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Antibacterial Activity of Tram Tron *Syzygium Glomerulatum* Extract against Methicillin-Resistant *Staphylococcus Aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has been increasingly spreading in hospitals. The discovery of a new antibiotic is increasingly difficult due to its huge amount of time and money requirements. Medicinal herbs were used in the treatment of infectious diseases for a long time. Binh Duong province has a rich source of indigenous plants. Traditional remedies of these plants have been used in treating infectious diseases. However, there are very few studies on natural antibiotics in the area. The main purpose of this study is to investigate antibacterial activity of *Syzygium glomerulatum* collected in Binh Duong on methicillin-resistant *Staphylococcus aureus*. Agar disc diffusion and micro dilution methods were performed to determine antibacterial activity against MRSA of *Syzygium glomerulatum* and its ability to combine with vancomycin antibiotic. In addition, the cell toxicity of *Syzygium glomerulatum* was determined by SRB (Sulforhodamine B). Results showed that the minimum inhibitory concentration (MIC) of *Syzygiumit glomerulatum* was 2.85714 µg/mL. Meanwhile, at the concentration of 35 times MIC, *Syzygium glomerulatum* did not give toxicity on hepatic cancer cells G2 and fibroblast cells. The FIC index of combination of *Syzygium glomerulatum* and vancomycin was 0.53544, indicated a high probability of partial combination of *Syzygium glomerulatum* and vancomycin. Furthermore, using disc diffusion method and measuring their zone of inhibition also indicated the possibility of combination of *Syzygium glomerulatum* and vancomycin against MRSA strains.

1. Introduction

Antibiotic resistance is one of the most important public health concerns worldwide (Franklyne et al., 2019). Addition, *Staphylococcus aureus* was a gram-positive, facultatively anaerobic bacteria which was the leading cause of inflammatory diseases, pimples and scabies in humans. All over the world, 20 % of the population was permanent carrier of *S.aureus*. Currently, the bacteria had developed a special antibiotic-resistant strain methicillin-resistant *Staphylococcus aureus* (MRSA). According to the American Medical Association (AMA), there were at least 94,360 cases of MRSA and 18,650 deaths since 2005 (Hadler et al., 2012). MRSA infections currently occurred exclusively in hospitals. It remained a great challenge to discover new antibiotic because it required a lot of time and money. There were two mechanisms of MRSA antimicrobial resistance: semisynthetic beta-lactamase–resistant penicillin and acquisition of a gene that encodes a shomologue of the penicillin-binding protein (PBPs) (Klevens et al., 2007). Previously, vancomycin antibiotic, subgroup of Glycopeptide, had been considered drug of choice for treating and preventing severe MRSA infections (Courvalin, 2006). Vancomycin prevented enzyme transpeptidase from making cross-linking of the peptidoglycan layer. By doing so, vancomycin prevented of cell-wall biosynthesis of bacteria. But emergence of vancomycin-intermediate resistant (VISA) and vancomycin-resistant (VRSA) strains has been documented due to its overuse (Devi et al., 2015). *Syzygium glomerulatum* was an indigenous plant in Thu Dau Mot City, Binh Duong Province that belongs to Kingdom: Plantae, Clade: Angiosperms, Order: Myrtales, Family: Myrtaceae, Tribe: Syzygieae, Genus: *Syzygium*, Species: *Syzygium glomerulatum*. This plant was collected and categorized at Ecology and Evolutionary Biology Department, University of Science, Vietnam National University HCMC. All over the world, there were some studies on *S. aureus* antibacterial activity of species of *Syzygium* genus (Chikowe et al., 2013). Myrtaceae family is one of the largest and most important of Viet Nam. In folk medicine the leaves of many species of this family are used in the most different diseases (Moniz

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et al., 2019). People in Binh Duong Province usually used *Syzygium glomerulatum* in traditional remedies to treat bacterial infection diseases. However, there were very few studies on natural antibiotics in the area. Therefore, the main purpose of this study was to investigate the antibacterial activity of *Syzygium glomerulatum* on MRSA. Moreover, the abuse of antibiotics might lead to resistance phenomenon, especially MRSA. On the other hand, the number of newly developed antibiotics has decreased dramatically in recent years. Instead, a reexamination of traditional medicines has become more common and has already provided several new antibiotics. Traditional medicine plants are likely to provide further new antibiotics in the future. However, the use of plant extracts or pure natural compounds in combination with conventional antibiotics may hold greater promise for rapidly providing affordable treatment options (Cheesman et al., 2017). Therefore, it was necessary to study the combination of *Syzygium glomerulatum* and vancomycin to reduce the usage of antibiotics and antibiotic resistance. So, the main aim of this study was collecting and investigating the antibacterial activity of *Syzygium glomerulatum* on MRSA, as well as to determine combined ability of *Syzygium glomerulatum* ethanol (SCTT E+ D) and vancomycin.

2. Methods and materials

2.1 Preparation of materials

Methicillin-sensitive *Staphylococcus aureus* strain (MSSA) ATCC 6538 used as a control strain, Methicillin-resistance *Staphylococcus aureus* strain (MRSA) ATCC 33591 provided by ATCC, USA. Both strains, were cultured on Tryton Soy Broth (Himedia, India) and Tryton Soy agar (Himedia, India), incubated at 37 °C for 18 – 24 h in an incubator (DaiHan, Korea).

Fresh biomass of *Syzygium glomerulatum* (leaves and young branches) was collected from March to August 2018, in Thu Dau Mot City, Binh Duong Province, 11000’33.02" North; 106039’00.37" East at an altitude of 11m ± 3m, pressure of 0.999 atm. The biomass was homogenized in distilled water to remove dirt, then well grinded using a 0.074-0.25 mm mesh size blender FY 130 at 34,000 rpm. The powder was then deeply soaked in ethanol at 50 °C, and the mixture was then shaken at 100 rpm by Ika KS 4000i machine (Germany). The mixture was collected after every 24 h, and extract completed after one week. The ratio of ethanol extraction and dry plant biomass was: 2 L in 50 g, repeated 3 times. The obtained solution filtered through Whatman 110 mm diameter filter paper with a filter size of 20-25 µm. The filtrate was then concentrated by EYELA N1110 (Japan) rotary evaporator at 50 °C, within 2-3 h. The residue extract then placed in drying oven at 50 °C and weighed to a constant weight. The crude extract of *Syzygium glomerulatum* weighed by analytical balance Sartorius (Germany) and dissolved in 10 % dimethyl sulphoxide (DMSO) (Merck, Germany). The soluble solution was then centrifuged by Hermle cold centrifuge (Germany) at 10,000 rpm speed in 5 min. This solution then filtered through 0.22 µm filter paper and determined concentration by concentrated 1 mL of solution to a constant mass in DaiHan drying oven (Korea) at 50 °C. The weight determined mass of soluble in 1 mL of solution. On the other hand, DMSO is negative control (not present in this paper). Testing is carried to check 20 % DMSO for MSSA and MRSA. The results show that 20 % DMSO does not affect the growth of bacteria.

2.2 Antibacterial susceptibility test (AST)

Muller-Hinton Agar (Neogen, USA) was the standard medium for investigating the antimicrobial ability of extracts. Kirby-Bauer method was applied, each plate was supplemented with 100 µL of 10^6 CFU/mL concentration standardized inoculum suspension, 3 wells were created with 4 mm diameter. A total of 100 µL of vancomycin (MIC 9.766 µg/mL), 100 µL of the crude extract of *Syzygium glomerulatum (*5.255 µg/mL), 100 µL of the crude extract of *Syzygium glomerulatum* (10.51 µg/mL) was then added to well No. 1, 2, 3. Plates incubated at 37 °C for 18 h. The experiments repeated 3 times. Size of inhibition zones in millimeter (mm) was measured after 18 h (Bauer et al., 1966), CLSI documents were used as references to validate results (Wayne, 2010).

2.3 Combination ability of *Syzygium glomerulatum* ethanol (SCTT E+ D) and vancomycin

The minimum inhibitory concentration (MIC) is defined as the lowest concentration which inhibits visible growth of bacterium following 18 h incubation. The MIC was determined by broth microdilution method. 96-well microplates were used, each well contained (1) 10 µL of 10^6 CFU/mL bacterial suspension, (2) 100 µL range of SCTT E+D concentrations in doubling dilution steps down from 10.51 µg/mL to 0,082 µg/mL, (3) 100 µL Muller Hinton Broth medium (MHB). The two control wells were used, one negative control contained only MHB media, and one positive control contained MHB and bacteria. The plate was then aerobically incubated at 37 °C for 18 h. The lowest concentration of the dilution series that inhibited the growth of bacteria was identified as MIC (Wayne, 2010). DMSO is negative control (not present in this paper), but we carried out to check 20 % DMSO for MSSA and MRSA. The results show that 20 % DMSO does not affect the growth of

bacteria. The synergistic interactions between the plant extracts and antibiotics against both MRSA and MSSA were quantified by The fractional inhibitory concentration (FIC) index. A series 2-fold dilution was used for both *Syzygium glomerulatum* ethanol (SCTT E+ D) (from 12 to 0.09375 µg/mL) and vancomycin (from 9.766 to 0.305 µg/mL). The mixing volume ratio was 1:1. The FIC index was calculated with Eq(1).

$$FIC\ Index\ = \frac{MIC\ of\ antibiotic\ in\ combination}{MIC\ of\ antibiotic\ alone} + \frac{MIC\ of\ plant\ extract\ in\ combination}{MIC\ of\ plant\ extract\ alone} \quad (1)$$

Where FICI <0.5 is synergistic, FICI from 0.5 to 0.75 is partially synergistic, FICI from 0.76 to 1 is additive effect and FICI >2 is antobonistic effect (Kuok et al., 2017).

A modification of Kirby-Bauer method was applied. Three wells were created in each MHA plate. The first well contained 100 µL of extract (A). The second well contained 100 µL of vancomycin (B). The third well contained the mixing of 100 µL extract (A) and 100 µL vancomycin (B). Then, plates incubated at 37 °C for 18 h. The experiments repeated 3 times. Size of inhibition zones in millimeter (mm) was measured after 18 h (Bauer et al., 1966), CLSI documents were used as references to validate results (Wayne, 2010) .

Diameter of inhibition zone of combination (D) was calculated though radius of inhibition zone of extract A (rA) (mm) and radius of inhibition zone of extract B (rB) (mm). If diameter of extended inhibition zone (C) of well No. 3 (mm) is larger than diameter of inhibition zone of combination (D) or they are equals, there is synergistic effect. In contrast, If C of well No. 3 (mm) is smaller than D, they are no difference (Aquil et al., 2006).

2.4 **The cell toxicity of Syzygium glomerulatum**

Sulforhodamine B (SRB) was a simple and sensitive method for testing toxicity of a drug to cells. SRB is a negatively charged dye. The negative charged of SRB would bind to the positively charged portions of the protein. The amount of remaining dye after washing indicated the total protein of the cells. In the study, cells were fixed, washed and dyed with SRB. The binding solution was crystal-clear with pink color. The optical density of this solution correlated with quantity of total protein of total number of cells. The difference between number of cells and control samples indicated the toxicity of drug tested. Hepatic cancer cells G2 and fibroblast cells provided by ATCC (USA) were cultured in E'MEM medium supplemented with L-glutamine (2 mM), HEPES (20 mM), amphotericin B (0,025 µg/mL), penicillin G (100 UI/mL), streptomycin (100 µg/mL), 10 % (v/v) fetal bovine serum (FBS) and incubated at 37 °C, 5 % CO_2. Cell was cultured in 96-well mircoplate, each well contained 10^4 cells. After 24 h, it was added to the culture medium and incubated for 48 h. The total protein by cold Trichloroacetic acid 50 % (Sigma) and dyed with Sulforhodamine B 0.2 % (Sigma) were fixed. Completed assay plates measured by ELISA at two wavelengths were 492 nm and 620 nm.

The percentage of growth inhibition (Inh %) was calculated according to Eq(2):

$$Inh\ \% = (1 - [\frac{ODt}{ODc}]\ x\ 100)\ \% \quad (2)$$

In which ODt and ODc are the optical density value of the test sample and the control sample. Camptothecin (Calbiochem) was used as a positive control. Prism software was used to identify IC50 using non-linear regression method multivariable and R2>0.9 (Nguyen and Ho Huynh, 2016). Experiments repeated 3 times, and results were presented by mean and ± standard deviation and data were analyzed using STATGRAPHICS Centurion XV software (Statgraphics Centurion, 2006).

3. Results and discussion

3.1 Antibacterial susceptibility test (AST) using agar diffusion method

The results indicated that with vancomycin at double concentration, MIC = 9.766 µg/mL, antibacterial activity was still low with a size of inhibition zone of MSSA and MRSA were 5 mm and 0 mm (Figure 1). Meanwhile, with *Syzygium glomerulatum* extract at a concentration of 10.51 µg/mL, inhibition zone was 10 mm for MSSA and 18 mm for MRSA. Furthermore, we investigated the AST of *Syzygium glomerulatum* extract with MIC at 6 µg/mL or 0.6 µg on disk (similar to vancomycin MIC). The inhibition zone of MSSA and MRSA were 10mm and 8mm. This result indicated the similarity of antibacterial activity of *Syzygium glomerulatum* extract and vancomycin, although the compounds from *Syzygium glomerulatum* were not isolated. Merradi Manel et al reported the antibacterial activity of other plants extract, at 1mg/mL concentration the inhibition zone was 14mm (Manel et al., 2018). With the concentration at 9.766 µg/mL, the antibacterial activity against MRSA of *Syzygium glomerulatum* collected from Binh Duong was 166 times higher than other plants extract reported by Merradi Manel et al. (in both MSSA and MRSA strains). Addition, anti-MRSA activity of *Syzygium glomerulatum* is higher than Ethanol Extract of *Ulmus pumila* Root Bark, of which antibacterial activity has the inhibition zone was 12 mm at concentration of 5mg/mL (You et al., 2013). The antibacterial activity of

Syzygium glomerulatum in *E. Coli* has not seen in previous study. A hypothesis has been made that *Syzygium glomerulatum*l can affect the cell wall synthesis, similar to mechanism of vancomycin.

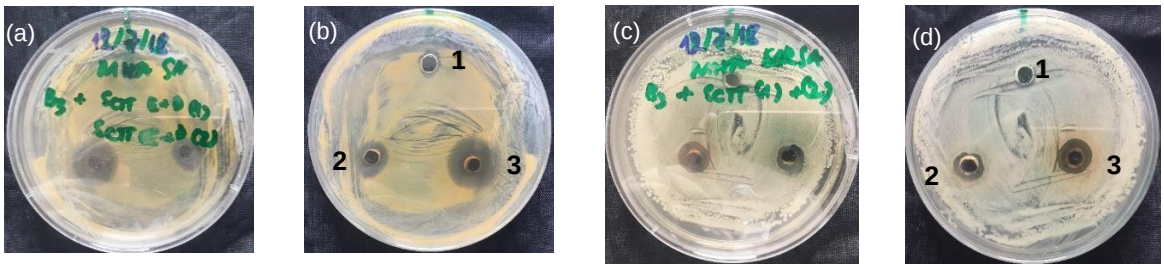

Figure 1: AST of MSSA ATCC 6538 using agar plate diffusion method, (a) front end (b) backed of plate, AST of MRSA ATCC 33591 using agar plate diffusion method, (c) front end (d) backend of plate, where sample 1 is MIC B3 vancomycin 0.9766 µg, sample 2 is Syzygium glomerulatuml 0.6 µg, sample 3 is Syzygium glomerulatuml 1.051 µg

On the other hand, the use of vancomycin is still very common; however, inadequate doses and prolonged therapy pose a risk of increasing minimum inhibitory concentrations (MICs), resulting in subtherapeutic levels, treatment failures and toxicity. So, further studies should be conducted to optimize the administration of vancomycin, monitoring treatments from the beginning in order to ensure safe and effective use of the drug (Bruniera et al., 2015). Therefore, we investigated the combination ability of Syzygium glomerulatuml and vancomycin using two methods: broth microdilution and agar plate diffusion. These results were presented in Table 1 and Table 2

3.2 Identification of *Syzygium glomerulatum* MIC and the combination ability with vancomycin

The MIC of vancomycin of both MSSA ATCC 6538 and MRSA ATCC 33591 strains using dilution method in 96-well microplates were 4.65 µg/mL. Based on Table 1, FIC index of the combination of *Syzygium glomerulatum* and vancomycin at well A6B3 was 0.62500. Concentration of *Syzygium glomerulatum* SCTT (E+D) was 0.3128 µg/mL compared to *Syzygium glomerulatum* MIC 2.5 µg/mL, in addition, concentration of vancomycin was 2.32515 µg/mL compared to vancomycin MIC 4.65 µg/mL. Similarly, combination FIC index of well A5B4 was 0.53544, and well A5B3 was 0.75000. Following the calculation formula of Kuok et al. (2017) the results indicated that *Syzygium glomerulatum* and vancomycin have a partial combination, which means there was synergy between *Syzygium glomerulatum* and vancomycin.

Table 1: FIC index of Syzygium glomerulatum SCTT E+D and vancomycin

Solutions	A3B3	A3B4	A4B3	A5B3	A5B4	A6B3
Concentration of vancomycin	2.32515	1.16257	2.32515	2.32515	1.16257	2.32515
Concentration of *Syzygium glomerulatum*	2.50238	2.50238	1.25119	0.62560	0.71429	0.31280
FIC plant extract	1.00000	1.00000	0.50000	0.25000	0.28544	0.12500
FIC vancomycin	0.50000	0.25000	0.50000	0.50000	0.25000	0.50000
FIC index	1.50000	1.25000	1.00000	0.75000	0.53544	0.62500

However, this synergy was only partially. This could be explained by there were many components of organic compounds without antibacterial activity in ethanol extract, which possibly inhibited the combination activity of compounds against MRSA in plant extract and even combination activity with vancomycin. Therefore, another experiment was conducted to determine the combination ability of *Syzygium glomerulatum* SCTT E + D and vancomycin, considered as a control method for dilution method on 96-well microplate. The experiment applied agar plate diffusion method and used a radius of inhibition zone for calculation following formula of Aqil et al. (2006). The concentration of SCTT in the study was 10.51 µg/mL, and concentration of vancomycin was 1,250 µg/mL (100 times higher than concentration of SCTT). This result indicated that *Syzygium glomerulatum* had strong antibacterial activity against MRSA. Table 2 and Figure 2 showed interactive combination of *Syzygium glomerulatum* SCTT E+D and vancomycin by using agar disc diffusion method. In table 2, radius of inhibition zone of *Syzygium glomerulatum* SCTT E+D was rA, radius of inhibition zone of vancomycin was rB, radius of inhibition zone of combination was D and diameter of extended inhibition zone

was C. Two concentrations of vancomycin were used in experiment, 1,250 µg/mL and 625 µg/mL, the concentration of *Syzygium glomerulatum* SCTT E+D was 10.51 µg/mL in two plates.

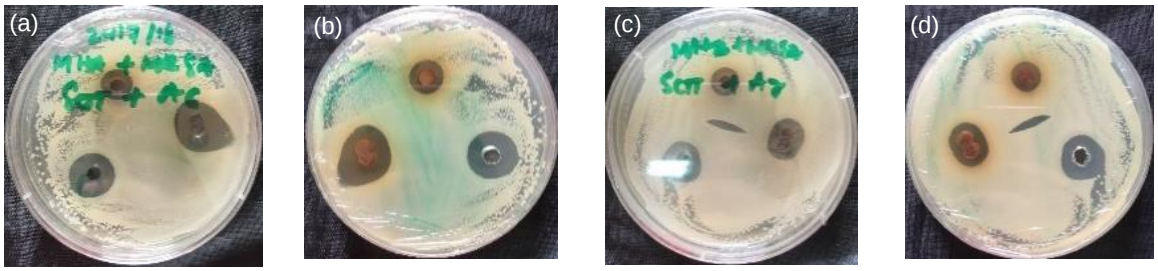

Figure 2: Interactive combination of (a) Syzygium glomerulatum SCTT E+D and vancomycin (A6) by using agar plate diffusion method,(b) backside of plate, (c) Syzygium glomerulatum SCTT E+D and vancomycin (A7), (d)backside of plate

Table 2: Interactive combination of Syzygium glomerulatum SCTT E+D and vancomycin

Syzygium glomerulatum SCTT (E+D) A	Radius of inhibition zone A	Vancomycin B	Radius B	Radius of combination inhibition zone D=rA+rB	Diameter of extended inhibition zone C	Synergistic
SCTT	5.5	A6	7.5	13	16	+
SCTT	4.5	A7	6.5	11	12	+

The results showed that size of extended inhibition zone was bigger than the radius of combination inhibition zone, specifically, $C_{SCTT\text{-}A6}$ 16 mm > $D_{SCTT\text{-}A6}$ 13 mm and $C_{SCTT\text{-}A7}$ 12 mm > $D_{SCTT\text{-}A7}$ 11 mm. This result demonstrated there was a combination of *Syzygium glomerulatum* and vancomycin in the antibacterial activity against MRSA. *Syzygium glomerulatum* may act by inhibiting bacterial cell wall synthesis similar to vancomycin. Moreover, this result was consistent with investigation of the combination ability using dilution method on 96-well microplate. Interesting, the results presented that partial combination of *Syzygium glomerulatum* and vancomycin against MRSA and combinational therapy is preferred over monotherapy in multiple life-threatening infectious diseases due to its ability to target multiple facets of disease and to curb resistance (WHO, 2016). On the other hand, Articles authored by researchers from developing countries were poorly represented in the findings (Salim et al., 2018), however, in this research the first time *Syzygium glomerulatum* extract is presented anti-MRSA activity.

3.3 **The cell toxicity of Syzygium glomerulatum**

The result showed that at the concentration of 100 µg/mL (10 times higher than the concentration of Syzygium glomerulatum against MRSA 10.51 µg/mL), there were no toxicity on hepatic cancer cells G2 and fibroblast cells. The average proportion of dead cells was 32.81 % on hepatic cancer cells and 37.37 % on fibroblast cells (shown in Table 3).

Table 3: Proportion (%) of cell toxicity on hepatic cancer cells G2 and fibroblast cells at concentration 100 µg/mL using SRB method

Type of cell	Sample	Proportion of cell toxicity (%)			
		1st time	2nd time	3rd time	Median ± Standard Deviation
Hep G2	SCTTED	36.20	35.60	26.62	32.81 ± 5.36
Fibroblast cell	SCTTED	36.17	36.40	39.53	37.37 ± 1.88

4. Conclusion

The study determined that *Syzygium glomerulatum* SCTT E+D at concentration of 10.51 µg/mL and 6 µg/mL showed strong antibacterial activity against MRSA similar to vancomycin at concentration of 9.766 µg/mL. Moreover, partial combination of *Syzygium glomerulatum* and vancomycin against MRSA was investigated by using dilution method on 96-well microplate and agar disc diffusion methods. Because the target of vancomycin action was cell wall which could be the target of *Syzygium glomerulatum*. In addition, at high concentrations of *Syzygium glomerulatum* extract, it did not show toxicity on fibroblast cells and hepatic

cancer cells Hep G2. Therefore, further studies to examine the specifc effects of the components present in *S. glomerulatum* and identify the target of antibacterial substances against MRSA are necessary.

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