment as negative control. The Absorbance (A₅₈₀) readings were taken every one hour for 4 h incubation, starting the MRSA cells dissolved in Lysis buffer (0.05M Tris-HCl solution containing 0.05% (v/v) Triton X-100).

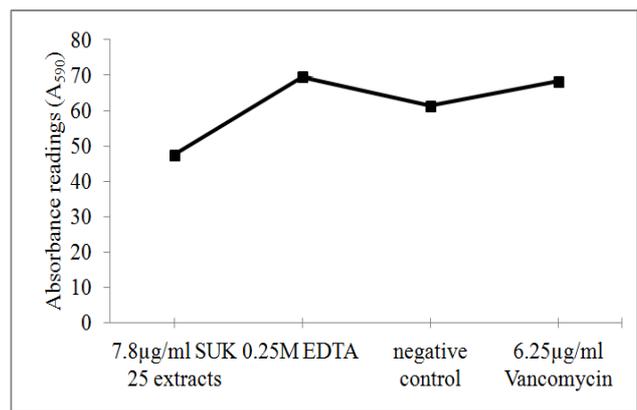


Figure 3: Crystal Violet uptake assay. The percentage of crystal violet uptake of MRSA ATCC 43300 treated with 7.8 µg/mL SUK 25 extracts was 47.62%. The MRSA cells treated with 0.25M EDTA and 6.25 µg/mL Vancomycin resulting Crystal Violet uptake percentage were 69.71% and 68.4% respectively. On the other hand, the MRSA cells without any treatments (control negative) produced 61.47 % crystal violet uptake.

The autolysis process was influenced by petidoglycan hydrolases enzyme or autolysin, which was secreted by *S. aureus* in nature. This process could probably break down the bacterial cell wall.^{13,23} In addition, the secretion of this enzyme functions for cell division and cell extension in their life cycle. Triton-X was a detergent used to degrade the resistance of Methicillin in bacteria without changing any structure of *mecA* gene.^{26,9} In this study, the abnormal lysis pattern of MRSA ATCC 43300 cells could be due to activation of autolysis. Consequently, it would cause the cells to be ruptured while the Vancomycin treatment against bacteria could halt the cell wall synthesis and lysis of the cells as shown in Figure 5 (c, f).²⁸

Besides, the percentage of Crystal Violet uptake by MRSA ATCC 43300 (Figure 3) without treatment was 58.73%, and the percentage declined significantly (p -value < 0.05) to 47.62% when the MRSA cells were treated with SUK 25 extracts. The increase in Crystal Violet uptake was seen in the MRSA cells exposed to 0.25M EDTA and 6.25 $\mu\text{g}/\text{mL}$ Vancomycin, which had 69.64% and 68.4%, respectively. Statistical analysis of one-way ANOVA and post-hoc Turkey test revealed that these data differ significantly from each other (p -value < 0.05).

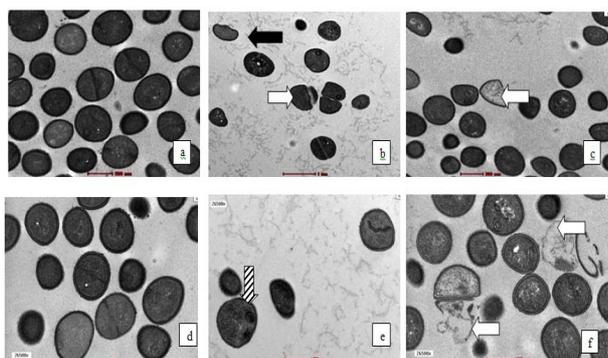


Figure 5: The MRSA ATCC 43300 with no treatment was incubated for 3 h. These photos were taken under magnification of 16 500X (a, b, c) and 26 500X (d, e, f), respectively. There was an action of 7.8 $\mu\text{g}/\text{mL}$ SUK 25 extracts against MRSA ATCC 43300 after 3 h incubation at 37 °C. White arrow (b) showed the cells lysed, whereas black arrow (b) and striped arrow (e) showed irregular shape and 0.5X enlargement respectively. Both pictures showed decrease in number of cells compared to normal (a, d). The 6.25 $\mu\text{g}/\text{mL}$ Vancomycin action was against MRSA ATCC 43300 after 3 h incubation at 37 °C (c, f). The white arrow (c, f) showed the lysed cells, and no septa in present cells represented any cell division.

The presence of EDTA in the Crystal Violet uptake assay could cause changes in permeability of outer membrane layer of bacterial cell wall, since the EDTA is a chelating agent. Therefore, the EDTA was used for external positive control whereas Vancomycin was used as an internal positive control in this test.¹⁵ In the present study, the membrane layer of MRSA ATCC 43300 cell wall represented a significant decrease in crystal violet uptake after treatment with SUK 25 extracts, as seen by when compared to normal MRSA ATCC 43300. In contrast, both

positive controls portrayed an increased uptake of crystal violet dye after the treatment. The difference in uptake of Crystal Violet from the bacterial cell wall after treatment compared to normal was due to defective cell wall, as there were permeability changes.¹¹ The reduction in Crystal Violet uptake could probably be attributed to enlargement of the treated cell and atypical cells presented in SUK 25 treatment cell (Figure 5 (b, e)). Hence, the permeability and structure of the cell wall membrane layer changed compared to normal cells (Figure 5 (a, d)). Sianglum reported that MRSA cells represented atypical and irregular shapes after treated with Rhodomymrtone extracts.²⁸ This is in accordance with the claim of Basri that the irregular shape of the MRSA cells treated with the combination of gall extract from *Quercus infectoria* with vancomycin was due to loss of cellular content from the cells.³

The release of UV absorbing materials 260 nm and 280 nm from MRSA ATCC 43300 after absorbing materials at 260 nm was 0.073 ± 0.02 and at 280 nm, it was 0.074 ± 0.080 , respectively; after treatment with 7.8 $\mu\text{g}/\text{mL}$ SUK 25 extracts. Meanwhile, the UV absorbing materials at 260 nm and 280 nm from the cells incubated with Vancomycin at 6.25 $\mu\text{g}/\text{mL}$ were 0.036 ± 0.0001 . For the untreated cells, very few UV absorbing materials at 260 nm and 280 nm were detected, which were 0.004 ± 0.001 and 0.026 ± 0.002 , respectively. Statistical analysis of one-way ANOVA for both tests and post-hoc Turkey tests showed that these data were significant to each other (p -value < 0.05). There were no cells grown on MHA overnight at 37 °C from the samples treated with 6.25 $\mu\text{g}/\text{mL}$ Vancomycin and 7.8 $\mu\text{g}/\text{mL}$ SUK 25 extracts, while the untreated cells grown on MHA at 1.65×10^4 CFU/mL were detected by colony count after incubation at 37 °C and tenfold dilution was performed.

The UV absorbing materials A_{260} were defined as the capability of nucleic acids such as DNA and RNA to absorb UV light at 260 nm wavelength.⁶ Whereas the UV absorbing materials A_{280} were known as protein molecules capable of absorbing the UV light at 280 nm wavelength.²³ The release of UV absorbing materials A_{260} and A_{280} (Figure 4) showed the effect of SUK 25 extracts against MRSA ATCC 43300. Oonmetta-aree proved that these UV absorbing materials were released due to antimicrobial action of ethanolic extracts from *Alpinia galangal* Linn.²⁵ In addition, Figure 5 exhibited that the action of SUK 25 extracts against MRSA ATCC 43300 caused the rupture of cytoplasmic membrane, which in turn led to cells' death.

The release of UV absorbing materials (such as nucleic acid and amino acid) are as a result of antibiotic actions, such as nisin and epidermin that caused cytoplasmic burst in Gram positive bacterial cells while the action of Vancomycin against MRSA ATCC 43300 only caused the lysis of the bacterial cells after 3 h treatment (Figure 5(c, f)) and thus caused the release of the UV absorbing materials, A_{260} and A_{280} (Figure 4).⁵

CONCLUSION

Conclusively, the SUK 25 extracts against MRSA ATCC 43300 brought about the internal changes in cells.

Therefore, permeability of cells changed to decrease the Crystal Violet uptake, decrease shape of cell changes, and also cause the lysis of cells. Consequently, this potential extracts have proved medicinal value to be purified and developed as antibiotic, especially as treatment against MRSA infection.

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