CHAPTER 3

Antibacterial Activity from Thai Medicinal Plants

3.1 Introduction

Skin diseases can be caused by a variety of the microbes and the most infections are usually caused by bacteria. The most common bacterial species which are infected human skin are *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pyogenes* (Group A hemolytic streptococcus) and *Micrococcus* sp. Broad panels of microbial pathogens are associated with various skin infections including wound infections, furuncles, carbuncles, abscesses, impetigo and erysipelas (Oumeish *et al.*, 2001; Sadick, 2002). However, the treatment of bacterial infections is increasingly complicated by the ability of bacteria to develop resistance to antimicrobial agents. Therefore, it is important to find new antibacterial compounds from natural sources that are effective for treatment of pathogenic bacteria.

S. aureus is one of the bacterium that belongs to family *Staphylococcaceae*. Althought, *S. aureus* is described in normal microflora found in skin, intestine, upper respiratory tract and vagina but this organism may become pathogenic. *S. aureus* cause a wide range of diseases from minor skin and soft tissue infection such as impetigo, cellulitis, folliculitis and furuncle to severe diseases such as pneumonia, meningitis, osteomyelitis, endocartitis, toxic shock syndrome (TSS), bacteremia and sepsis (Stryjewski and Chambers, 2008). However, the treatment of *S. aureus* infection is quiet difficult because this become to resistance to more than one classes of antibiotic especially methicillin and other member of β-lactam family (Gomes *et al.*, 2006; Pantosti *et al.*, 2007). Many drugs were used for treatment of this bacterial infection but the use may become limit due to the rapid development of drug resistance after longterm therapy. The most common resistance is methicillin which represented nosocomial infection in community (CA-MRSA) and hospital (HA-MRSA). There are

various tools to identify MRSA such as biochemical identification, disc diffusion susceptibility testing, automated method including the Vitex (bioMerieux, France) and Microscan (Dade Microscan, West Sacramento, USA) and other commercial methods such as latex agglutination assay kits (Brown *et al.*, 2005). However, the detection and identification of MRSA by these assays is time consuming and high cost. Therefore, the molecular identification is another choice for rapid detection of MRSA based on the amplification of *mecA* gene, which encode the modified penicillin binding protein (PBP2a). The expression of PBP2a was controlled by two regulatory genes on *mec* DNA, *mecI* and *mecR1* located upstream, which encoded *mecA* repressor and transducer protein, respectively (Song *et al.*, 1987). Thus, the deletion or mutation on *mec* DNA might be result to the level of resistance in these bacteria.

For a long period of time, plants were used medicinally in different countries and had many potent and powerful biological activities for maintenance of human health. In recent years, a number of studies have been conducted in different countries to prove such efficacy of plants against various microorganisms. Rao *et al*. (2012) reported the antibacteria and anti-inflammatory of various extracts of *Rauvolfia tetraphylla* including hydro-alcoholic, methanolic, chloroform and hexane extracts against four Gram positive bacteria; *St. pneumonia, S. aureus, B. cereus* and *B. pumilis* and four Gram negative bacteria; *E. coli, Enterobacter aerogenes, Ps. aeruginosa* and *St. marienensis.* Moreover, a total of 58 traditional medicines were screened for antibacterial activity against the growth of various microorganisms including *Aspergillus fumigates*, *Candida albicans*, *Acenetobacter baumannii*, *Ps. aeruginosa* and *S. aureus.* The antimicrobial activities indicated that 15 plant extracts showed antifungal activity, 23 plant extracts showed antibacterial activity and only 8 plant extracts showed both antifungal and antibacterial activity. The extracts of *Eucommia ulmoides, Polygonum cuspidatum, Poria cocos* and *Uncaria rhyncophylla* could exhibit both bacterial and fungal strains (Zhang *et al.*, 2013).

In Thailand, several medicinal plants have been used as antibacterial substances. Many Thai plants are sources of many beneficial compounds against microbial infection. Chamuangone, a new polyprenylated benzophenone from *Garcinia cowa* could exhibit satisfactory antibacterial activity against *St. pyogenes, St. viridians,*

H. pylori, *S. aureus*, *B. subtilis* and *Enterococcus* sp. (Sakunpak and Panichayupakaranant, 2012). The prenylated xanthone, α-mangostin from *Garcinia mangostana* have also isolated and this compound could inhibit the growth of *S. aureus*, *S. epidermidis* and MRSA (Chomnawang *et al.*, 2009). Therefore, medicinal plants were sources of many beneficial compounds that used as remedies for treatment of infectious diseases in many tropical countries against microbial infection. The aim of this study was to detect *mecA* gene from the resistant bacteria polymerase chain reaction and nucleotide mutation of *mecA* was determined. Moreover, twenty two medicinal plants were screened for their antibacterial activity by disc diffusion and broth dilution methods. The partial purified of the active plants also investigated by partition and chromatography techniques. In addition, the plant which had the highest antibacterial activity was further evaluated antibacterial activity on the bacterial cell morphology alteration using scanning electron microscope (SEM). The effect of active plants on the bacterial gene that involved resistant level, biofilm formation and toxin production were also investigated.

3.2 Materials and Methods

3.2.1 Detection of methicillin resistance of *Staphylococcus aureus*

Macroscopic and microscopic morphological characterization of *S. aureus* was observed and described. Biochemical characterizations including catalase, coagulase, glucose fermentation and mannitol fermentation tests were tested.

Antibiotic susceptibility testing of *S. aureus* was performed by disc diffusion method on Mueller Hinton agar (MHA). A suspension of each isolate was adjusted to 1x 10^8 CFU/ml by comparing to McFarland standard No. 0.5. Then, the culture was swabbed onto MHA and commercial antibiotic disc included oxacillin (1 μg) and cefoxitin (30 μg) were applied in each plate and incubated at 37°C for 24 hours. After incubating, inhibition zone around the disc was measured. The antibiotic resistant level was determined and compared with standard breakpoint values (Table 3.1).

Table 3.1 Interpretive standards breakpoint values for *Staphylococcus* spp. *mecA*-mediated resistance (CLSI, 2012)

Antimicrobial Agents	Disc Content	Zone Diameter (mm)			MIC Interpretive Standard (µg/ml)		
		R	I	S	R	I	S
Oxacillin	1 ug	≤ 10	11-12	≥ 13	≥4	-	≤2
Cefoxitin	30 ug	≤ 21	-	≥ 22	≥8		≤4

R= resistant; I = intermediate; S = sensitive

3.2.2 Detection of *mecA* gene by PCR

1) Isolation of genomic DNA

Total bacterial DNA was extracted using the modified phenol chloroform extraction method (Salloum *et al*., 2002). Briefly, the 5 ml bacterial culture was precipitated by centrifugation and the cells were resuspended in 560 µl TE buffer, and lysed by 30 µl of SDS (10%). After that, 3 µl of proteinase K (20 mg/ml) was added and incubated at 37°C for 1 hour. Then, 100 µl of NaCl (5 M) was added and incubated at 65°C for 10 minutes. To extract DNA, an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and centrifuged at 14,000 rpm for 10 minutes. Subsequently, the upper layer was collected, equal volume of phenol: chloroform (50: 50) was added, and centrifuged at 14,000 rpm for 10 minutes. The upper layer was transferred to new tube. The DNA in supernatant was precipitated by 95% ethanol overnight and the pellet was washed with 70% ethanol and resuspended in 50 ml of TE buffer. The DNA quality and quantity were determined using agarose gel electrophoresis.

2) Polymerase chain reaction (PCR) of *mecA* gene and sequencing

Polymerase chain reaction was used to amplify *mecA* gene with amplicon size 922 bp by specific forward primer (GGCTATCGTGTCACAATCG) and reverse primer (GTTCTGCAGTACCGGATTTG). Each reaction contained 10 ng template DNA, 0.05 mM dNTPs, 0.05U Taq polymerase, 0.3 µM primer and 1x buffer with $MgCl_2$ in a total volume of 50 µl. The PCR amplification cycle was 30 seconds at 94°C, then 30 cycles

of denaturation at 94°C for 45 seconds, annealing at 63°C for 45 seconds, extension at 72°C for 1 minute, and a final extension of at 72°C for 5 minutes. A sample of 5 µl from each reaction was analyzed by agarose gel electrophoresis. The PCR products were sent to First BASE Laboratories Sdn Bhd (Malaysia) for sequencing (Kaewkod, 2011).

3.2.3 Medicinal plants and extraction procedure

Medicinal plants during March - July, 2010 used in this study were purchased from Lampang Herb Conservation, Thailand. Plant materials were collected and then washed with tap water, reduced their size and dried at 55 °C for 48-72 hours. The dried powder of plant material was extracted with two solvents including distilled water and 95% ethanol with ratio of 1:10 (w/v). First, the ground plant (250 g) was extracted with distilled water for 3 hours at 45ºC. Another portion (250 g) was macerated with 95% ethanol for 72 hours with frequent agitation (Houghton and Raman, 1998). The plant extracts were filtered with Whatman No.1 and then concentrated by evaporation at 45 ºC under reduced pressure in rotary evaporator (Buchi^{TM}) and lyophilized to obtain the crude extract. After that, the crude extract was dissolved in dimethylsulfoxide (DMSO) to give concentration of 500 mg/ml before testing.

3.2.4 Investigation of antibacterial activity

1) Bacteria

The tested bacterial strains, *Escherichia coli* O157:H7 DMST12743 and *Propionibacterium acnes* DMST14916 were obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, methicillin resistant *S. aureus* (MRSA) isolate number 32, 43, 50, 64, 65, 66, 67, 72, 80, and 82 and *Pseudomonas aeruginosa* were obtained from Microbiology section, Department of Medical Technology, Faculty of Associated Medical Science, Chiang Mai University, Chiang Mai, Thailand.

2) Agar disc diffusion assay

The antibacterial activity was performed using agar disc diffusion method (Collins *et al.,* 1995). The tested bacteria were cultured in Mueller-Hinton Broth (MHB) at 37°C for 18-24 hours. Turbidity of the bacterial culture was adjusted with medium comparing to Mc Farland standard No. 0.5 to obtain bacterial cells approximately 1.0 x 10^8 CFU/ml. The culture of bacteria was swabbed on Mueller-Hinton Agar (MHA). Then, a sterile paper disc (Macherey-Nagel®) with 6 mm diameter was soaked in 500 mg/ml of each crude plant extracts and the discs were placed on the agar compared with DMSO which was used as a solvent control. These plates were incubated at 37°C for 24 hours.

P. acnes was cultured in Brain Heart Infusion Broth (BHI) at 37°C for 72 hours and adjusted to approximately 1.0 x 10^8 CFU/ml. The extracts were also tested against *P. acnes* with the procedure mentioned above and the plates were incubated at 37°C for 72 hours under anaerobic condition. Diameters of the inhibition zone around the discs were measured to access antibacterial activity. All experiments were performed in triplicates and the mean of inhibition zone was calculated.

3) Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The minimum inhibitory concentrations (MIC) were measured by broth dilution method (Collins *et al.,* 1995). Two-fold serial dilutions of crude plant extracts were prepared in 0.5 ml MHB or BHI before inoculating with 0.5 ml of bacterial culture as shown in Table 3.2. The test tubes were incubated at 37°C, 72 hours under anaerobic condition for *P. acnes* and incubated at 37°C, 24 hours for other bacterial strains. MIC was recorded as the lowest concentration of crude extracts, which bacterial growth was inhibited. For MBC evaluation, the tubes with no growth were streak plated on MHA or BHI agar and incubated under above condition for different bacterial strains. The MBC was recorded as the lowest concentration showing no visible growth of bacterial strains.

Table 3.2 Amount of the samples in test tubes for determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

sample	volume (ml) in each tube									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
extract or antibiotic	1.0	1.0 →	1.0 →	1.0 →	1.0 →	1.0 →	1.0 →	1.0	-	-
medium	-	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	2.0
tested bacteria	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	-

→ = Two fold serial dilution

4) Time killing assay

Time killing assay was carried out using modification method of Rukholm *et al.* (2006). Bacterial culture was adjusted a turbidity to approximately 1.0 x 10^8 CFU/ml. Then, each culture was treated with plant extracts at MIC concentrations and incubated at 37°C. Then, 0.1 ml of every dilution was spread on Brain Heart Infusion Agar (BHA) at 0, 6, 12, 18, 24, 30, 36, 48, 54, 60, 66 and 72 hours for *P. acnes* and spread on Nutrient Agar (NA) after 0, 2, 4, 6, 8, 10, 12 and 24 hours for other bacteria and then, incubated at 37 °C for 24 hours. The CFU for each strain at different time points were counted after 24 hours. Plates with 30-300 colonies were used for colony counts. Then, the percentage of bacterial inhibition by medicinal plant extracts was calculated by following equation.

$$\text{Percentage inhibition (\%)} = \frac{\text{untreated bacteria - treated bacteria}}{\text{untreated bacteria}} \text{ X } 100$$

3.2.5 Effect of plant extracts on bacterial cell morphology using scanning electron microscopy (SEM)

Scanning electron microscopy was performed by method of Yenugu *et al.* (2004) and Agizzio *et al.* (2006) with slightly modification. In brief, each bacterial species was incubated with plant extracts with different concentration at 1MIC, 2MIC and 4MIC, respectively. After incubating at 37 °C for 24 hours, the suspension was washed with phosphate buffer saline (PBS), pH 7.2 and filtrated through cellulose acetate membrane 0.2 mm (Sartorius®). The specimens were fixed overnight at 4 °C with 2.5% (v/v)

lutaraldehyde. Subsequently, the bacterial cells were rinsed three times with PBS, incubated for 3 hours at room temperature with 1.0% osmium tetroxide (OsO_4), which diluted in PBS and rinsed with distilled water. After that, the bacterial cells were dehydrated through a grade series of ethanol at 40, 50, 60, 70, 80, 85, 90, 95 and 100%, respectively. Specimens in 100% ethanol were critical point dried in a CO_2, mounted on aluminum stubs and coated with gold using a sputter coater. Samples were examined using a scanning electron microscope JSM5910LV (JEOL Ltd., Japan).

3.2.6 Effect of ethanolic extracts of *C. fenestratum* and *S. venosa* on gene expression in *S. aureus* and MRSA

1) RNA isolation

The total RNA was extracted using NucleoSpin® RNA/Protein (MACHEREY-NAGEL) according to the manufacturer's instructions. MRSA isolates number 80 was treated with the ethanolic extract of *C. fenestratum* and *S. venosa* at 4 MIC concentrations. After incubating at 37 °C for 24 hours, the suspension was centrifuged at 11,000 x g for 10 minutes, twice. Then, the pellet was washed with phosphate buffer saline (PBS), pH 7.2 twice by centrifugation for 5 minutes at 11,000 x g. The bacterial cell pellet was resuspended in 100 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) which containing 2 mg/ml lysozyme by vigorous vortex and then incubated at 37 °C for 10 minutes. 350 µl of lysis buffer (RP1) and 3.5 µl of β-mercaptoethanol were added to the suspension in each tube by vortex for 30 seconds for disruption of protein disulfide bonds. The suspension was then filtered through NucleoSpin® filter and centrifuged for 1 minute at 11,000 x g. 350 µl of 70% ethanol was added in the filtrate to the homogenized lysate and mixed by pipetting up and down. After that, the mixture was filtrated through NucleoSpin® RNA/Protein column and then centrifuged at 11,000 x g for 30 seconds. RNA and DNA were bounded to the column membrane. After that, 350 µl of membrane desalting buffer (MDB) was added to the column and then centrifuged at 11,000 x g for 1 minute to dry the membrane. To digest DNA, rDNase reaction mixture was directly added on the membrane and incubated at room temperature for 15 minutes. The membrane was washed by adding RA2 buffer (200 µl), centrifuged at 11,000 x g for 30 seconds. After adding RA3 (600 µl), the tubes were centrifuged at 11,000 x g for 30 seconds and RA3 (250 µl) buffer was added

and centrifuged at 11,000 x g for 2 minutes, respectively. Finally, RNA was eluted by adding RNase-free water (40 µl) by centrifugation at 11,000 x g for 2 minutes. The RNA concentrations were measured at OD 260 using Nanodrop (Thermoscientific Nanodrop 2000c spectrophotometer). Finally, RNA sample was kept at -80 ºC for further study.

2) cDNA synthesis

The mRNA was reverse transcribed into cDNA using the ReverTra Ace® qPCR RT Kit according to the manufacturer's instructions. Briefly, the 20 ng of RNA was denatured by incubating at 65 °C for 5 minutes, and keep on ice afterwards. After that, the reaction solution was prepared as described in Table 3.3. The reaction was performed at 37°C for 30 minutes followed by heating at 98 °C for 5 minutes.

Table 3.3 Chemical component for reverse transcription reaction

Reagents	**Volume (µl)**
Nuclease-free water	x
5 x RT Buffer	2
RT Enzyme Mix	0.5
Primer Mix	0.5
RNA	20 ng
Total	**10**

3) Quantitative PCR amplification

The interested gene and oligonucleotide amplification primers were shown in Table 3.4 and Table 3.5. Real time quantitative PCR amplification was performed in a 20 µl reaction mixture using the THUNDERBIRD™ SYBR® qPCR Mix and 0.2 µM of each specific primer. Chemical components for quantitative polymerase chain reaction (qPCR) reaction were shown in Table 3.6. The PCR reactions were carried out in 96-well microplates using iCycler iQ5 (Bio-Rad, Hercules, CA). The amplification was programmed as follows: 95°C for 5 minutes followed by 45 cycles of 94°C for 45 seconds, 62°C for 45 seconds and 72°C for 1 minute. Fluorescence was measured repeatedly each cycle during the annealing step. This procedure was followed by a

melting curve dissociation analysis to confirm product size. The amplification results were expressed as the threshold cycle (Ct) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. All samples were analyzed in triplicate, and the malonyl CoA-acyl carrier protein transacylase (*fabD*) was served as an internal control to normalize the expressional levels between samples (Theis *et al.*, 2007). The relative expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 3.4 Gene and their protein of *S. aureus* and MRSA analyzed in this study

Gene	**Protein product**
fabD	malonyl CoA-acyl carrier protein transacylase
hla	α-toxin
mecA	penicillin-binding protein (PBP) 2a
mecR1	*mecA* transducer protein
mecI	*mecA* repressor protein
nucA	nuclease A protein

Table 3.5 Oligonucleotide primers for real-time quantitative PCR

Primer name	**Sequence 5 ' to 3 '**	**Product size**	**References**
fabD - F	CCT TTAGCA GTATCTGGA CC	103	Theis *et al.*, 2007
fabD-R	GAA ACTTAG CATCACGCC		
hla-F	CCATATACC GGGTTCCAA GA	165	-
hla-R	TGCAAATGT TTCGATTGGTC		
mecA-F	GGC TAT CGT GTC ACA ATC G	922	Kaewkod, 2011
mecA-R	GTT CTG CAG TAC CGG ATT TG		
mecR1-F	AAGCACCGTTACTATCTGCAC A	235	Lee *et al.*, 2007
mecR1-R	GAGTAAATTTTGGTC GAATGCC		
mecI-F	CTGCAGAATGGGAAGTTA TG	268	Lee *et al.*, 2007
mecI-R	ACAAGTGAATTGAAACCGCC		
nucA-F	GCGATTGATGGTGATACGGTT	270	Lee *et al.*, 2007
nucA-R	AGCCAAGCCTTGACGAACTAAAGC		

Table 3.6 Chemical components for quantitative polymerase chain reaction (qPCR) reaction

Reagents	**Final concentration**	**Volume (µl)**
Distilled water		7.8
THUNDERBIRD™ SYBR® qPCR Mix	1x	10
Forward Primer	0.2 mM	0.4
Reverse Primer	0.2 mM	0.4
50X ROX reference dye	1x	0.4
DNA solution		1
Total		**20**

3.2.7 Effect of plant extracts on PBP2a protein in MRSA

1) Bacterial protein extraction

Bacterial proteins were extracted by NucleoSpin® (MACHEREY -NAGEL, Germany) according to the manufacturer's instructions. MRSA isolates number 80 was treated with the ethanolic extract of *C. fenestratum* extract (CF) at concentration of 0.001 MIC (0.031 mg/ml), 0.01 MIC (0.313 mg/ml), 0.1 MIC (3.13 mg/ml) and *S. venosa* extract (SV) at concentration of 0.001 MIC (0.0039 mg/ml), 0.01 MIC (0.039 mg/ml) and 0.1 MIC (0.39 mg/ml). After incubating at 37 °C for 24 hours, the suspension was centrifuged at 11,000 x g for 10 minutes, twice. Then, the pellet was washed with phosphate buffer saline (PBS), pH 7.2 twice by centrifugation for 5 minutes, 11,000 x g. The bacterial cell pellet was resuspended in 100 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) which containing 2 mg/ml lysozyme by vigorous vortexing and then incubated at 37 °C for 10 minutes. Lysis buffer RP1 (350 µl) and β-mercaptoethanol (3.5 µl) were added to the suspension in each tube by vortex for 30 seconds for disruption of protein disulfide bonds. The suspension was then filtered through NucleoSpin® filter and centrifuged for 1 minute at 11,000 x g. After that, 70% ethanol (350 µl) was added in the filtrate to homogenize lysate and mixed by pipetting up and down. After that, the mixture was filtrated through NucleoSpin® RNA/Protein Column and then centrifuged at 11,000 x g for 30 seconds. The flow-through was used to protein isolation. Then, protein precipitation buffer (700 µl) was

added and mixed vigorously. The mixture was incubated at room temperature for 30 minutes. After incubation, the mixture was centrifuged at 11,000 x g for 5 minutes to remove the supernatant. The protein pellet was washed with 50% ethanol (500 µl) and then centrifuged at 11,000 x g for 1 minute. The supernatant was discarded to obtain protein pellet and the pellet was dried at room temperature for 15-20 minutes. The protein solving buffer (50 µl) containing reducing agent (PSB-TCEP) was added to the pellets and incubated for 3 minutes at 95-98°C for completely protein dissolving and denaturation. The protein samples were cool down to room temperature and then, the tubes were centrifuged for 1 minute at 11, 000 x g. Protein concentrations were determined by Bradford protein assay.

2) Determination of total protein by SDS-PAGE

Protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-PROTEIN Tetra Handset. Protein (50µg) was separated by 10% acrylamide-SDS gels. SDS-PAGE gel electrophoresis was performed at 100 volts for 1.30 hours to determine proteins from samples.

3) Detection of PBP-2a protein by Western blot analysis

PBP2a expression was determined by Western blot analysis using anti-PBP2a mouse monoclonal IgG (US biological Life Sciences®). Proteins on polyacrylamide gel were transferred to nitrocellulose membrane using semi-dry blotting method, which the gel and membrane were placed as shown in Figure 3.1. Nitrocellulose membrane and filter papers were soaked in Towbin transfer buffer, pH 8.3 for 15-30 minutes and assembled three filter paper, membrane, gel and filter paper from anode to cathode. Air bubbles were removed using rolling clean tube and the top was closed with the cover transfer apparatus. Protein transferring was performed at 15 volts for 4 hours. The membranes were washed in Tris Buffer Saline with 0.1% Tween 20 (TBS-T) for 5 minutes, 5 times and incubated 1 hour with frequent agitation in 5% skim milk to block non-specific binding on the membrane. After treatment, the membrane was rinsed with TBS-T and then incubated over night at 4°C with anti-PBP2a with ratio of 1: 100 by dilution in 5% skim milk. Then, the membrane was washed 5 times for 5 minutes each in TBS-T and goat anti mouse antibody (Millipore®) with ratio 1: 3000

by dilution in 1% skim milk was added and incubated for 1 hours. Finally, the membranes were incubated with DAB peroxidase substrate system (Amresco®). The colorimetric substrate was converted by the enzyme to color product that was visible the membrane as shown in brown color. The membranes were scanned by scanner, and protein band intensity was analyzed and quantified by Gene Tools Match (LAB Focus, Co.ltd).

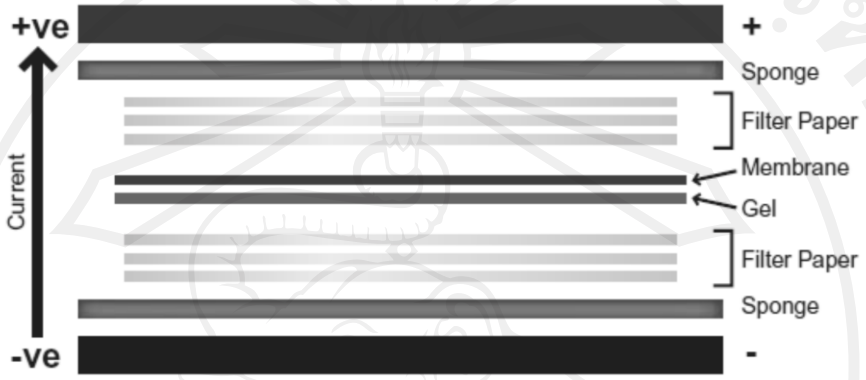


Figure 3.1 Western blotting

3.2.8 Preparation of plant extracts

1) Preparation of crude plant extracts

C. fenestratum and *S. venosa* powder (2.5 kg) were saparately macerated using methanol as a solvent with the ratio of 1:4 (w/v) for 72 hours at room temperature with frequent agitation. The extract was collected and filtered through Whatman No. 1 filter paper. The methanol extracts of each was concentrated to dryness under reduce pressure at 45ºC using rotary evaporator.

2) Isolation of *C. fenestratum*

For preliminary screening, the crude methanolic extract of *C. fenestratum* was separated using partition technique. Four kinds of solvents, *n*- hexane, chloroform, *n*-butanol and water, were selected to separate their chemical constituents based on their polarity. The methanolic extract (25.23 g) was initially dissolved in 56 ml of water and then partitioned with 50 ml of *n*-hexane four times to obtain the *n*-hexane fraction. After that, the water layer was partitioned with 50 ml of chloroform four times to obtain

chloroform fraction. Next, the water layer was partitioned with 50 ml of *n*-butanol four times to obtain *n*-butanol fraction. Supernatants were collected and evaporated to obtain each extract from *n*-hexane, chloroform, *n*-butanol and water. The extracts were subsequently tested for their antioxidant activity.

Methanolic extract (100 g) of *C. fenestratum* was coarsely isolated by column chromatography using celite® 545 (Fluka, Switzerland) as stationary phase. The crude extract was mixed with celite thoroughly. After that, celite: crude extracts mixture was packed into glass column (7 cm diameter) to 13 cm height and then eluted with dichloromethane, dichloromethane: 95% ethanol and 95% ethanol, respectively. The eluted samples were collected in 3 fractions depending on their TLC pattern, dried under evaporator and then antibacterial activity was tested. The sample with the highest antibacterial activity was further determined phytochemical constituents.

3) Isolation of *S. venosa*

The crude methanolic extract of *S. venosa* was separated by partition technique. Four kinds of solvents, *n*- hexane, chloroform, *n*-butanol and water, were selected to separate chemical constituents based on their polarity. The properties of hexane, chloroform, n-butanol and water were used to extract constituents from low to high polarity, consecutively. The crude methanolic extract (25.51 g) was initially dissolved in 56 ml of water and then partitioned with 50 ml of *n*-hexane four times to obtain the *n*-hexane fraction. After that, the water layer was partitioned with 50 ml of chloroform four times to obtain chloroform fraction. Next, the water layer was partitioned with 50 ml of *n*-butanol four times to obtain *n*-butanol fraction. Supernatants were collected and evaporated to obtain each fraction from partition by hexane, chloroform, n-butanol and water. Each fraction obtained from partition technique was subsequently tested for their antibacterial activity. The sample with the highest antibacterial activity was further determined phytochemical constituents.

4) Phytochemical screening of plant extracts

The plant extract was evaluated for phytochemical constituents including alkaloids, glycosides, tannins and phenolics. The methods of phytochemical screening were as follows (Trease and Evans, 1972; Tyler *et al.*, 1988; Houghton and Raman, 1998).

Detection of alkaloids

Screening of alkaloids was performed using general reagent, Dragendorff testing solution, and then confirmed by three kinds of chemical reagent such as Mayer, Wagner and Hager. First, sample (300 mg) was extracted in 15 ml of 2N HCl. An acidic extract solution was filtered for the primary testing. Dragendorff, Mayer, Wagner and Hager testing solution were applied for the primary testing. An acidic extract solution at volume of 0.5 ml was placed into five test tubes. The general reagent of Dragendorff's and specific reagents such as Mayer's reagent, Wagner's reagent and Hager's reagent were dropped into each test tube, respectively. The positive results, which indicated the present of alkaloids in the extract exhibited the present of turbidity and yellow-brown precipitation.

Detection of flavonoids

Flavonoid glycoside compounds in the extract were tested by Shibata's reaction. Sample (300 mg) was dissolved in 1 ml of 95% ethanol and placed in evaporation dish. Then, one small thin piece of magnesium metal was put and 5-6 drops of the concentrated HCl was added. However, the color of solution was red, flavonol compounds were presented in the extract. But if the color of solution was orange, that is referred to the presence of flavanone compounds.

Detection of coumarins testing

The coumarins were identified by the Coumarin's test. Sample (300 mg) was added to the test tube and dissolved in 0.1 ml of water. Then, filter paper was dipped with 1N NaOH and was placed upper the extract while the tube was boiled in the water bath for 3-5 minutes. After that, the filter paper was visualized under UV 365 nm. The blue-green fluorescence were detected under UV lamp due to the conjugated coumarin ring indicated the presence of coumarins.

Detection of saponins

The saponin glycosides were identified by a froth test. Briefly, sample (300 mg) was dissolved in 10 ml of distilled water and then filtered. Appearance of froth after shaking indicated the presence of saponin glycosides.

Detection cardiac glycosides

Plant extract (1000 mg) was dissolved in 10% ethanol and filtered through Whatman No.1. The filtrate was partitioned with chloroform three times and then dried on hot plate. The filtrate was divided into two tubes for testing of cardiac glycosides. Lieberman-Burchard test was used to evaluate the presence of steroidal nucleus in the tested sample. Briefly, few drops of acetic anhydride were added to extract followed by few drops of H_2SO_4. The color change from pink to green indicated the presence of steroidal nucleus. Moreover, Keller- Kiliani's test was used to evaluate the presence of deoxy sugar. The evaporated extract was dissolved in few drops of chloroform. Then, 3 ml of 10% Ferric chloride in acetic acid was added to the extract solutions followed by few drops of H_2SO_4. The brown ring between layers indicated the presence of deoxy sugar.

Detection of antraquinone glycosides

The Borntrager's test was used to determine antraquinone glycosides in plant extracts. Briefly, sample (300 mg) was dissolved with 20 ml of HCl in water bath at 95-98 ºC for 15 minutes, cooled and filtered. The filtrate was extracted with 10 ml chloroform twice. Then, NaOH was added to the chloroform layer. Pink coloration in base layer was produced if antraquinone glycosides were presented.

Detection of tannins

Sample (300 mg) was dissolved with 20 ml of distilled water, cooled and filtered. The filtrate was divided into 7 tubes for detection of tannins (Table 3.7).

Table 3.7 Amount of the samples in test tubes for tannin detection

Test	Reactions	Positive result	Compounds
1	control	-	-
2	Gelatin (1%) was added for 2-3 drops	white precipitate	tannin
3	$FeCl_3$ was added for 2-3 drops	blue precipitate green precipitate	hydrolysable tannin condensed tannin.
4	Formaldehyde (40%) was added for drops and HCl (10%) 6 drops were added and boiled for 2	pink precipitate	condensed tannin.
5	The filtrated was dried. Then, 1 ml of vanillin and few drops of HCl were added.	pink precipitate	condensed tannin.
6	Lime water (1ml) was added	dark - grey and blue precipitated	hydrolysable tannin
7	Lead acetate (10%) was added for 2 ml.	showed white precipitate in 15 minutes.	hydrolysable tannin

Phenolics testing

Sample (500 mg) was extracted with 70% ethanol (6 ml) in water bath at 95-98 ºC for 2 minutes, cooled and filtered. Then, aqueous iron (III) chloride solution (5%) was added. A blue green or green color was produced if phenolic substances were presented.

3.2.9 Identification of volatile constituents found in *C. fenestratum* extract by Gas chromatography/ Mass spectrometry (GC/MS)

The samples were sent to Central Laboratory, Chiang Mai, Thailand. The crude ethanolic extracts was chromatographed by capillary GLC and fitted with MS, which were used for the analysis of volatile constituents. In brief, the sample was prepared by dissolving plant extract (0.25 g) with methanol (1ml) and then analyzed on a Hewlett Packard model 6890N gas chromatography (Agilent Technologies, Germany) equipped with an HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25μm) and

coupled with a HP 5973N mass selective detector (Agilent Technologies, USA). The oven temperature was initially held at 35°C, 3 minutes and then increased by 10°C/minute to 320°C. Both injector and detector temperatures were 280°C. Purified Helium was used as the carrier gas at a flow rate 1 ml/minute. The mass spectrometer was scanned over the 40-400 m/z with an ionizing voltage of 70 eV. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The identification of the compounds in plant extract was based on the comparison of their mass spectra with reference mass spectra from Wiley version 7 database.

3.3 Results and Discussion

3.3.1 Characterization of methicillin resistant *S. aureus*

The identification of *S. aureus* is based on phenotypic and genotypic investigation (Fluit *et al.,* 2001). The phenotypic identification of *S. aureus* includes Gram staining, catalase, coagulase and DNAse test. Culturation of *S. aureus* on mannitol salt agar or blood agar and sugar fermentation test were also performed (Waldvogel, 2000). According to the Clinical and Laboratory Standards Institute (CLSI), oxacillin and cefoxitin disc susceptibility test was recommended for phenotypic detection of MRSA strains. Oxacillin was used to detect MRSA isolates instead of methicillin because it was more stable and most likely to detect heteroresistant strains. Moreover, cefoxitin is a strong inducer of *mecA* gene expression so MRSA with a *mecA* mediated resistance can be detected (Swenson, 2005). The result of cefoxitin susceptibility test can be used to predict the presence of *mecA*-mediated oxacillin resistant in *S. aureus*; and oxacillin was reported as susceptible or resistant based on cefoxitin results (CLSI, 2013). Recently, there are various molecular techniques that implemented for rapid identification and characterization of resistant strains. These include genotypic identification of antibiotic resistance in *S. aureus* based on the amplification of *mecA* gene, which conferred resistance to methicillin and oxacillin (Murakami *et al.,* 1991; Chongtrakool *et al.,* 2006; McClure *et al.,* 2006). In the current study, various biochemical tests including catalase, coagulase, mannitol fermentation and glucose fermentation was used to identify *S. aureus* both sensitive and resistant strains. It was found that all ten isolates of bacteria had a positive result in all biochemical test, thus, they were identified as *S. aureus* (Table 3.8). Moreover, disc susceptibility test was

used to confirm resistant ability using standard antibiotics including oxacillin and cefoxitin. The result showed that all ten isolates were resistance to oxacillin and cefoxitin antibiotics with the inhibition zone ≤ 10 mm for oxacillin and ≤ 21 mm for cefoxitin, respectively (Table 3.9, Figure 3.2). Therefore, it was indicated that conventional disc susceptibility testing method could detect resistant isolates from the community. The determination of antibiotic resistance bacteria by antibiotic susceptibility test is a crucial step in the prognosis of *S. aureus* infections. However, many reports have highlighted the difficulties and error in the identification of MRSA when using phenotypic identification. Therefore, molecular methods including conventional PCR and real time PCR was an alternate choice for the rapid and accurate identification and characterization resistance isolates (Felten *et al.,* 2002; Kaka *et al.,* 2006).

The molecular techniques are often applied for routine diagnosis of bacterial resistance along with antimicrobial susceptibility testing methods, because susceptibility testing alone is not enough to confirm *S. aureus* resistant due to the sensitivity of test conditions (Trindade *et al,* 2003). The use of PCR for detection of *mecA* gene has been previously described by many researchers (Khan *et al.,* 2012). Moreover, other genes such as *femA*, *femB* and *nuc* gene may be detected in MRSA isolates but these genes may be absent in some MRSA strains (Jonas *et al.*, 1999). In our study, polymerase chain reaction (PCR) was used to detect clinical MRSA isolates. The PCR result in Figure 3.3 showed that all MRSA isolates carried *mecA* gene (922 bp) when compare to *S. aureus*. The detection of antibiotic resistance in *S. aureus* by PCR assay is reliable, as PCR assay contributes rapid and faster diagnosis of MRSA than the disc diffusion method. Therefore, it was suggested that PCR technique can be chosen comparing to a culture technique to detect resistance in *S. aureus*.

Table 3.8 Biochemical test of ten isolates of *S. aureus*

Bacterial isolates	**Coagulase test**	**Catalase test**	**Mannitol fermentation**	**Glucose fermentation**
S 32	+	+	+	+
S 43	+	+	+	+
S 50	+	+	+	+
S 64	+	+	+	+
S 65	+	+	+	+
S 66	+	+	+	+
S 67	+	+	+	+
S 72	+	+	+	+
S 80	+	+	+	+
S82	+	+	+	+
S. aureus	+	+	+	+
S. epidermidis	-	+	-	+

Table 3.9 Antibiotic susceptibility testing of clinical isolates MRSA using oxacillin and cefoxitin disc

Strains	**1 µg Oxacillin (mm)**	**30µg Cefoxitin (mm)**	**Susceptibility result**
S 32	0	0	resistant
S 43	0	0	resistant
S 50	0	0	resistant
S 64	0	0	resistant
S 65	0	0	resistant
S 66	0	0	resistant
S 67	0	0	resistant
S 72	0	11.0±0.0	resistant
S 80	0	15.3±1.2	resistant
S 82	0	0	resistant
S. aureus	15.8 ±0.7	30.3 ±2.0	susceptible
S. epidermidis	26.0 ±1.0	37.7 ±2.8	susceptible

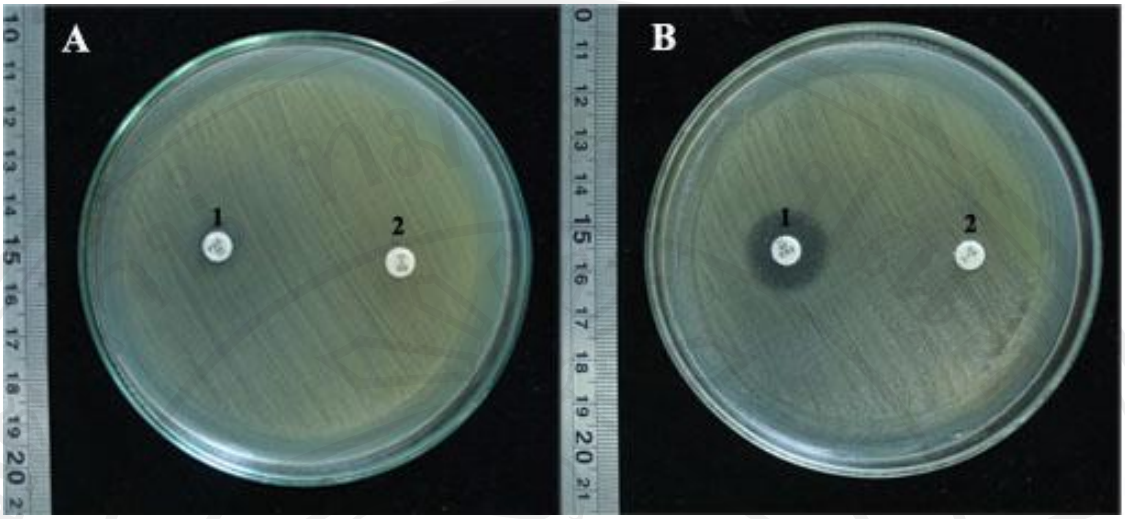


Figure 3.2 Antibiotic susceptibility testing of S 32 and S 72 using oxacillin (1μg) and cefoxitin (30μg) antibiotic

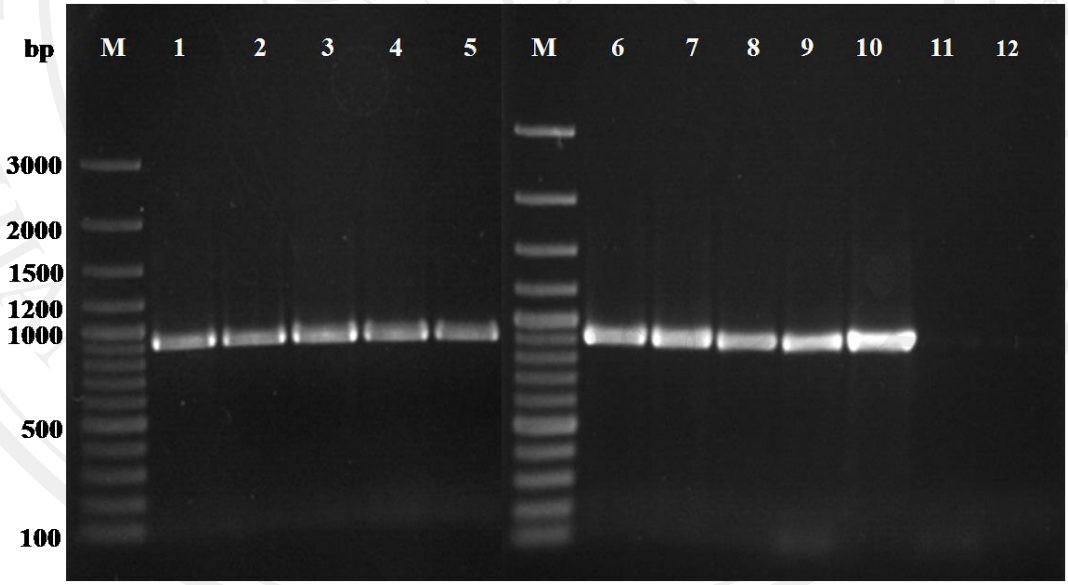


Figure 3.3 PCR product of methicillin resistant gene (*mecA*) of ten methicillin resistant *S. aureus* (MRSA) isolates (lane 1-10); *S. aureus* (lane 11); negative control (lane 12); 100 bp marker (M)

3.3.2 *mecA* gene mutation analysis by DNA sequencing technique

Methicillin-resistant *S. aureus* (MRSA) is defined by the production of a specific penicillin-binding protein (PBP2a), which has a reduced binding affinity for β-lactam antibiotics. PBP2a is encoded by the structural gene *mecA* on the chromosome, which has been detected in methicillin-resistant strains of staphylococcal species. More than 90% of clinical MRSA isolates carry *mecA* on their chromosomes. However, the mutation in the *mec* complex may affect the function of these genes and result in methicillin resistance. There are many researchers found some mutation in *mecA* and *mecI* gene which affected the increasing of resistance against β-lactams. In our study, the nucleotide sequencing was conducted in MRSA isolates 50, 64, 66, 67, 72 and 80. The locations and sequences of the primers in *mec* DNA were depicted in Figure 3.4. The sequences of MRSA isolates number 50, 64, 66, 67, 72 and 80 were shown in Figure 3.5-3.10. The nucleotide sequences of the *mecA* gene of MRSA strains employed in this study were compared with those of *S. aureus* subsp. *aureus* USA300_ TCH1516 accession number: CP000730.1. The result showed that sequence of these MRSA in this study was similar to the reference sequence with 100% identity. Thus, mutation was not found in this *mecA* segment (922 bp).

Previously study of *mecA* RFLP pattern of MRSA isolates number S16, S18, S21, S46, S49, S50, S53, S54, S64 and S81 using four restriction enzymes including *Cla*I, *Eco*RII, *Nde*I and *Pfe*I found that all isolates had the same RFLP pattern that might be concluded that there are no mutation in 922 bp *mecA* gene (Kaewkod, 2011). However, there were some limitations in this study that the primer for *mecA* sequencing generated only 922 bp product and it could not sequence all whole *mecA* genome of 2007 bp. However, some researchers reported that in some MRSA isolates, point mutation was found in other locations including the *mecI* gene or *mecA* promoter/ operator region of MRSA isolates. A single base substitution was detected in *mecI* with three different positions and *mecA* operator with two different positions, while a 28-base deletion in *mecI* was found in only one isolate. On the other hand, no mutation was detected in these *mecA* gene sequences of methicillin resistant *S. epidermidis* (MRSE) (Kobayashi *et al.*, 1998).

Moreover, Rosato *et al.* (2003) reported the mutation in *mecI* gene with various types including nonsense mutation with the base substitutions C to T at position 202 in 12 isolates, frameshift mutation in 4 isolates and missense mutation in 11 isolates from 65 clinical isolates. Recently, Shukla *et al*. (2004) studied polymorphisms in three *mec* genes including *mecA*, *mecI* and *mecR1* in MRSA isolated from Wisconsin, U.S.A. It was found that there were18 mutation types identified with 8, 7 and 2 mutation in *mecA*, *mecI* and *mecR1*, respectively. Moreover, another researcher had described in their study of isolates from Zurich, Switzerland and found the mutation in *mecA* promoter/operator region which containing the binding site for *mecI* and *blaI*. Expression studies showed that this mutation had significant effects on both *mecA* transcription and corresponding PBP2a production, but only small effects on β-lactam resistance levels. Besides, the mutations in *mecA* ribosomal binding site have no effect on *mecA* transcription and PBP2a content, and only minimal effects on β-lactam resistance (Ender *et al.*, 2007).

Forward Primer *mecA* (886-904)

GGCTATCGTGTCACAATCGTTGACGATAATAGCAATACAATCGCACATACATTAA
TAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAACTATTGATGCTAAAGT
TCAAAAGAGTATTTATAACAACATGAAAAATGATTATGGCTCAGGTACTGCTATC
CACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACCTTCATATGACGTCT
ATCCATTTATGTATGGCATGAGTAACGAAGAATATAATAAATTAACCGAAGATAAA
AAAGAACCTCTGCTCAACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAA
AAAATATTAACAGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAA
GTTATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACA
ACGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAGA
ATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGCAGTAAGAAA
TTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATATACCAAGTGATTAT
CCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATAATGAAATATTATTAGC
TGATTCAGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCTTTC
AATCTATAGCGCATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAG
ACACGAAAAACAAAGTTTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCT
ATTAACTGATGGTATGCAACAAGTCGTAAATAAAACACATAAAGAAGATATTTATA
GATCTTAATTGG**CAAATCCGGTACTGCAGAAC**TCAAAATGAAA

Reverse primer *mecA* (1788-1807)

Figure 3.4 Specific primer on *mecA* gene in *Staphylococcus aureus* subsp. *aureus* USA300_TCH1516 (886-1807bp) (Kaewkod, 2011)

CACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACT
AACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATG
ATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCA
CTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGT
AACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCA
ACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATTAAC
AGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTTATA
AAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAA
CGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAA
TAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGC
AGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATA
TACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAG
ATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGA
TTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGC
AATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGA
AGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGC
AACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTT

Figure 3.5 The 843 bp DNA sequence of MRSA 50 (929-1769 bp)

GCACATACATTAATAGAGAAAAAGAAAAAAAGATGGCAAAGATATTCAA
CTAACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAA
ATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTA
GCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATG
AGTAACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGC
TCAACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATT
AACAGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGT
TATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTA
CAACGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAA
GCAATAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATT
AGGCAGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGA
AGATATACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAA
ATTTAGATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAA
ATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAA
TAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAA
GTTTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGA
TGGTATGCAACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGAT
CTT

Figure 3.6 The 844 bp DNA sequence of MRSA 64 (928-1767 bp)

GCACATACATTAATAGAGAAAAAGAAAAAAAGATGGCAAAGATATTCAA
CTAACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAA
ATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTA
GCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATG
AGTAACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGC
TCAACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATT
AACAGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGT
TATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTA
CAACGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAA
GCAATAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATT
AGGCAGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGA
AGATATACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAA
ATTTAGATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAA
ATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAA
TAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAA
GTTTGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGA
TGGTATGCAACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGAT
CTT

Figure 3.7 The 844 bp DNA sequence of MRSA 66 (928-1768 bp)

CACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACT
AACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATG
ATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCA
CTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGT
AACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCA
ACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATTAAC
AGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTTATA
AAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAA
CGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAA
TAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGC
AGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATA
TACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAG
ATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGA
TTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGC
AATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGA
AGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGC
AACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTT

Figure 3.8 The 843 bp DNA sequence of MRSA 67 (929-1771 bp)

CACATACATTAATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACT
AACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATG
ATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCA
CTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGT
AACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCA
ACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATTAAC
AGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTTATA
AAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAA
CGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAA
TAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGC
AGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATA
TACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAG
ATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGA
TTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGC
AATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGA
AGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGC
AACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAGATCTT

Figure 3.9 The 843 bp DNA sequence of MRSA 72 (929-1771 bp)

CACATACATTAATAGAGAAAAAGAAAAAAAGATGGCAAAGATATTCAACT
AACTATTGATGCTAAAGTTCAAAAGAGTATTTATAACAACATGAAAAATG
ATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCA
CTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGT
AACGAAGAATATAATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCA
ACAAGTTCCAGATTACAACTTCACCAGGTTCAACTCAAAAAATATTAAC
AGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTTATA
AAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAA
CGTTACAAGATATGAAGTGGTAAATGGTAATATCGACTTAAAACAAGCAA
TAGAATCATCAGATAACATTTTCTTTGCTAGAGTAGCACTCGAATTAGGC
AGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGATA
TACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAG
ATAATGAAATATTATTAGCTGATTCAGGTTACGGACAAGGTGAAATACTGA
TTAACCCAGTACAGATCCTTTCAATCTATAGCGCATTAGAAAATAATGGC
AATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGA
AGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGC
AACAAGTCGTAAATAAAACACATAAAGAAGATATTTATAG

Figure 3.10 The 838 bp DNA sequence of MRSA80 (929-1766 bp)

3.3.3 Plant extraction

Twenty two medicinal plants that related in the use of Thai folklore medicine to treat skin diseases were selected to evaluate bioactive activity in this study (Table 3.10). The yields of aqueous and ethanolic extracts of medicinal plants were shown in Table 3.11. Yield of aqueous extracts were ranging from 2.25-15.54 % while the yields of ethanolic extracts were ranging from 3.03-13.20 %. The variation of the yields depended on their chemical nature, extraction method and solvents. Moreover, it was found that the aqueous extract of *Stemona* sp. gave the highest percentage yield with 15.54 % whereas the aqueous extract of *Hiptage* sp. gave the lowest with 2.25%. The color of the extract was green to brown varying in each plant extracts (Figure 3.11). Then, the aqueous and ethanolic extracts were screening for their antibacterial activity by agar disc diffusion and broth dilution methods.

There are various parameters influencing the quality of extract. The different extraction methods, temperature of extraction, time of extraction and polarity of solvents could affect quantity and secondary metabolite composition in the plant extracts. Moreover, geographical locations of plants, collection period, drying methods and storage condition were also influenced on the extracts (Tiwari *et al.,* 2011). The selection of solvent extraction depended on the specific nature of the bioactive compound being targeted. Different solvents were available to extract the various compounds from natural products. The extraction of hydrophilic compounds usually used polar solvents such as methanol, ethanol or ethyl acetate. Moreover, dichloromethane was used to extracted lipophilic compounds (Sasidharan *et al.,* 2010). In this study, water and 95% ethanol were selected for plant extraction. Water was a universal solvent which used to extract the polar constituent in medicinal plants. Moreover, ethanol was not only used for extraction various polar compounds but also solubilized non polar compounds (Tiwari *et al.,* 2011).

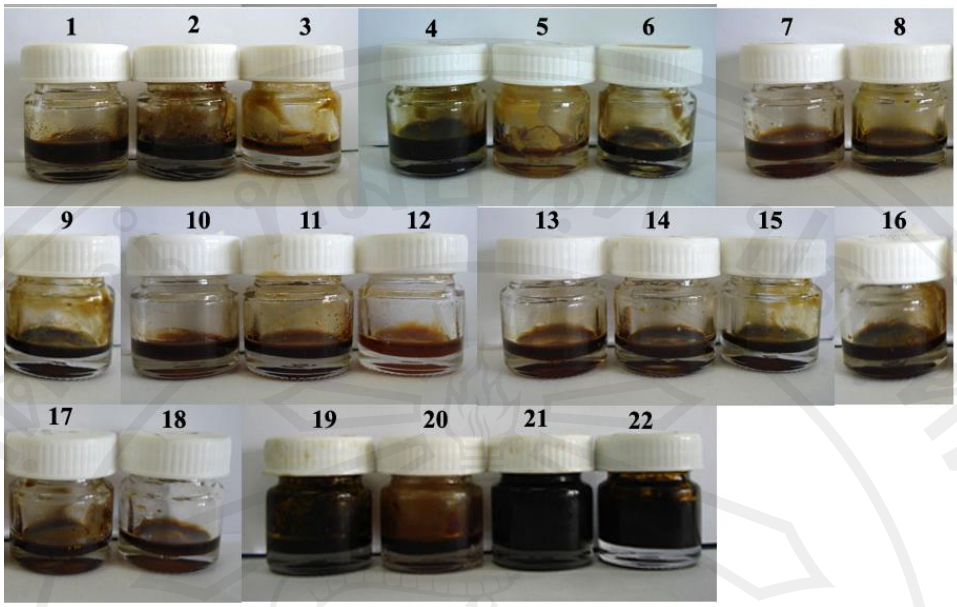
Table 3.10 Plant materials used in this study

Scientific name	**Family**	**Part used**
Andrographis paniculata Nees	Acanthaceae	Whole plant
Cissus quadrangularis L.	Vitaceae	Stem
Coscinium fenestratum (Gaertn.) Colebr.	Menispermaceae	Stem
Derris scandens (Roxb.) Benth.	Fabaceae	Leaf
Eclipta prostrata (L.) L.	Compositae	Whole plant
Glycyrrhiza glabra L.	Fabaceae	Leaf
Gynostemma pentaphyllum (Thunb.) Makino	Cucurbitaceae	Whole plant
Hiptage cf. *benghalensis* ssp. *benghalensis*	Malphigiaceae	Leaf
Houttuynia cordata Thunb.	Saururaceae	Whole plant
Momordica charantia L.	Cucurbitaceae	Whole plant
Phyllanthus amarus Schumach.	Euphorbiaceae	Whole plant
Pluchea indica (L.) Less.	Compositae	Leaf
Pseuderanthemum palatiferum (Nees) Radlk. ex Lindau	Acanthaceae	Leaf
Rhinacanthus nasutus Kuntze	Acanthaceae	Leaf
Schefflera leucantha R.Vig.	Araliaceae	Leaf
Senna alata (L.) Roxb.	Fabaceae	Leaf
Stemona sp.	Stemmonaceae	Whole plant
Stephania venosa (Blume) Spreng.	Menispermaceae	bulbs
Thunbergia laurifolia Lindl.	Acanthaceae	Whole plant
Tinospora crispa (L.) Hook.f. & Thomson	Menispermaceae	Stem
Vernonia cinerea (L.) Less.	Asteraceae	Whole plant
Zingiber montanum Link ex A. Dietr.	Zingiberaceae	Rhizome

Table 3.11 Percentage yield of plant extracts

Plant species	**Yield (%)**	
	Aqueous extract	**Ethanolic extract**
Andrographis paniculata	3.41	13.18
Cissus quadrangularis	14.04	6.12
Coscinium fenestratum	3.84	7.98
Derris scandens	3.07	7.68
Eclipta prostrata	6.64	9.11
Glycyrrhiza glabra	3.54	10.42
Gynostemma pentaphyllum	4.42	3.22
Hiptage sp.	2.25	5.34
Houttuynia cordata	5.00	6.68
Momordica charantia	3.20	3.14
Phyllanthus amarus	5.36	5.46
Pluchea indica	4.16	6.01
Pseuderanthemum palatiferum	3.61	3.87
Rhinacanthus nasutus	10.65	6.00
Schefflera leucantha	13.79	12.19
Senna alata	10.90	12.63
Stemona sp.	15.54	13.20
Stephania venosa	7.82	5.37
Thunbergia laurifolia	2.33	5.40
Tinospora crispa	7.70	3.03
Vernonia cinerea	6.50	3.28
Zingiber montanum	3.40	4.99

A



B

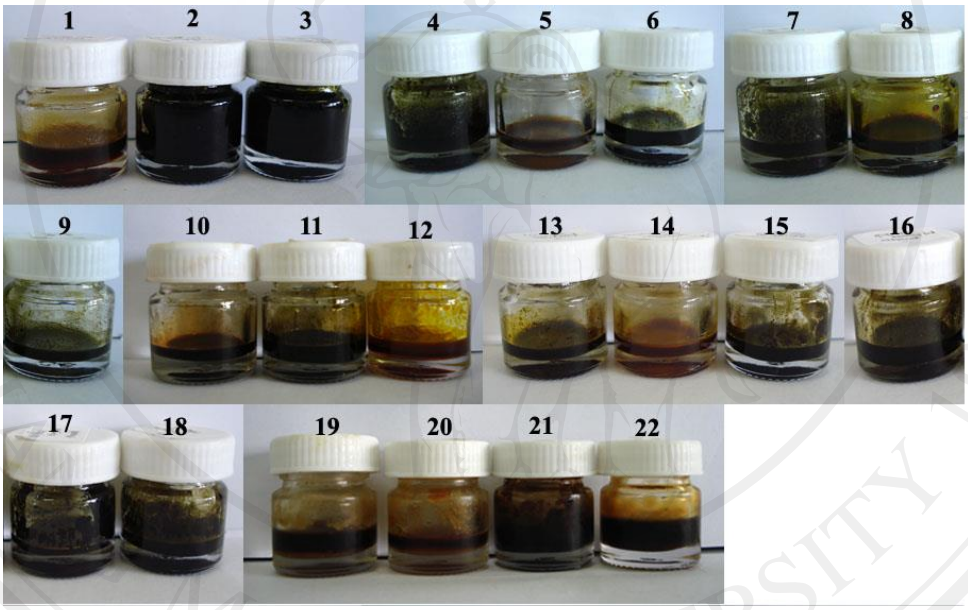


Figure 3.11 Aqueous (A) and ethanolic (B) extract of 22 medicinal plants;

1. *Stemona* sp.	2.*T. laurifolia*	3. *G. pentaphyllum*	4. *A. paniculata*
5. *S. venosa*	6. *E. prostrata*	7. *V. cinerea*	8. *C. fenestratum*
9. *S. leucantha*	10. *Hiptage* sp.	11. *S. alata*	12. *Z. montanum*
13. *T. crispa*	14. *D. scandens*	15. *R. nasatus*	16. *P. amarus*
17.*M. charantia*	18. *P. indica*	19. *H. cordata*	20. *G. glabra*
21. *P. palatiferum*	22.*C. quadrangularis*		

3.3.4 Antibacterial activity

1) Agar disc diffusion method

Aqueous and ethanolic extracts of 22 medicinal plants were screened for antibacterial activity using agar disc diffusion method against *E. coli* O157: H7, *Ps. aeruginosa*, *S. aureus*, *S. epidermidis*, *St. pyogenes*, *P. acnes* and 10 isolates of methicillin resistant *S. aureus*. The diameters of inhibition zones obtained were presented in Table 3.12-3.13. The inhibitory effect of some medicinal plants using agar disc diffusion methods were shown in Figure 3.12-3.13. The results showed that almost plant extracts could inhibit growth of Gram positive bacteria higher than Gram negative bacteria. The sensitivity between Gram positive and Gram negative bacteria after treatment with the plant extracts could be ascribed in difference cell morphology. The cell wall of Gram negative bacteria had the complex structure more than Gram positive bacteria. It contained an outer phospholipids membrane carrying the structural lipopolysaccharide components, which made the cell wall of Gram negative bacteria impermeable to antimicrobial substances (Nostro *et al.*, 2000; Tadeg *et al.*, 2005). Thus, the Gram negative bacterial cells might be difficult to destroy by the plant extracts.

Water and ethanol are commonly solvents for extraction of phytochemical compounds. Water is considered to have large dipole molecules and a high dielectric constant. Thus, water is very polar and only miscible in itself. Furthermore, ethanol is also classified as a polar solvent, although it is not very polar as water. This means that this solvent is miscible in water and it can extract mostly the ionic compounds from plant materials. Ethanol has better dissolving capabilities than water because it has a slightly lower dipole and dielectric constant than water, thus it is slightly polar (Scheflan and Jacobs 1953). From our study, almost ethanolic extracts showed efficacy in antibacterial activity higher than aqueous extracts. Many researchers reported that aqueous extract usually gave little or no inhibitory effect on microbial pathogens (Siripongvutikorn *et al.*, 2005; Afolayan and Aliero, 2006). However, the antibacterial activity of each plant extracts depended on the ability of active components miscibility in water so some aqueous extracts had high antibacterial activity such as *C. fenestratum, D. scandens, E. prostrata, G. glabra, P. amarus, P. indica* and *S. alata*

while some extracts could not inhibit any pathogenic bacteria including *A. paniculata*, *G. pentaphylum* and *S. leucantha*.

Ethanolic extract of *S. venosa* had the highest antibacterial activity against all tested bacterial strains in both Gram positive and Gram negative bacteria with inhibition zones ranging between 8.3-35.0 mm and the highest inhibitory effect was found against *P. acnes* with inhibition zone 35.0 mm. Moreover, ethanolic extract of *C. fenestratum* also had high antibacterial activity against all Gram positive bacteria with inhibition zone ranging between 17.0-50.0 mm. The ethanolic extract of *P. palatiferum* could inhibit five tested bacterial including *E. coli* O157: H7, *Ps. aeruginosa*, *S. aureus*, *S. epidermidis* and *St. pyogenes* with inhibition zone ranging from 9.7-19.7 mm. The ethanolic extracts of *A. paniculata*, *D. scandens*, *P. amarus* and *S. alata* inhibited four tested bacteria including *S. aureus*, *S. epidermidis*, *St. pyogenes* and *P. acnes* with inhibition zone ranging between 9.0-26.3 mm. The ethanolic extract of *E. prostrata*, *M. charantia* and *T. lauriferia* showed moderate activity against three tested bacteria with inhibition zone ranging between 8.0-19.7 mm. The ethanolic extract of *G. pentaphylum*, *P. indica*, *R. nasatus*, *Hiptage* sp. and *T. crispa* could inhibit two tested bacterial strains with inhibition zone ranging between 6.3-11.0 mm. The ethanolic extract of *C. quadrangularis*, *Stemona* sp., *V. cineria* and *Z. montanum* could inhibit only one bacterium with inhibition zone ranging between 9.0-12.0 mm. However, the ethanolic extracts of *G. glabra* and *S. leucantha* could not inhibit growth of any pathogenic bacteria.

Moreover, the aqueous extract of *C. fenestratum* also had high antibacterial activity against all Gram positive bacteria with inhibition zone ranging between 15.0-52.0 mm. The aqueous extract of *H. cordata* could inhibit four tested bacteria including *E. coli* O157: H7, *Ps. aeruginosa*, *S. epidermidis* and *St. pyogenes* with inhibition zone ranging between 7.0-13.3 mm. Furthermore, the aqueous extract of *S. alata* could exhibit four tested bacteria including *S. aureus*, *S. epidermidis*, *St. pyogenes* and *P. acnes* with inhibition zone ranging between 11.7-23.0 mm. The aqueous extract of *P. amarus* and *S. venosa* showed moderate activity against three tested bacteria with inhibition zone ranging between 7.3-12.0 mm. The aqueous extracts of *C. quadrangularis*, *G. glabra*, *M. charantia*, *R. nasatus*, *Hiptage* sp., *Stemona* sp.,

T. laurifolia, *T. crispa*, *V. cineria* and *Z. montanum* had low antibacterial activity against one or two bacterial strains with inhibition zone ranging between 8.3-18.3 mm. However, the aqueous extracts of *A. paniculata*, *E. prostata*, *G. pentaphylum*, *P. palatiferum* and *S. leucantha* could not inhibit growth of any pathogenic bacteria.

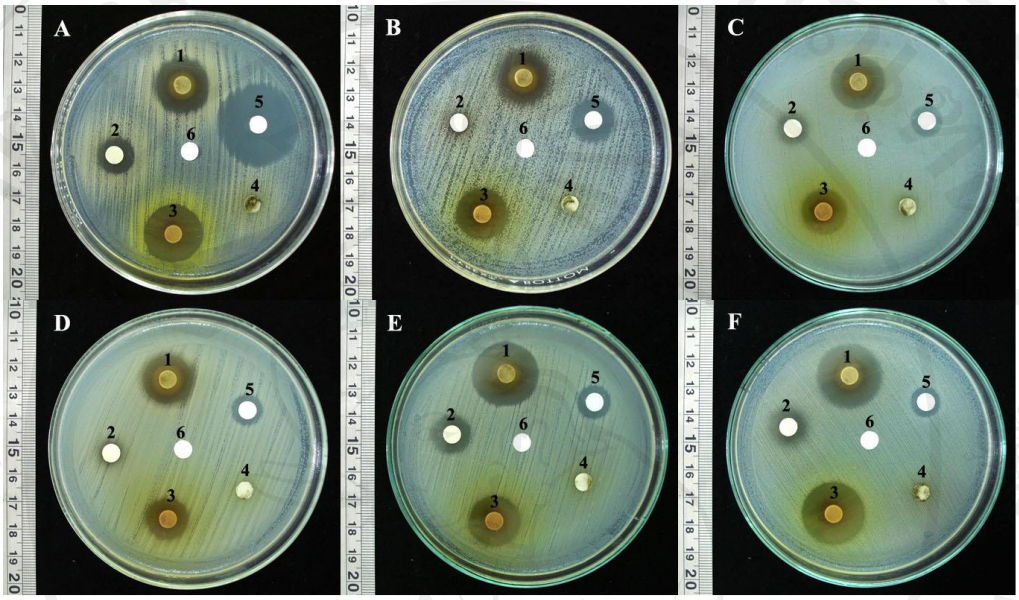


Figure 3.12 Inhibitory effect of ethanolic extract of *V. cinerea* (1), *E. prostrata* (2), *C. fenestratum* (3), *S. leucantha* (4) and gentamycin (40 mg/ml) (5) on growth of *S. aureus* (A), *S. epidermidis* (B), MRSA 64 (C), MRSA 65 (D), MRSA 66 (E) and MRSA 67 (F) by agar disc diffusion method

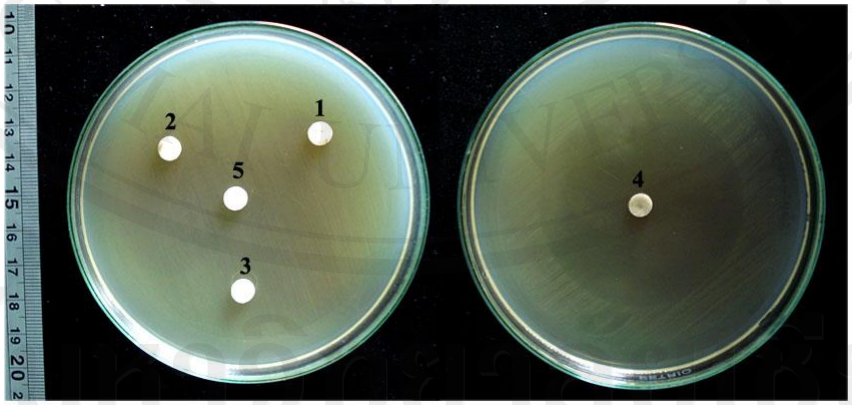


Figure 3.13 Inhibitory effect of ethanolic extract of *V. cinerea* (1), *E. prostrata* (2), *S. leucantha* (3), *C. fenestratum* (4) on growth of *P. acnes* and DMSO was used as negative control (5) by agar disc diffusion method

Table 3.12 Inhibitory effect of plant extracts on pathogenic bacteria using agar disc diffusion method

Plant species	Extracts (500mg/ml)	Zone of inhibition (mm)					
		Bacterial strains					
		***E. coli* O157: H7**	***Ps. aeruginosa***	***S. aureus***	***S. epidermidis***	***St. pyogenes***	***P. acnes***
Andrographis paniculata	Water	0	0	0	0	0	0
	Ethanol	0		9.0 ± 1.0	12.3 ± 1.5	13.0 ± 1.7	26.3 ± 0.6
Cissus quadrangularis	Water	9.0 ± 0.6	10.0 ± 0.0	0	0	0	0
	Ethanol	0	0	0	0	9.0 ± 0.0	0
Coscinium fenestratum	Water	0	0	15.0 ± 0.0	16.7 ± 0.6	27.0 ± 0.0	52.0 ± 1.0*
	Ethanol	0	0	17.0 ± 0.0	17.7 ± 0.6	31.0 ± 0.0*	50.0 ± 0.0
Derris scandens	Water	0	0	20.7 ± 0.6*	18.7 ± 1.5	0	0
	Ethanol	0	0	10.7 ± 0.6	10.0 ± 0.0	10.7 ± 0.6	12.7 ± 1.2
Eclipta prostrata	Water	0	0	0	0	0	0
	Ethanol	0	0	0	10.3 ± 0.6	15.0 ± 0.0	12.0 ± 0.0
Glycyrrhiza glabra	Water	0	0	14.0 ± 1.0	11.0 ± 1.0	0	0
	Ethanol	0	0	0	0	0	0
Gynostemma pentaphylum	Water	0	0	0	0	0	0
	Ethanol	0	0	8.0 ± 0	0	10.7 ± 0.6	0
Hiptage sp.	Water	0	0	0	8.3 ± 0.6	10.7 ± 0.6	0
	Ethanol	0	0	11.0 ± 0.0	6.3 ± 0.6	0	0

Table 3.12 (Continued)

Plant species	Extracts (500 mg/ml)	Zone of inhibition (mm)					
		Bacterial strains					
		E. coli O157: H7	*Ps. aeruginosa*	*S. aureus*	*S. epidermidis*	*St. pyogenes*	*P. acnes*
Houttuynia cordata	Water	9.3 ± 0.6	11.7 ± 1.2*	0	7.0 ± 0.0	13.3 ± 1.5	0
	Ethanol	7.3 ± 0.6	0	0	0	0	0
Momordica charantia	Water	0	0	0	10.7 ± 0.6	0	0
	Ethanol	0	0	10.0 ± 0.0	0	15.3 ± 0.6	17.3 ± 0.6
Phyllanthus amarus	Water	0	0	10.0 ± 1.0	11.3 ± 1.2	9.3 ± 1.2	0
	Ethanol	0	0	17.7 ± 0.6	18.7 ± 1.5	10.7 ± 0.6	14.7 ± 0.6
Pluchea indica	Water	0	0	9.0 ± 1.0	0	0	0
	Ethanol	0	0	7.3 ± 0.6	0	8.0± 0.0	0
Pseuderanthemum palatiferum	Water	12.0 ± 1.0*	0	0	0	0	0
	Ethanol	9.7 ± 0.6	12.0 ± 1.0*	15.0 ± 1.0	19.7 ± 1.2	16.0 ± 1.0	0
Rhinacanthus nasutus	Water	0	0	11.7 ± 0.6	0	0	0
	Ethanol	0	0	8.0 ± 0.0	0	9.3 ± 0.6	0
Schefflera leucantha	Water	0	0	0	0	0	0
	Ethanol	0	0	0	0	0	0
Senna alata	Water	0	0	14.7 ± 0.6	11.7 ± 0.6	15.0 ± 0.0	23.0 ± 1.0
	Ethanol	0	0	11.3 ± 0.6	15.0 ± 0.0	10.3 ± 0.6	14.7 ± 0.6

Table 3.12 (Continued)

Plant species	Extracts (500 mg/ml)	Zone of inhibition (mm)					
		Bacterial strains					
		E. coli O157: H7	*Ps. aeruginosa*	*S. aureus*	*S. epidermidis*	*St. pyogenes*	*P. acnes*
Stemona sp.	Water	0	0	0	0	15.0 ± 1.0	0
	Ethanol	0	0	0	0	12.0 ± 0.0	0
Stephania venosa	Water	0	0	7.3 ± 0.6	10.3 ± 0.6	0	12.0 ± 0.0
	Ethanol	9.3 ± 0.6	8.3 ± 0.6	12.7 ± 0.6	20.0 ± 0.1*	26.0± 1.0	35.0 ± 0.0
Thunbergia laurifolia	Water	0	0	0	0	18.3 ± 1.5	0
	Ethanol	0	0	10.0 ± 0	8.0 ± 1.7	19.7 ± 0.5	0
Tinospora crispa	Water	0	0	0	0	11.7 ± 0.6	0
	Ethanol	0	0	0	6.7 ± 0.6	10.0 ± 1.0	0
Vernonia cinerea	Water	0	0	13.0 ± 0.0	0	0	0
	Ethanol	0	0	0	0	0	9.7 ± 0.6
Zingiber montanum	Water	0	0	0	0	8.3 ± 0.6	0
	Ethanol	0	0	0	0	11.3 ± 1.5	0
gentamycin 40 mg/ml	-	30.3 ± 0.6	36.0 ± 0.0	29.3 ± 0.6	39.7 ± 1.2	38.3 ± 0.6	53.0 ± 2.6

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using Post hoc Duncan test. (*, $P<0.05$) represent the significantly highest antibacterial activity in each column.

Interestingly, almost plant extracts could inhibit the growth of methicillin resistant *S. aureus* (MRSA) clinical isolates. The ethanolic extract of *C. fenestratum, D. scandens, E. prostrata, P. amarus, P. palatiferum, S. alata, Hiptage* sp. and *S. venosa* had high activity against all ten isolates with the inhibition zone ranging between 9.0-26.7 mm. The ethanolic extract of *A. paniculata and T. laurifolia* also showed high activity on inhibiting nine isolates exclude MRSA isolate number 64 with inhibition zone ranging between 7.0-24.3 mm. Additionally, the ethanolic extract of *G. glabra* could inhibit eight MRSA isolate number 32, 43, 50, 64, 66, 72, 80 and 82 with inhibition zone ranging between 11.7-19.0 mm. The ethanolic extract of *R. nasatus* could inhibit MRSA isolate number 32, 43, 50, 67, 72, 80 and 82 with inhibition zone ranging between 8.7-20.7 mm while the ethanolic extract of *V. cinerea* could inhibit MRSA isolate number 32, 50, 64, 65, 72, 80 and 82 with inhibition zone ranging between 10.0-24.0 mm. The ethanolic extract of *M. charantia* could inhibit MRSA isolate number 32, 43, 50, 72, 80 and 82 with inhibition zone ranging between 14.0-18.3 mm while the ethanolic extract of *T. crispa* could inhibit six isolates number 32, 43, 64, 65, 66 and 67 with inhibition zone ranging between 7.3-12.3 mm. The ethanolic extract of *Z. montanum* could inhibit the growth of MRSA isolate number 32, 43, 50, 72 and 80 with inhibition zone ranging between 10.7-12.7 mm. Moreover, *G. pentaphylum*, *H. cordata* and *P. indica* had low antibacterial activity against few MRSA isolates with inhibition zone ranging between 7.7-16.3 mm. However, the ethanolic extracts of *C. quadrangularis* and *S. leucantha* could not inhibit any MRSA isolates.

Furthermore, the aqueous extract of *C. fenestratum, G. glabra, P. amarus* and *P. indica* had high antibacterial activity against all MRSA isolates with inhibition zone ranging between 8.0-22.7 mm. The aqueous extract of *E. prostrata* and *S. alata* against almost MRSA isolates exclude isolate number 65 and 64, respectively with inhibition zone ranging between 10.0-20.3 mm. The aqueous extract of *D. scandens* could inhibit seven MRSA isolate number 32, 43, 50, 65, 66, 67 and 80 with inhibition zone ranging between 16.0-24.7 mm. The aqueous extract of *R. nasatus* could inhibit MRSA isolate number 64, 65, 66 and 67 with inhibition zone ranging between 10.7-15.7 mm while aqueous extract of *Hiptage* sp. could inhibit MRSA isolate number 32, 65, 66 and 67 with inhibition zone ranging between 8.0-15.3 mm. In addition, the aqueous extract of

C. quadrangularis, H. cordata, Stemona sp., *S. venosa* and *Z. montanum* had low antibacterial activity against few MRSA isolates with inhibition zone ranging between 8.3-19.7 mm. However, the aqueous extracts of *A. paniculata, G. pentaphylum, P. palatiferum, S. leucantha* and *T. crispa* could not exhibit any MRSA isolates.

For considering on each tested bacteria, the aqueous extract of *P. palatiferum* had significant highest activity against *E. coli* O157: H7 with inhibition zone of 12.0 mm while the ethanolic extract of this plant gave significant highest activity against *Ps. aeruginosa* with inhibition zone of 12.0 mm. The ethanolic extract of *C. fenestratum* gave the highest inhibitory effect against *St. pyogenes*, MRSA isolate number 82 with inhibition zone of 31.0 mm and 22.3 mm while the aqueous extract had significant highest inhibition on *P. acnes* and MRSA isolate number 43 and 80 with inhibition zone of 52.0, 24.7 and 21.7 mm, respectively. Moreover, the ethanolic extract of *S. venosa* gave significant highest activity aginst *S. epidermidis*, MRSA isolate number 65 and 82 with inhibition zone of 20.0, 20.7 and 21.0 mm. The aqueous extract of *D. scandens* had the highest inhibition zone of on *S. aureus* 20.7 mm. In addition, the ethanolic extract of *E. prostrata* gave the highest activity against MRSA number 50 and 82 with inhibition zone 26.7 and 21.7 mm, respectuvely. The aqueous extract of *P. amarus* gave the highest activity on MRSA number 64 while the ethanolic extract could inhibit MRSA number 66 with inhibition zone of 20.0 and 24.3 mm, respectively.

Table 3.13 Inhibitory effect of plant extracts on methicillin resistant *S. aureus* (MRSA) using agar disc diffusion method

Plant species	Extracts (500 mg/ml)	Zone of Inhibition (mm)									
		Bacterial strains									
		MRSA 32	MRSA 43	MRSA 50	MRSA 64	MRSA 65	MRSA 66	MRSA 67	MRSA 72	MRSA 80	MRSA 82
Andrographis	Water	0	0	0	0	0	0	0	0	0	0
paniculata	Ethanol	18.0± 1.0	17.7± 1.2	21.0± 1.0	0	9.7 ± 1.2	7.3 ± 0.6	9.0 ± 1.0	24.3± 0.6	18.0± 1.0	15.3± 1.5
Cissus	Water	0	0	0	0	0	9.7 ±0.6	0	0	0	0
quadrangularis	Ethanol	0	0	0	0	0	0	0	0	0	0
Coscinium	Water	14.3±1.7	13.0±1.7	14.7± 0.6	17.3 ± 0.6	12.0 ± 0.0	19.7 ± 0.6	20.0 ± 0.0	16.0± 1.0	16.0± 1.0	9.0± 0.0
fenestratum	Ethanol	20.3± 1.5	20.3± 1.2	22.7± 0.6	12.0 ± 0.0	12.0 ± 0.0	11.3 ± 0.6	17.0 ± 0.0	24.0± 1.1	21.0± 1.0	22.3± 1.2*
Derris scandens	Water	16.8± 1.5	24.7±1.5*	24.3± 1.5	0	16.0 ± 0.0	23.0 ± 1.0	21.3 ± 1.2	0	24.0± 1.0*	0
	Ethanol	15.0 ± 1.0	13.0± 1.0	15.7± 1.2	13.7 ± 1.2	11.0 ± 1.0	14.7 ± 0.6	14.0± 1.7	13.3± 1.5	9.0± 0.0	9.0± 0.0
Eclipta prostrata	Water	15.8 ± 1.7	11.0± 1.0	20.3± 0.6	10.0 ± 0.0	0	15.7 ± 0.6	10.0 ± 0.0	11.7± 0.6	11.3± 0.6	10.0± 1.0
	Ethanol	20.3± 0.5	20.3± 1.2	26.7±0.5*	12.3 ± 0.6	12.3 ± 0.6	13.3 ± 0.6	12.0 ± 0.0	25.0± 0.0	20.7± 0.6	21.7± 1.2*
Glycyrrhiza	Water	10.7 ± 0.6	11.3 ± 0.6	11.7 ± 0.6	14.7 ± 1.0	13.3 ± 0.5	16.0 ± 1.0	10.0 ± 1.0	11.7 ± 0.6	11.3 ± 0.6	11.3 ± 0.7
glabra	Ethanol	15.7± 0.6	19.0± 1.0	11.7 ±0.6	12.0 ±0.6	0	14.0 ±0.6	0	14.7± 0.6	16.0± 1.0	16.7± 0.6
Gynostemma	Water	0	0	0	0	0	0	0	0	0	0
pentaphylum	Ethanol	12.3± 0.6	0	0	0	0	0	0	0	0	0
Hiptage sp.	Water	8.0± 1.2	0	0	0	9.3 ± 0.5	15.3 ± 0.6	11.0 ± 1.0	0	0	0
	Ethanol	16.3± 0.6	10.3± 0.6	20.3± 1.5	16.3 ± 0.6	11.7 ± 0.6	19.7 ± 0.6	17.0 ± 1.0	17.7± 0.6	9.0± 0.0	9.0± 0.0

Table 3.13 (Continued)

Plant species	Extracts (500 mg/ml)	Zone of Inhibition (mm) Bacterial strains									
		MRSA 32	MRSA 43	MRSA 50	MRSA 64	MRSA 65	MRSA 66	MRSA 67	MRSA 72	MRSA 80	MRSA 82
Houttuynia cordata	Water	8.7 ± 0.6	8.7 ± 0.6	8.3 ±0.6	0	0	0	0	0	0	0
	Ethanol	8.0± 0.0	8.0± 0.0	7.7 ±0.6	0	0	0	0	0	0	0
Momordica charantia	Water	0	0	0	12.0 ±1.0	8.7 ± 0.5	14.0 ± 1.0	0	0	0	0
	Ethanol	15.7± 1.2	18.3± 1.5	15.7± 0.6	0	0	0	0	16.7± 0.6	14.0± 1.0	15.7± 1.2
Phyllanthus amarus	Water	22.0± 1.0	20.7 ± 0.6	17.7 ± 1.2	20.0±1.0*	14.3 ± 0.5	22.7 ± 0.6	21.0 ± 1.0	20.3 ±0.6	14.0 ± 2.6	15.3 ± 0.0
	Ethanol	19.0± 1.0	21.0± 1.0	18.3± 0.6	18.3 ± 0.6	16.7± 0.6	24.3±1.2*	21.7 ± 1.5	15.3± 0.6	20.3± 0.6	15.7± 1.2
Pluchea indica	Water	13.0 ± 1.7	8.0 ± 0.0	8.0 ± 0.0	14.3 ±0.6	10.7 ± 0.9	16.3 ± 1.5	13.7 ± 0.6	8.0 ± 0.0	8.0 ± 0.0	8.0 ± 0.0
	Ethanol	10.0± 1.0	8.0± 0.0	0	0	0	0	0	9.0± 0.0	0	0
Pseuderanthemum palatiferum	Water	0	0	0	0	0	0	0	0	0	0
	Ethanol	19.7± 0.6	16.7± 2.1	17.7 ± 1.2	16.7 ±0.6	15.3 ±0.6	19.3 ±0.6	16.3 ±0.6	23.0± 1.0	16.3± 0.6	13.3± 0.6
Rhinacanthus nasutus	Water	0	0	0	15.7 ± 0.6	10.7 ± 0.5	15.0 ± 1.0	15.3 ± 0.6	0	0	0
	Ethanol	20.7± 1.2	20.3± 0.6	18.3± 0.6	0	0	0	8.7 ± 1.2	15.0± 2.0	19.7± 1.2	18.7± 0.6
Schefflera leucantha	Water	0	0	0	0	0	0	0	0	0	0
	Ethanol	0	0	0	0	0	0	0	0	0	0
Senna alata	Water	9.0± 0.0	13.0± 1.7	13.3± 1.2	0	15.7 ± 0.6	12.3 ± 0.6	14.7 ± 0.6	16.0± 1.0	13.0± 0.0	11.3± 0.6
	Ethanol	13.7± 0.6	13.3± 0.6	17.3± 0.6	14.3 ± 0.6	9.3 ± 0.5	19.7 ± 0.6	17.7 ± 1.2	17.3± 1.5	12.0± 1.0	11.0± 0.0

Table 3.13 (Continued)

Plant species	Extracts (500 mg/ml)	Zone of Inhibition (mm) Bacterial strains[a]									
		MRSA 32	MRSA 43	MRSA 50	MRSA 64	MRSA 65	MRSA 66	MRSA 67	MRSA 72	MRSA 80	MRSA 82
Stemona sp.	Water	0	0	0	0	8.3 ± 0.5	0	0	0	0	0
	Ethanol	0	0	0	0	0	0	0	0	0	0
Stephania venosa	Water	0	0	0	12.0 ± 0.0	8.7 ± 0.5	0	19.3 ± 0.6	0	0	0
	Ethanol	20.7± 0.6	20.3± 1.2	24.0± 1.7	17.7 ± 0.6	20.7±0.6*	19.0 ± 1.0	18.0 ± 0.0	25.3± 1.2	21.7± 1.2	21.0± 1.0*
Thunbergia laurifolia	Water	0	0	0	0	0	8.0 ± 1.0	7.3 ± 0.6	0	0	0
	Ethanol	16.7 ± 0.6	17.3± 0.6	20.3± 0.6	0	7.0 ± 0.0	7.0 ± 0.0	7.7 ± 0.6	21.7± 0.6	19.0± 1.0	11.3± 0.6
Tinospora crispa	Water	0	0	0	0	0	0	0	0	0	0
	Ethanol	15.3± 1.5	9.0± 0.0	0	7.3 ± 0.6	10.0± 0.0	12.7 ± 0.6	11.7 ± 1.5	0	0	0
Vernonia cinerea	Water	10.0±1.2	0	10.0± 1.0	10.0 ± 0.0	0	14.7 ± 0.6	15.0 ± 0.0	0	0	0
	Ethanol	16.3± 0.6	0	21.7± 0.6	10.0 ± 0.0	10.0 ± 0.0	0	0	24.0± 1.0	15.3± 0.6	21.0± 1.7*
Zingiber montanum	Water	0	0	0	0	0	0	0	12.7± 2.1	0	0
	Ethanol	12.7± 0.6	10.7± 0.6	11.0± 1.0	0	0	0	0	12.3± 0.6	12.3 ±0.6	0
gentamycin 10mg/ml	-	14.7 ± 0.6	16.3 ± 2.3	14.7 ± 2.1	11.7 ± 0.6	11.7 ± 0.6	13.0 ± 1.0	11.0 ± 0.0	16.0 ± 1.7	16.3 ± 1.2	15.0 ± 0.0

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using Post hoc Duncan test. (*, $P<0.05$) represent the significantly highest antibacterial activity in each column.

2) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The plant which showed positive antibacterial activity in agar disc diffusion method was further determined for their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values. MIC and MBC of Thai medicinal plants were determined by broth dilution method. MIC was defined as the lowest concentration that completely inhibited bacterial growth for 24 hours. As the same concept, MBC was defined as the lowest concentration at which no growth of bacteria was observed after incubation at 37 ºC for 24 hours (Hammer *et al.*, 1999; Delaquis *et al.,* 2002). The results of MIC and MBC values were summarized in Table 3.14-3.15. From the present study, the ethanolic extract of *S. venosa* had high antibacterial efficacy against all tested bacteria with MIC and MBC values ranging between 0.12-62.5 mg/ml. However, the highest inhibitory effect was found on *St. pyogenes* with MIC and MBC values of 0.12 mg/ml. Moreover, the ethanolic extract *C. fenestratum* also showed high antibacterial activity against all Gram positive bacteria with MIC and MBC values ranging between 0.03-7.8 mg/ml. The highest activity of the ethanolic extract of *C. fenestratum* was found on susceptibility bacteria; *P. acnes* with MIC of 0.03 mg/ml and MBC of 0.06 mg/ml. Furthermore, the ethanolic extracts of *P. amarus* and *S. alata* also showed high inhibitory on all Gram positive bacteria with MIC and MBC values ranging between 3.9-62.5 mg/ml.

The 10 clinical MRSA isolates were also tested with 22 medicinal plant extracts. The data showed that almost plant extracts could inhibit the growth of clinical isolates of MRSA and the ethanolic extract of *S. venosa* showed higher inhibition against all MRSA isolates than other extracts with MIC ranging between 0.48-31.3 mg/ml and MBC ranging between 0.97-62.5 mg/ml. Additionally, the ethanolic extract of *C. fenestratum* also showed high inhibition against all MRSA isolates with MIC ranging between 1.9-62.5 mg/ml and MBC ranging between 7.8-125 mg/ml. According to the previous study, Nair *et al.* (2005) found that methanolic extract of *C. fenestratum* had antibacterial activity higher than aqueous extract against *B. subtilis, Enterococcus* sp., *E. coli, Ps. aeruginosa, Sal. typhi* and *S. aureus.* In 2007, Kumar *et al.* reported that this plant also had the greatest antimicrobial effect against *S. epidermidis* and *P. acnes*.

For phytochemical screening, they revealed that alkaloid was the main bioactive compound which could be responsible for these activities. Thailandine, alkaloids which isolated from *S. venosa* had strong activity against some microorganisms such as *Plasmodium falciparum* (K1 strain), *Mycobacterium tuberculosis* H (37) Ra, *St. pneumoniae* and *S. aureus.* Moreover, this compound also showed strong anticancer activity against lung carcinoma cell (A549). Additionally, other compounds, oxostephanine and dehydrocrebanine also exhibited inhibition activity against breast cancer (BC) and acute lymphoblastic leukemia cells (MOLT-3) and promyelocytic leukemia cells (HL-60), respectively (Makarasen *et al.,* 2011). Therefore, our result indicated that the ethanolic extract of *C. fenestratum* and *S. venosa* exhibited high antibacterial activity against almost tested bacteria so these plants were further extracted with other solvents including methanol and dichloromethane to elucidate the suitable extractants to obtain the extract with high antibacterial ability.

Table 3.14 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of crude plant extracts against pathogenic bacteria using broth dilution method

Plant species	Extracts	MIC and MBC (mg/ml)											
		Bacteria											
		E. coli O157: H7		*Ps. aeruginosa*		*S. aureus*		*S. epidermidis*		*St. pyogenes*		*P. acnes*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Andrographis paniculata	Water	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	250	250	125	250	125	125	125	125
Cissus quadrangularis	Water	125	250	125	250	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	0.98	0.98	-	-
Coscinium fenestratum	Water	-	-	-	-	15.6	31.3	1.9	3.9	1.9	1.9	0.24	0.24
	Ethanol	-	-	-	-	1.9	7.8	1.9	3.9	0.12	0.49	0.03	0.06
Derris scandens	Water	-	-	-	-	62.5	62.5	31.3	62.5	-	-	-	-
	Ethanol	-	-	-	-	15.6	25.6	15.6	31.3	0.49	0.49	125	125
Eclipta prostrata	Water	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	31.3	31.3	3.9	7.8	125	125
Glycyrrhiza glabra	Water	-	-	-	-	62.5	250	125	250	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-
Gynostemma pentaphylum	Water	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	125	125	-	-	15.6	31.3	-	-
Hiptage sp.	Water	-	-	-	-	-	-	62.5	125	62.5	62.5	-	-
	Ethanol	-	-	-	-	1.9	3.9	15.6	31.3	-	-	-	-

Table 3.14 (Continued)

Plant species	Extracts	MIC and MBC (mg/ml)											
		Bacteria											
		E. coli O157:H7		*Ps. aeruginosa*		*S. aureus*		*S. epidermidis*		*St. pyogenes*		*P. acnes*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Houttuynia cordata	Water	62.5	250	62.5	125	-	-	62.5	125	62.5	125	-	-
	Ethanol	62.5	62.5	-	-	-	-	-	-	-	-	-	-
Momordica charantia	Water	-	-	-	-	-	-	62.5	62.5	-	-	-	-
	Ethanol	-	-	-	-	31.3	62.5	-	-	15.6	31.3	0.49	0.49
Phyllanthus amarus	Water	-	-	-	-	62.5	62.5	31.3	62.5	7.8	7.8	-	-
	Ethanol	-	-	-	-	31.3	62.5	31.3	62.5	7.8	15.6	7.8	7.8
Pluchea indica	Water	-	-	-	-	125	250	-	-	-	-	-	-
	Ethanol	-	-	-	-	31.3	62.5	-	-	15.6	31.3	-	-
Pseuderanthemum palatiferum	Water	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	62.5	125	62.5	125	62.5	125	62.5	125	15.6	31.3	-	-
Rhinacanthus nasutus	Water	-	-	-	-	62.5	125	-	-	-	-	-	-
	Ethanol	-	-	-	-	31.3	31.3	-	-	0.12	0.24	-	-
Schefflera leucantha	Water	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-
Senna alata	Water	-	-	-	-	62.5	62.5	62.5	125	0.12	0.12	0.49	0.49
	Ethanol	-	-	-	-	31.3	62.5	15.6	15.6	3.9	7.8	62.5	7.8

Table 3.14 (Continued)

Plant species	Extracts	MIC and MBC (mg/ml)											
		Bacteria											
		E. coli O157: H7		*Ps. aeruginosa*		*S. aureus*		*S. epidermidis*		*St. pyogenes*		*P. acnes*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Stemona sp.	Water	-	-	-	-	-	-	-	-	125	125	-	-
	Ethanol	-	-	-	-	-	-	-	-	125	125	-	-
Stephania venosa	Water	-	-	-	-	62.5	125	31.3	62.5	-	-	125	250
	Ethanol	15.6	62.5	31.3	62.5	31.3	62.5	1.9	3.9	0.12	0.12	1.9	3.9
Thunbergia laurifolia	Water	-	-	-	-	-	-	-	-	31.3	31.3	-	-
	Ethanol	-	-	-	-	31.3	31.3	31.3	62.5	7.8	7.8	-	-
Tinospora crispa	Water	-	-	-	-	-	-	-	-	31.3	62.5	-	-
	Ethanol	-	-	-	-	-	-	31.3	62.5	3.9	7.8	-	-
Vernonia cinerea	Water	-	-	-	-	62.5	250	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	31.3	125
Zingiber montanum	Water	-	-	-	-	-	-	-	-	125	125	-	-
	Ethanol	-	-	-	-	-	-	-	-	3.9	15.6	-	-
gentamycin	-	0.039	<0.039	0.039	<0.039	0.039	<0.039	0.15	0.30	0.12	0.12	0.06	0.06

Table 3.15 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of crude plant extracts against methicillin resistant *S. aureus* (MRSA) using broth dilution method

Plant species	Extracts	MIC and MBC (mg/ml)																			
		Bacteria																			
		MRSA 32		MRSA 43		MRSA 50		MRSA 64		MRSA 65		MRSA 66		MRSA 67		MRSA 72		MRSA 80		MRSA 82	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Andrographis	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
paniculata	Ethanol	125	250	125	250	62.5	125	-	-	125	125	125	250	125	250	62.5	125	62.5	125	125	250
Cissus	Water	-	-	-	-	-	-	-	-	-	-	125	250	-	-	-	-	-	-	-	-
quadrangularis	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coscinium	Water	62.5	125	31.3	62.5	62.5	125	15.6	31.3	31.3	125	62.5	125	1.9	7.8	31.3	31.3	15.6	31.3	15.6	15.6
fenestratum	Ethanol	31.3	62.5	62.5	125	62.5	125	15.6	31.3	62.5	125	62.5	125	1.9	7.8	62.5	125	15.6	31.3	15.6	15.6
Derris	Water	125	250	125	250	31.3	62.5	-	-	15.6	31.3	31.3	62.5	125	250	31.3	62.5	31.3	62.5	62.5	125
scandens	Ethanol	125	250	125	250	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5	62.5	125	31.3	62.5	31.3	62.5	62.5	125
Eclipta	Water	125	250	125	250	125	250	125	250	-	-	62.5	125	31.3	31.3	125	250	62.5	125	125	250
prostrata	Ethanol	125	250	125	250	125	250	62.5	250	125	250	62.5	125	125	250	125	250	62.5	125	125	250
Glycyrrhiza	Water	125	250	125	250	125	250	125	250	125	250	125	250	125	250	125	250	125	250	125	250
glabra	Ethanol	125	250	125	250	125	250	125	250	-	-	125	250	-	-	125	250	125	250	125	250
Gynostemma	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pentaphylum	Ethanol	62.5	125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hiptage sp.	Water	31.3	62.5	-	-	-	-	-	-	250	250	125	250	125	250	-	-	-	-	-	-
	Ethanol	62.5	125	31.3	62.5	62.5	125	62.5	125	7.8	7.8	31.3	62.5	62.5	125	62.5	125	15.6	31.3	31.3	62.5

Table 3.15 (Continued)

Plant species	Extracts	MIC and MBC (mg/ml)																			
		Bacteria																			
		MRSA 32		MRSA 43		MRSA 50		MRSA 64		MRSA 65		MRSA 66		MRSA 67		MRSA 72		MRSA 80		MRSA 82	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Houttuynia cordata	Water	125	250	62.5	125	125	250	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	125	250	62.5	125	125	250	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Momordica charantia	Water	-	-	-	-	-	-	125	250	250	250	62.5	250	-	-	-	-	-	-	-	-
	Ethanol	62.5	125	62.5	125	-	-	-	-	-	-	-	-	-	-	62.5	125	125	250	125	250
Phyllanthus amarus	Water	125	250	15.6	31.3	62.5	125	62.5	125	62.5	125	62.5	125	62.5	125	15.6	31.3	62.5	125	62.5	125
	Ethanol	31.3	62.5	31.3	62.5	15.6	62.5	31.3	62.5	31.3	62.5	31.3	62.5	62.5	125	15.6	31.3	7.8	15.6	62.5	125
Pluchea indica	Water	125	250	125	250	125	250	62.5	125	250	250	125	250	125	250	125	250	125	250	125	250
	Ethanol	62.5	125	62.5	125	-	-	-	-	-	-	-	-	-	-	62.5	125	-	-	-	-
Pseuderanthemum palatiferum	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	125	250	31.3	62.5	15.6	31.3	31.3	31.3	31.3	31.3	62.5	62.5	31.3	62.5	15.6	15.6	15.6	15.6	15.6	31.3
Rhinacanthus nasutus	Water	-	-	-	-	-	-	125	250	250	250	125	250	62.5	125	-	-	-	-	-	-
	Ethanol	15.6	31.3	15.6	31.3	15.6	31.3	-	-	-	-	31.3	62.5	31.3	62.5	15.6	31.3	15.6	31.3	31.3	62.5
Schefflera leucantha	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Senna alata	Water	15.6	31.3	7.3	15.6	7.3	15.6	-	-	15.6	15.6	31.3	62.5	31.3	31.3	7.3	15.6	62.5	125	31.3	31.3
	Ethanol	62.5	125	62.5	125	62.5	125	62.5	125	31.3	62.5	15.6	31.3	31.3	62.5	31.3	62.5	62.5	125	62.5	125

Table 3.15 (Continued)

Plant species	Extracts	MIC and MBC (mg/ml)																			
		Bacteria																			
		MRSA 32		MRSA 43		MRSA 50		MRSA 64		MRSA 65		MRSA 66		MRSA 67		MRSA 72		MRSA 80		MRSA 82	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Stemona sp.	Water	-	-	-	-	-	-	-	-	62.5	250	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stephania venosa	Water	-	-	-	-	-	-	62.5	250	250	250	-	-	62.5	125	-	-	-	-	-	-
	Ethanol	0.48	0.97	7.81	15.6	15.6	31.3	31.3	62.5	31.3	62.5	31.3	62.5	31.3	62.5	0.97	1.95	3.9	7.8	15.6	31.3
Thunbergia laurifolia	Water	-	-	-	-	-	-	-	-	-	-	62.5	62.5	31.3	62.5	-	-	-	-	-	-
	Ethanol	31.3	62.5	31.3	62.5	62.5	125	-	-	31.3	125	31.3	125	31.3	62.5	62.5	125	31.3	62.5	62.5	125
Tinospora crispa	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	31.3	62.5	15.6	31.3	-	-	31.3	62.5	62.5	125	31.3	62.5	62.5	125	-	-	-	-	-	-
Vernonia cinerea	Water	125	250	-	-	125	250	62.5	125	-	-	31.3	125	62.5	125	-	-	-	-	-	-
	Ethanol	31.3	62.5	-	-	62.5	125	31.3	62.5	62.5	125	-	-	-	-	62.5	125	31.3	62.5	62.5	125
Zingiber montanum	Water	-	-	-	-	-	-	-	-	-	-	-	-	-	-	125	250	-	-	-	-
	Ethanol	125	250	125	250	125	250	-	-	-	-	-	-	-	-	125	250	31.3	62.5	-	-
Gentamycin	-	10	20	10	20	10	20	10	20	10	20	10	20	10	20	5	10	5	10	5	10

3) Comparison of solvents extraction

The success of the determination of bioactive activity from plant materials largely depended on the type of solvents used in the extraction procedure which might be extracted different biochemical compounds (Tiwari *et al.*, 2011). From the preliminary screening, it was found that the ethanolic extracts of *C. fenestratum* and *S. venosa* had high inhibitory effect on the wide range of tested bacteria. Therefore, *C. fenestratum* and *S. venosa* were further extracted with others solvents including methanol and dichloromethane for comparing which solvent gave the higher potent antibacterial activity. Methanol was selected because it commonly solubilized the wide range of compounds in medicinal plants higher than ethanol. Mostly methanol is used for extraction various polar compounds but certain group of non polar compounds was also soluble in methanol. Moreover, dichloromethane was nonpolar solvent and had ability to dissolve a wide range of organic compounds thus usually used as useful solvent for many chemical processes (Tiwari *et al.*, 2011). The results of antibacterial activity of *C. fenestratum* and *S. venosa* by different solvents were shown in Table 3.16-3.19 and Figure 3.14-3.15. The antibacterial activity from different extracts of this plant varied against the tested bacteria. Both aqueous extracts gave less antibacterial activities than other organic solvents extraction.

For *C. fenestratum*, antibacterial activity of methanol and dichloromethane extract were higher than ethanolic and aqueous extracts, respectively. The methanolic extract of *C. fenestratum* had the highest inhibitory effect against all Gram positive bacteria with inhibition zone ranging between 12.3-9.0 mm. MIC and MBC values of the methanolic extract ranging between 0.06-62.5 mg/ml. Dichloromethane extract showed inhibition zone ranging between 18.0-55.7 mm. MIC and MBC values of dichloromethane extract ranging between 0.06-31.3 mg/ml. In addition, the ethanolic extract gave the inhibition zone ranging between 11.3-50.0 mm. MIC and MBC values of the ethanolic extract ranging between 0.03-125 mg/ml. On the other hand, the aqueous extract gave the lowest inhibitory effect with inhibition zone ranging between 12.0-52.0 mm. MIC and MBC values of the aqueous extract ranging between 0.24-125 mg/ml. However, all extracts of *C. fenestratum* could not inhibit growth of *E. coli* O157: H7 and *Ps. aeruginosa*.

For *S. venosa*, methanolic, dichloromethane and ethanolic extracts could inhibit all tested bacteria while the aqueous extract could not inhibit *E. coli* O157: H7, *Ps. aeruginosa, St. pyogenes* and MRSA isolate number 66. The dichloromethane extract gave the highest antibacterial activity with inhibition zones ranging between 11.0-45.3 mm. MIC and MBC values of dichloromethane extract ranging between 0.12-62.5 mg/ml. Methanolic extract also showed high antibacterial activity with inhibition zone ranging between 9.3-35.7 mm. MIC and MBC values of the methanolic extract ranging between 0.12-62.5 mg/ml. The ethanolic extract of *S. venosa* gave the inhibition zone ranging between 8.3-35.0 mm. MIC and MBC values of the ethanolic extract ranging between 0.12-62.5 mg/ml. The aqueous extract gave the lowest antibacterial activity the inhibition zone ranging between 7.3-19.3 mm. MIC and MBC values of the aqueous extract ranging between 31.3-250 mg/ml.

The extraction of active ingredient compounds from plant material depended on the type of solvent used in the extraction procedure. The criteria for solvent selection depended on the target compounds to be extracted and the solvents should be non toxic, and not interfere with the bioassay (Parekh *et al.,* 2005; Majhenic *et al.,* 2007). There were various solvents that were frequently used in the extraction procedure such as water, ethanol, acetone, chloroform and ether (Tiwari *et al.,* 2011). In the present study, four solvents; water, ethanol, methanol and dichloromethane were used to extract based on their polarity. Although, water was universal solvent, which was used to extract phytochemical compounds with antimicrobial activity but plant extract from organic solvent had been found to give more consistent antimicrobial activity higher than aqueous extract. The plant extraction in alcohol usually gave high amount of polyphenol, while the extraction using water usually gave water soluble flavonoids mostly anthocyanins, which showed lower antibacterial activity. However, some water soluble phenolics showed important antioxidant compounds (Das *et al.,* 2010). In addition, the decrease in activity of aqueous extract could be ascribed from the polyphenol oxidase enzyme, which degraded polyphenols in water extracts, whereas in methanol and ethanol they were inactive. Additionally, water was easier to be contaminated with microbes compared to organic solvent (Lapornik *et al.,* 2005). Moreover, dichloromethane was an organic solvent with had intermediate polarity and usually had been used as extractants in various plant species. Previous study found that

the dichloromethane extract gave high antimicrobial activity (Ayepola and Adeniyi, 2008; Borroto *et al.*, 2010; Maobe *et al.*, 2013). In the present finding, dichloromethane extract also gave high potent of antibacterial activity. In our study, this result confirmed the result of previous study which reported that the plant extractions with organic solvents provided antibacterial activity stronger than extraction with water. Therefore, the methanolic extracts of both plant species was chosen for further study. Although, dichloromethane extract showed high antibacterial activity but low percentage yields was obtained, thus, dichloromethane extract was not suitable for further study used as solvent for extraction.

Table 3.16 Antibacterial activity of *C. fenestratum* in four different solvent extractions

Bacterial strains	Inhibition zone (mm)			
	aqueous extract	**ethanolic extract**	**methanolic extract**	**dichloromethane extract**
E. coli O157: H7	0	0	0	0
Ps. aeruginosa	0	0	0	0
S. aureus	15.0 ± 0.0	17.0 ± 0.0	21.0 ± 1.0*	20.0 ± 0
S. epidermidis	16.7 ± 0.6	17.7 ± 0.6	20.3 ± 0.6*	19.3 ± 0.6*
St. pyogenes	27.0 ± 0.0	31.0 ± 0.0	36.7± 0.6*	30.7 ± 2.1
P. acnes	52.0 ± 1.0	50.0 ± 0.0	59.0± 1.0*	55.7 ± 1.2
MRSA 64	17.3 ± 0.6	12.0 ± 0.0	17.7 ± 0.6	20.0 ± 0*
MRSA 65	12.0 ± 0.0	12.0 ± 0.0	12.3 ± 0.6	19.0 ± 1.0*
MRSA 66	19.7 ± 0.6*	11.3 ± 0.6	13.7 ± 0.6	18.0 ± 1.7*
MRSA 67	20.0 ± 0.0	17.0 ± 0.0	22.0 ± 1.7	23.7± 1.5

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using Post hoc Duncan test. (*, $P<0.05$) represent the significantly highest antibacterial activity in each column.

Table 3.17 Antibacterial activity of *S. venosa* in four different solvent extractions

Bacterial strains	Inhibition zone (mm)			
	aqueous extract	**ethanolic extract**	**methanolic extract**	**dichloromethane extract**
E. coli O157: H7	0	9.3 ± 0.6	9.3 ± 0.6	11.0 ± 0.0*
Ps. aeruginosa	0	8.3 ± 0.6	9.7 ± 0.6*	10.3 ± 0.6*
S. aureus	7.3 ± 0.6	12.7 ± 0.6	20.0 ± 1.0	21.3 ± 0.6*
S. epidermidis	10.3 ± 0.6	20.0 ± 0.1	20.3 ± 0.6	23.3 ± 1.2*
St. pyogenes	0	26.0± 1.0	30.7± 1.2*	24.0± 1.7
P. acnes	12.0 ± 0.0	35.0 ± 0.0	35.7± 1.2	45.3 ± 0.6*
MRSA 64	12.0 ± 0.0	17.7 ± 0.6	19.0 ± 1.7	22.7 ± 2.1*
MRSA 65	8.7 ± 0.5	20.7 ± 0.6	19.3 ± 0.6	22.7 ± 0.6*
MRSA 66	0	19.0 ± 1.0	19.7 ± 0.6	23.0± 1.0*
MRSA 67	19.3 ± 0.6	18.0 ± 0.0	20.3 ± 0.6	23.3 ± 2.1*

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using Post hoc Duncan test. (*, $P<0.05$) represent the significantly highest antibacterial activity in each column.

Table 3.18 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of methanolic and dichloromethane extracts of *C. fenestratum*

Bacterial strains	MIC and MBC (mg/ml)							
	aqueous extract		ethanolic extract		methanolic extract		dichloromethane	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli O157: H7	-	-	-	-	-	-	-	-
Ps. aeruginosa	-	-	-	-	-	-	-	-
S. aureus	15.6	31.3	1.9	7.8	7.8	15.6	1.95	3.9
S. epidermidis	1.9	3.9	1.9	3.9	0.48	0.48	0.95	1.95
St. pyogenes	1.9	1.9	0.12	0.49	0.24	0.49	0.24	0.24
P. acnes	0.24	0.24	0.03	0.06	0.06	0.06	0.06	0.06
MRSA 64	15.6	31.3	15.6	31.3	15.6	31.3	15.6	31.3
MRSA 65	31.3	125	62.5	125	31.3	62.5	15.6	31.3
MRSA 66	62.5	125	62.5	125	31.3	62.5	15.6	31.3
MRSA 67	1.9	7.8	1.9	7.8	15.6	31.3	15.6	31.3

Table 3.19 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of methanolic and dichloromethane extracts of *S. venosa*

Bacterial strains	MIC and MBC (mg/ml)							
	aqueous extract		ethanolic extract		methanolic extract		dichloromethane	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli O157: H7	-	-	15.6	62.5	7.8	15.6	3.9	7.8
Ps. aeruginosa	-	-	31.3	62.5	31.3	62.5	31.3	62.5
S. aureus	62.5	125	31.3	62.5	7.8	15.6	1.95	3.9
S. epidermidis	31.3	62.5	1.9	3.9	0.98	1.95	0.48	0.48
St. pyogenes	-	-	0.12	0.12	0.12	0.12	0.12	0.12
P. acnes	125	250	1.9	3.9	1.9	3.9	1.9	3.9
MRSA 64	62.5	250	31.3	62.5	31.3	62.5	15.6	31.3
MRSA 65	250	250	31.3	62.5	31.3	62.5	15.6	31.3
MRSA 66	-	-	31.3	62.5	31.3	62.5	15.6	31.3
MRSA 67	62.5	125	31.3	62.5	31.3	62.5	15.6	31.3

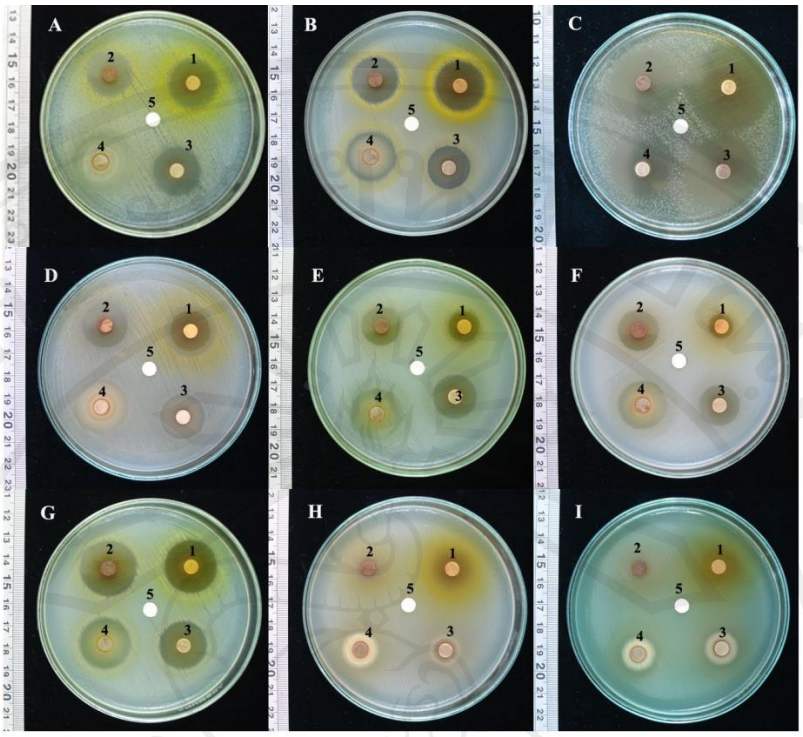


Figure 3.14 Antibacterial effect of methanolic and dichloromethane extracts of *C. fenestratum* (1, 2) and *S. venosa* (3, 4) on *S. aureus* (A), *S. epidermidis* (B), *St. pyogenes* (C), MRSA 64 (D), MRSA 65 (E), MRSA 66 (F), MRSA67 (G), *E. coli* O157: H7 (H) and *Ps. aeruginosa* (I) by agar disc diffusion method

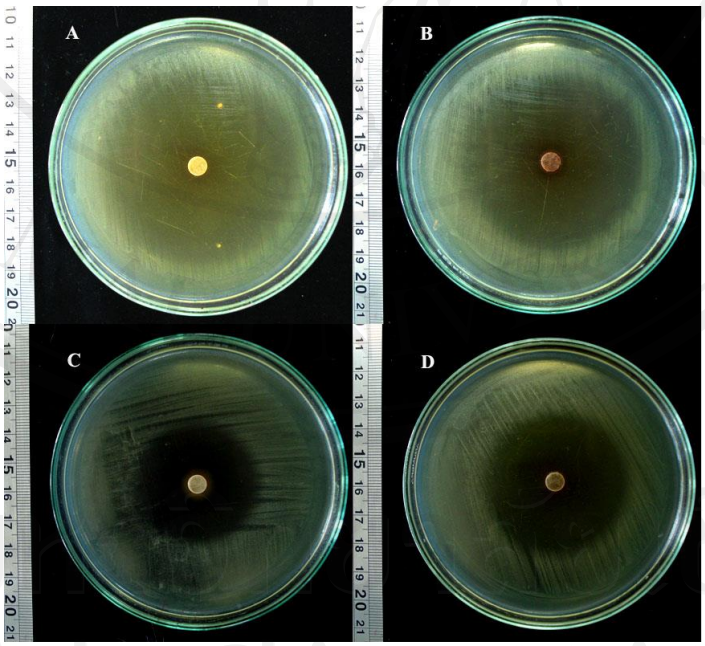


Figure 3.15 Antibacterial effect of methanolic and dichloromethane extracts of *C. fenestratum* (A, B) and *S. venosa* (C, D) on *P. acnes* by agar disc diffusion method

4) Time killing study of medicinal plant extracts against tested bacteria

Traditionally, dose and drug selection is based on a static *in vitro* laboratory MIC measurement which is used at a selected concentration and time. Although time-kill assays were labor intensive more than MIC and MBC assays, they were recognized to provide a greater degree of characterization of the bacterial cell eradication potential of antibacterial agent. However, time killing approach described the interaction between bacteria and antimicrobials in a dimensional way by a dynamic of integration of concentration of plant extracts and the exposure time on bacteria (Mueller *et al.*, 2004). In our study, the time-kill studies were performed during a period of 24 hours for *S. aureus*, *S. epidermidis*, *St. pyogenes* and MRSA or 72 hours for *P. acnes* at concentration of the plant extract at minimal inhibitory concentration (MIC). The plants which had high effective effect of antibacterial activity were selected to examine by time killing study. Three medicinal plants including *H. cordata*, *P. palatiferum* and *S. venosa* were selected to determine time kill kinetic against *E. coli* O157: H7, while *P. palatiferum* and *S. venosa* were used to inhibit *Ps. aeruginosa*. Moreover, five medicinal plants including *C. fenestratum, D. scandenns, P. amarus, S. alata* and *S. venosa* were selected to determine antibacterial activity of *S. aureus*, *S. epidermidis*, MRSA isolates number 64, 72 and 80, *St. pyogenes* and *P. acnes* by time killing study. Antibiotic, gentamycin was used as a positive control. Results were presented as a reduction of viable colony number and expressed as log CFU/ml (Figure 3.16-3.24). The percentages of inhibition after incubating with various plants extract were also shown in Table 3.20. All extracts exhibited different degrees of bactericidal and bacteriostatic activities of plant extracts on the tested bacteria.

The result showed that the log reduction in viable cell counted in all tested bacteria was decreased rapidly in different time after testing with the crude plant extracts. For time dependent inhibition, *E. coli* O157: H7 was completely inhibited after contacted with the ethanolic extracts of *H. cordata, P. palatiferum* and *S. venosa* within 4 - 6 hours, respectively. The ethanolic extracts of *P. palatiferum* and *S. venosa* extract could completely inhibit the growth of *Ps. aeruginosa* after incubating for 6 hours. The presence of ethanolic extracts of *C. fenestratum, D. scandens, P. amarus, S. alata* and *S. venosa* could inhibit the growth of *S.aureus* from 6-8 hours. The

ethanolic extract of *C. fenestratum, D. scandens, P. amarus, S. alata and S. venosa* displayed bactericidal activity on *S. epidermidis* from 4-10 hours after incubation. Resistant strain of *S. aureus*; MRSA isolates number 64, 72 and 80 were totally inhibited by the ethanolic extracts of the *C. fenestratum, D. scandens, P. amarus, S. alata* and *S. venosa* after incubating for 4-10 hours. Moreover, *St. pyogenes* was completely inhibited from 4-8 hours while *P. acnes* was completely inhibited after incubation 12-24 hours after incubating with *C. fenestratum, D. scandens, P. amarus, S. alata* and *S .venosa* (Table 3.20, Figure 3.16-3.24).

Table 3.20 Time killing of plant extract on pathogenic bacteria

Extract and tested bacteria / Hour	Inhibition (%)							
	0	**2**	**4**	**6**	**8**	**10**	**12**	**24**
***E. coli* O157:H7**								
H. cordata	0	99.60	99.88	100	100	100	100	100
P. palatiferum	0	99.97	100	100	100	100	100	100
S. venosa	0	95.83	99.94	100	100	100	100	100
Gentamycin	0	97.34	100	100	100	100	100	100
Ps. aeruginosa								
P. palatiferum	0	99.96	99.99	100	100	100	100	100
S. venosa	0	99.69	99.95	100	100	100	100	100
Gentamycin	0	100	100	100	100	100	100	100
S. aureus								
C. fenestratum	0	99.02	99.99	100	100	100	100	100
D. scandens	0	95.92	99.34	99.99	100	100	100	100
P. amarus	0	42.90	99.65	100	100	100	100	100
S. alata	0	98.03	99.72	100	100	100	100	100
S. venosa	0	96.41	99.38	99.99	100	100	100	100
Gentamycin	0	100	100	100	100	100	100	100

Table 3.20 (continued)

Extract and tested bacteria / Hour	Inhibition (%)							
	0	**2**	**4**	**6**	**8**	**10**	**12**	**24**
S. epidermidis								
C. fenestratum	0	96.98	100	100	100	100	100	100
D. scandens	0	83.77	99.74	99.89	99.99	100	100	100
P. amarus	0	92.80	99.88	99.99	99.99	100	100	100
S. alata	0	84.63	99.94	99.99	100	100	100	100
S. venosa	0	93.36	99.89	100	100	100	100	100
Gentamycin	0	99.97	100	100	100	100	100	100
MRSA 64								
C. fenestratum	0	99.91	100	100	100	100	100	100
D. scandens	0	97.15	99.84	99.99	100	100	100	100
P. amarus	0	97.50	99.90	100	100	100	100	100
S. alata	0	95.31	99.60	99.98	100	100	100	100
S. venosa	0	99.28	100	100	100	100	100	100
Gentamycin	0	96.74	99.78	100	100	100	100	100
MRSA 72								
C. fenestratum	0	85.73	99.92	99.99	100	100	100	100
D. scandens	0	98.05	100	100	100	100	100	100

Table 3. 20 (continued)

Extract and tested bacteria / Hour	Inhibition (%)							
	0	**2**	**4**	**6**	**8**	**10**	**12**	**24**
P. amarus	0	92.80	99.88	99.99	99.99	100	100	100
S. alata	0	84.63	99.94	99.99	100	100	100	100
S.venosa	0	93.36	99.89	100	100	100	100	100
Gentamycin	0	99.97	100	100	100	100	100	100
MRSA 80								
C. fenestratum	0	98.74	100	100	100	100	100	100
D. scandens	0	95.72	97.90	99.44	99.95	99.99	100	100
P. amarus	0	96.22	98.91	99.78	99.96	99.99	100	100
S. alata	0	95.49	99.87	100	100	100	100	100
S. venosa	0	84.63	99.48	99.98	100	100	100	100
Gentamycin	0	96.10	99.98	100	100	100	100	100
St. pyogenes								
C. fenestratum	0	72.73	97.43	99.96	100	100	100	100
D. scandens	0	99.47	99.98	100	100	100	100	100
P. amarus	0	99.64	100	100	100	100	100	100
S. alata	0	18.18	88.67	99.96	100	100	100	100
S. venosa	0	63.64	99.07	99.99	100	100	100	100
Gentamycin	0	82.55	99.99	100	100	100	100	100

Table 3.20 (continued)

Extract and tested bacteria / Hour	Inhibition (%)												
	0	**6**	**12**	**18**	**24**	**30**	**36**	**42**	**48**	**54**	**60**	**66**	**72**
P. acnes													
C. fenestratum	0	97.25	99.89	100	100	100	100	100	100	100	100	100	100
D. scandens	0	99.42	100	100	100	100	100	100	100	100	100	100	100
P. amarus	0	70.83	99.00	99.95	100	100	100	100	100	100	100	100	100
S. alata	0	99.89	100	100	100	100	100	100	100	100	100	100	100
S. venosa	0	90.83	99.94	100	100	100	100	100	100	100	100	100	100
Gentamycin	0	95.17	100	100	100	100	100	100	100	100	100	100	100

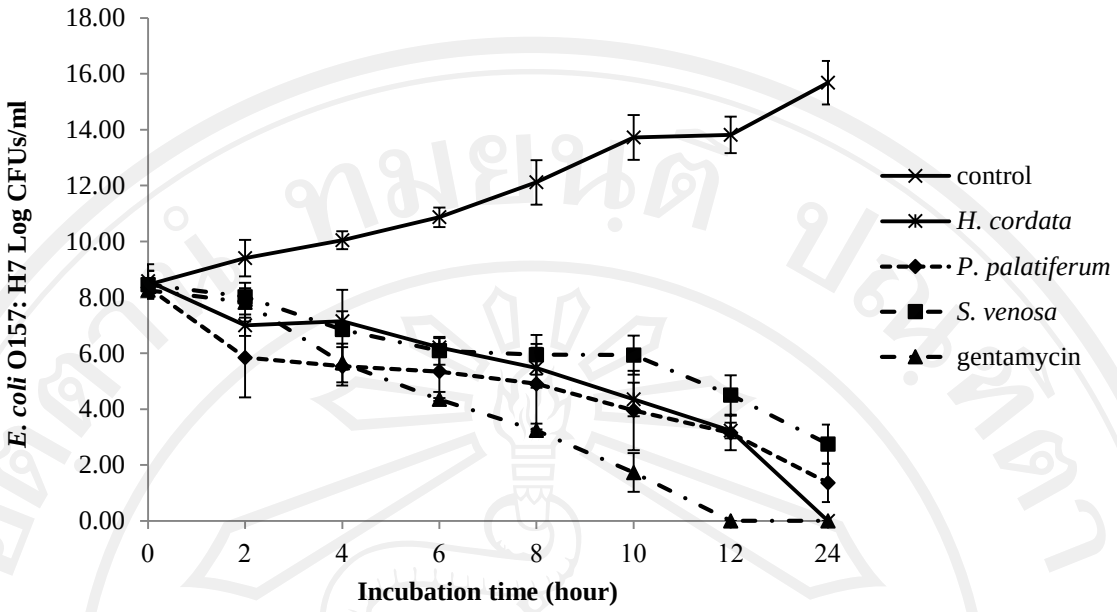


Figure 3.16 Time-killing curves of the bacterial growth of the *E. coli* O157:H7 after incubating with ethanolic extracts of plant extracts and gentamycin was used as a positive control

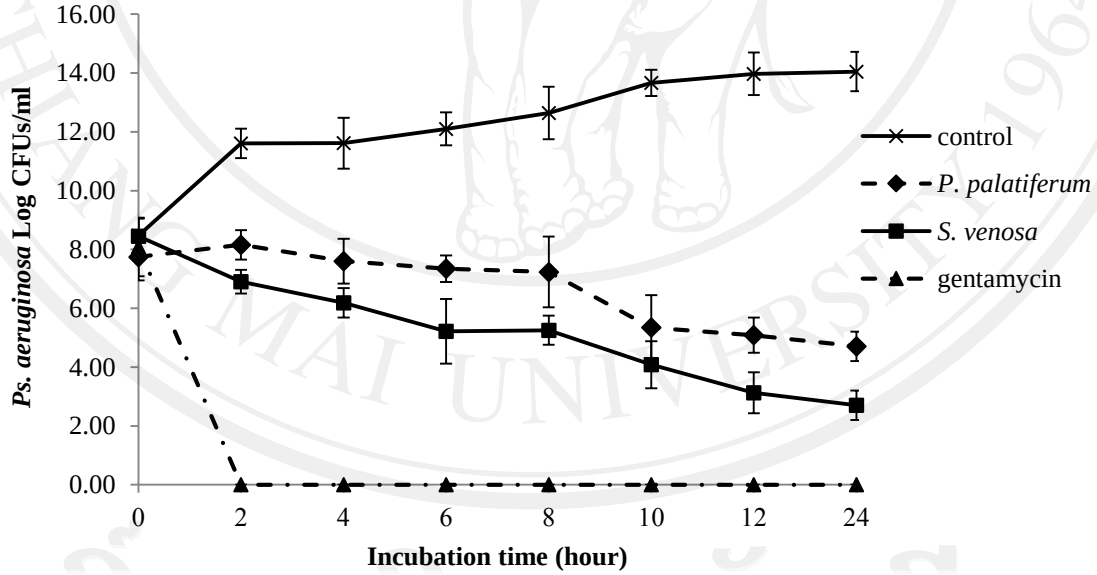


Figure 3.17 Time-killing curves of the bacterial growth of the *Ps. aeruginosa* after incubating with ethanolic extracts of plant extracts and gentamycin was used as a positive control

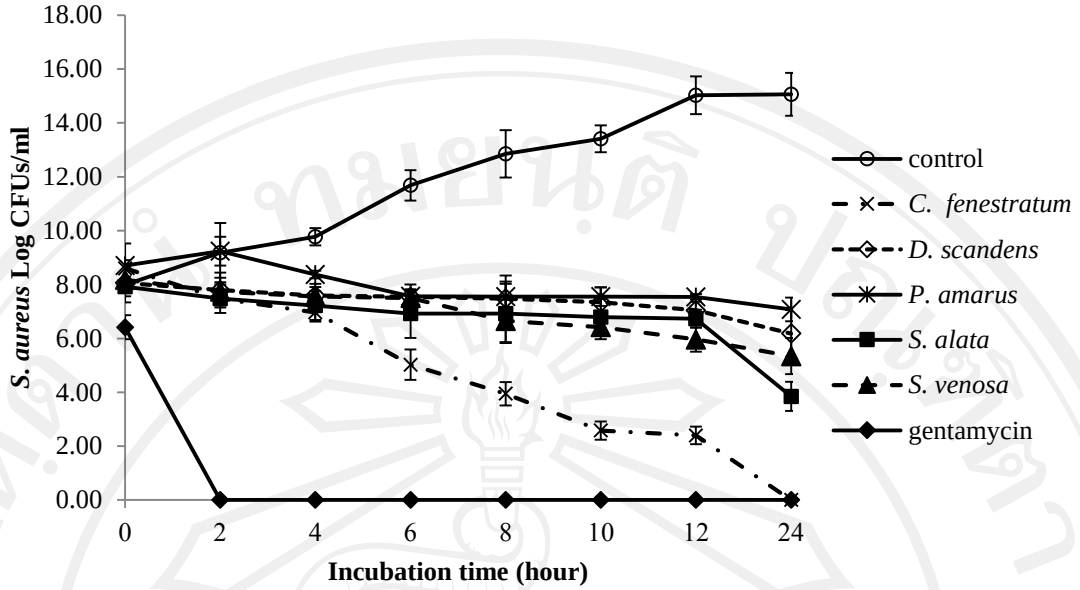


Figure 3.18 Time-killing curves of the bacterial growth of the *S. aureus* after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

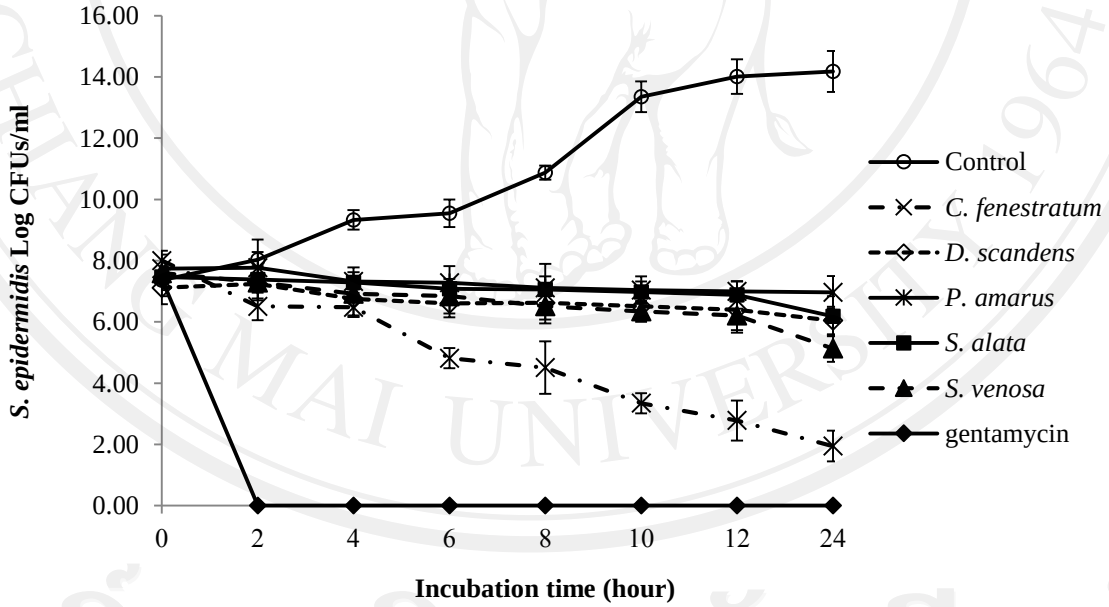


Figure 3.19 Time-killing curves of the bacterial growth of the *S. epidermidis* after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

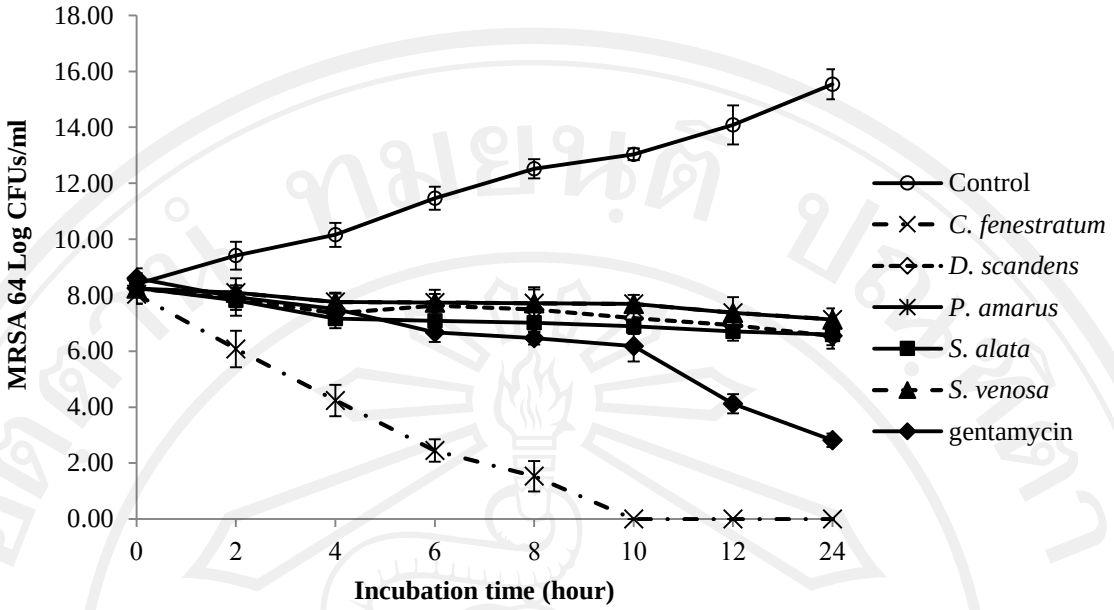


Figure 3.20 Time-killing curves of the bacterial growth of the MRSA 64 after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

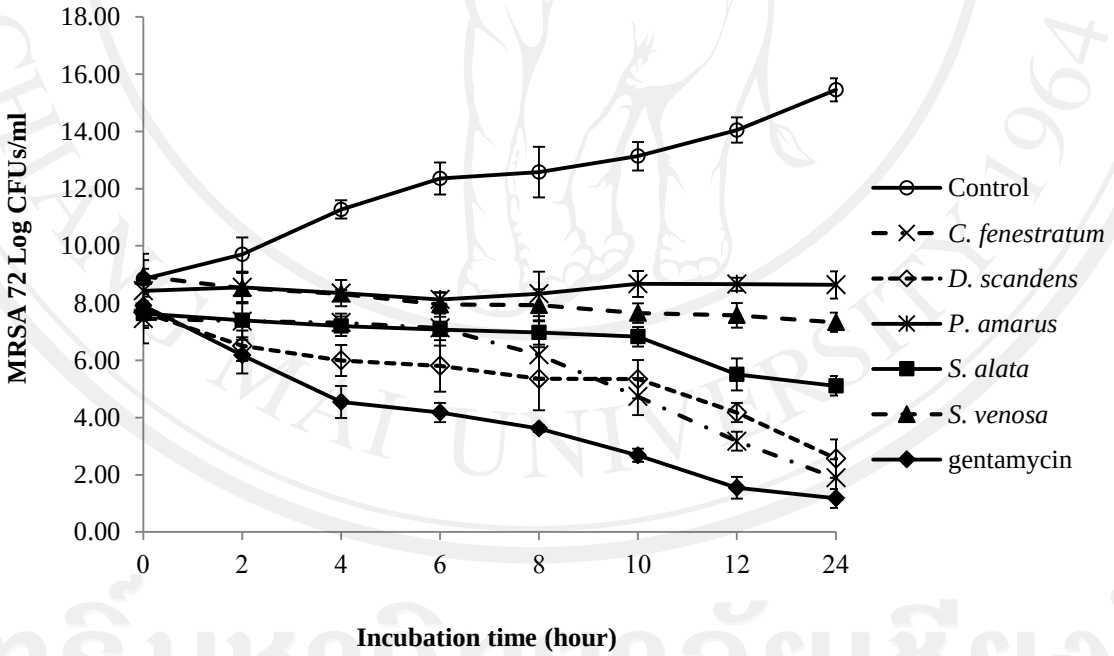


Figure 3.21Time-killing curves of the bacterial growth of the MRSA 72 after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

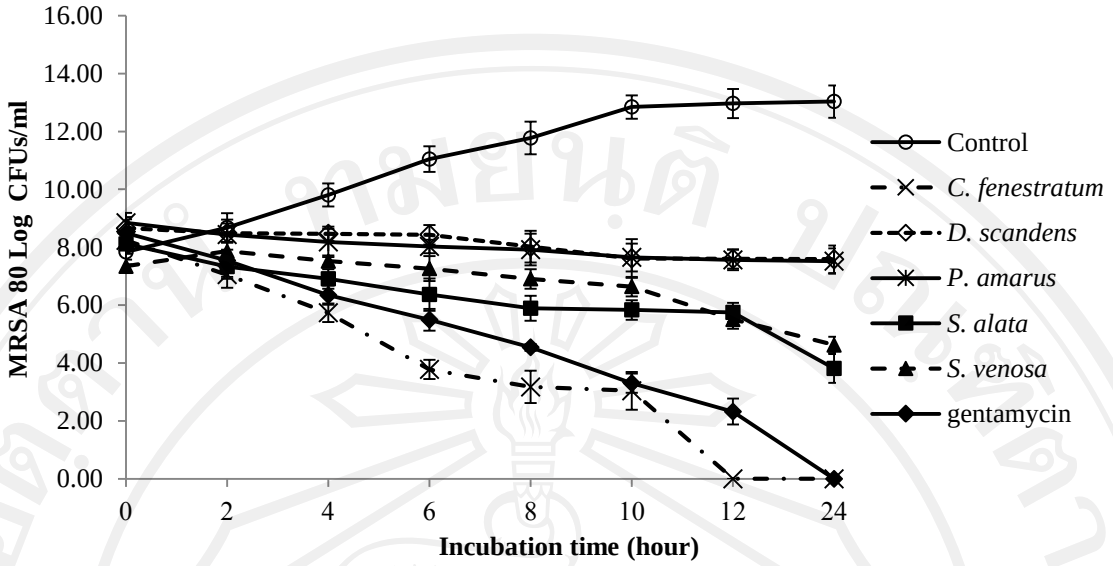


Figure 3.22 Time-killing curves of the bacterial growth of the MRSA 80 after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

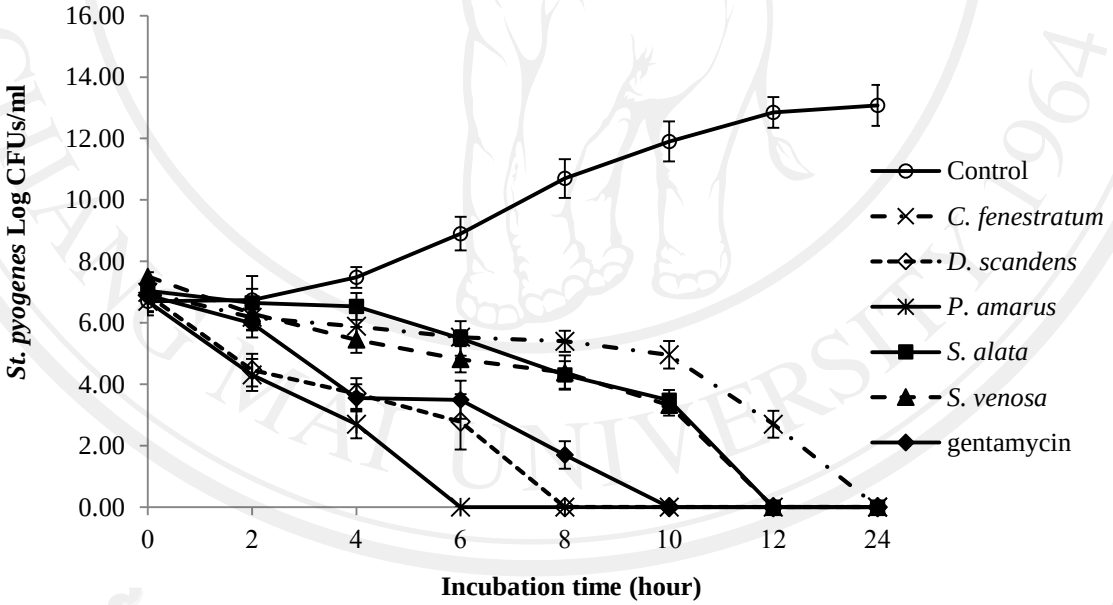


Figure 3.23 Time-killing curves of the bacterial growth of the *St. pyogenes* after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

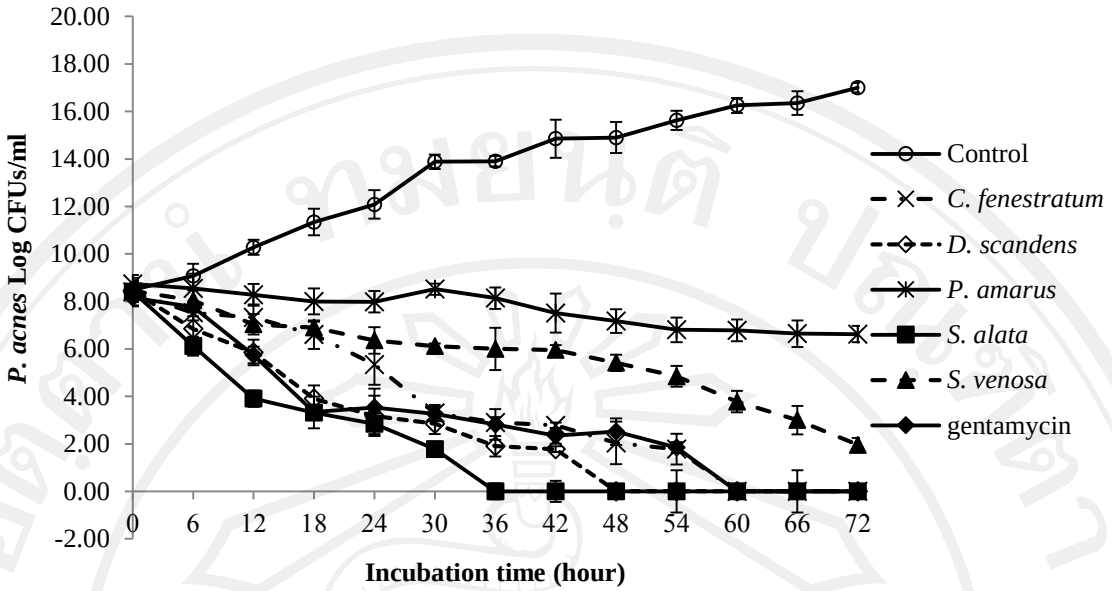


Figure 3.24 Time-killing curves of the bacterial growth of the *P. acnes* after incubating with ethanolic extracts of five plant extracts and gentamycin was used as a positive control

3.3.5 Determination of plant extracts on bacterial cell morphology using scanning electron microscope (SEM)

Scanning electron microscopy study was used to view general morphological changes of bacterial cells and surface alterations after exposure to plant extracts. Comparisons were made between bacterial cells control and the treated cells. In the present study, *S. aureus*, *S. epidermidis*, MRSA isolate number 64 and 80 were treated with ethanolic extract of *C. fenestratum* and *S. venosa* and only *E. coli* O157: H7 was treated with ethanolic extract of *S. venosa* at the concentration 1, 2 and 4 MIC, respectively. In the present study, it was found that the ethanolic extract of *S. venosa* at 4MIC concentration could affect to the cell morphology of *S. aureus* by showing membrane bleb or destruction (Figure 3.25). Moreover, after treatment *E. coli* O157: H7 with *S. venosa* extract, the significant morphological changes of this bacterium had been observed when compared to untreated control (Figure 3.29). The morphology of these cells revealed wrinkled abnormalities with numerous small clefts distributed around the cell surfaces. On the contrary, the cell size of *S. epidermidis*, MRSA isolate number 64 and 80 was not different between tested and controls after treatment of both plant extracts (Figure 3.26-3.28). Therefore, this research has shown

that crude extract of *S. venosa* killed *S. aureus* and *E. coli* O157: H7 by destroying their membranes. The main reason for this destruction was the severe alterations of the cell wall with the formation of holes, invaginations, and morphological disorganization caused by the extract. Many studies have reported that various antimicrobial agents have altered the morphology of bacterial cells. Anam *et al.* (2010) reported the effect of active component of *Terminalia muelleri* on *S. aureus* and MRSA cell morphology using SEM and TEM methods when compared with tetracycline HCl, penicillin G and vancomycin HCl. For SEM experiment, the active component caused shrinkage and thinning of the cell wall. The cell damage pattern which was caused by the active compound was similar to the damage caused by vancomycin HCl. Moreover, the TEM experiment showed appearance of MRSA bacterial cell after treated by the compound. The MRSA cell morphology such as larger nucleotide, the bigger vacuole, thickened cell wall and some lost part of the cells were observed.

In another study, the effect of some essential oils on bacterial cell was observed by SEM. The result showed formation of blebs, coagulation of cytoplasmic constituents, cell structure damage, and cytoplasmic material devoid (Becerril *et al.*, 2007). Similarly, SEM study of bacterial cells showed damage to the outer membrane and morphological changes of the cells when treated with eicosapen-taenoic acid and potassium salt of conjugated linoleic acid (Byeon *et al.*, 2009; Shin *et al.*, 2007). Bacterial cells exposed to carvacrol and thymol showed the disintegration of outer membrane of *E. coli* and *Sal. typhimurium*. Disruption of the cell wall with roughness and lacking of cytoplasm have been reported in *Listeria monocytogenes* when treated with thyme essential oil (Fisher and Phillips, 2008). Physiological and morphological changes of *E. coli* and *Sal. typhi* were observed by electron microscopy by mustard essential oil, which suggesting permeability of bacterial cells (Turgis *et al.*, 2009).

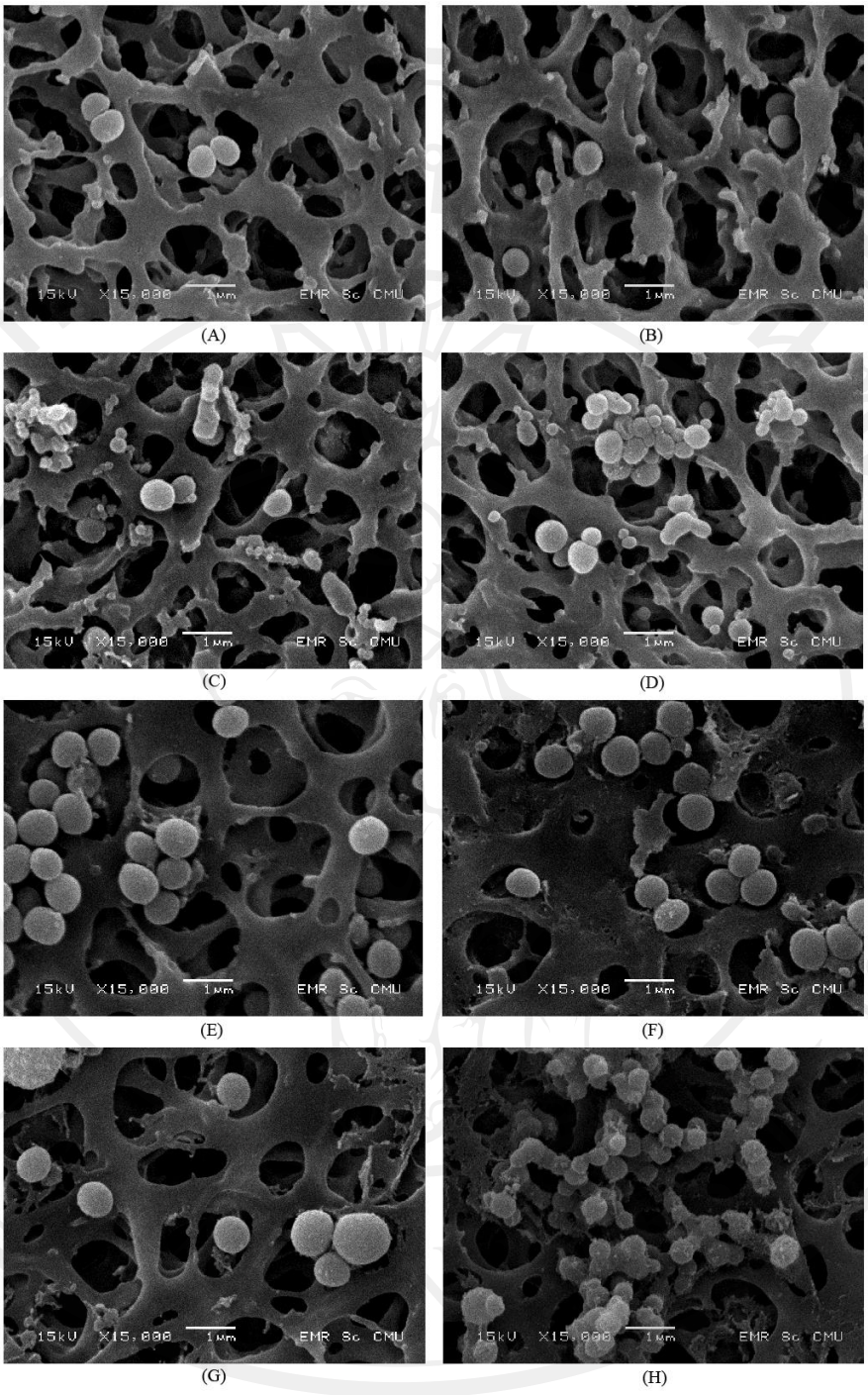


Figure 3.25 Scanning electron micrograph of *S. aureus* treated with ethanolic extract of *C. fenestratum* (A-D) and *S. venosa* (E-H) at 37 °C for 24 hours from different concentration; untreated control (A, E), 1MIC (B, F), 2MIC (C, G) and 4MIC (D, H)

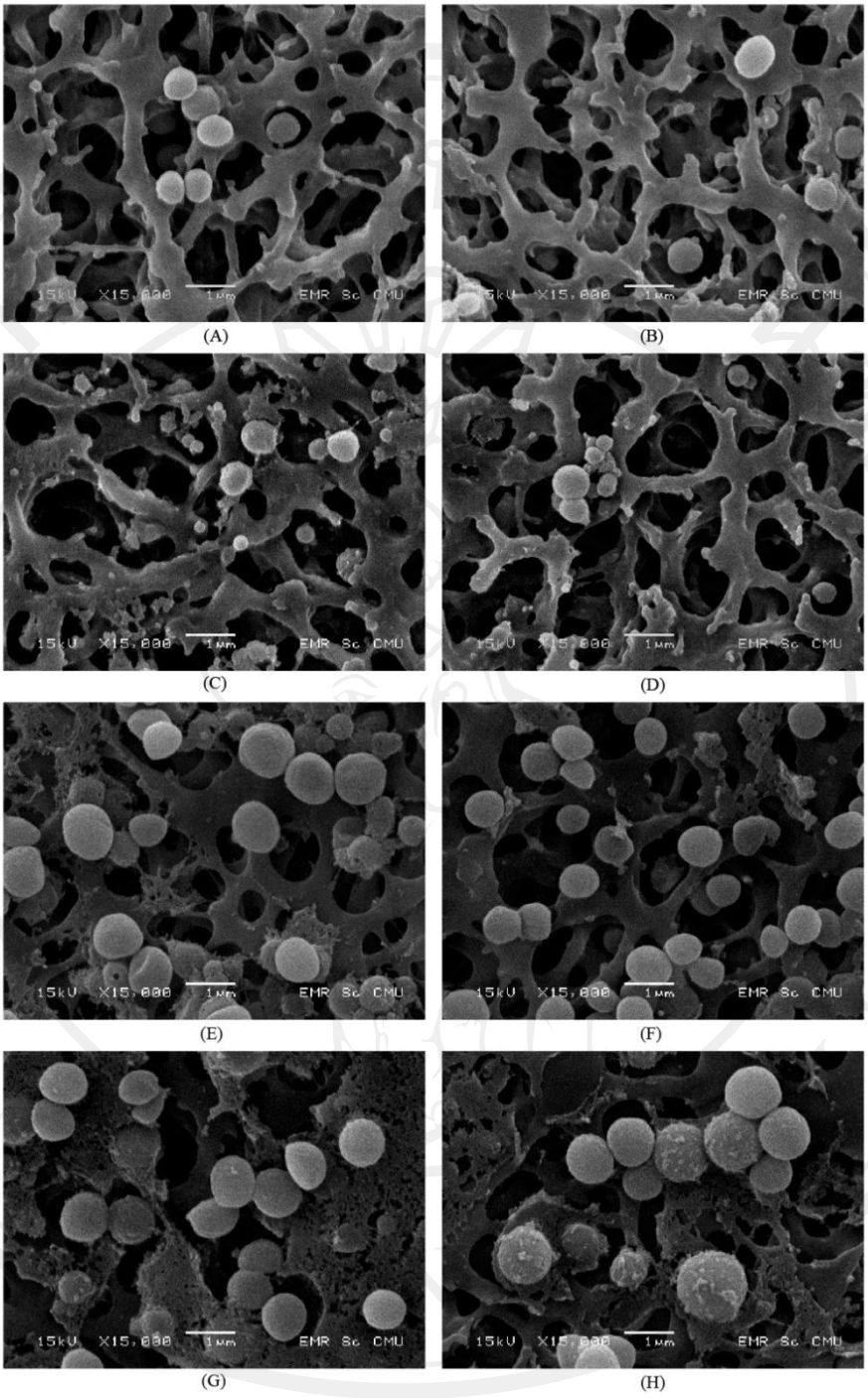


Figure 3.26 Scanning electron micrograph of *S. epidermidis* treated with ethanolic extract of *C. fenestratum* (A-D) and *S. venosa* (E-H) at 37 °C for 24 hours from different concentration; untreated control (A, E), 1MIC (B, F), 2MIC (C, G) and 4MIC (D, H)

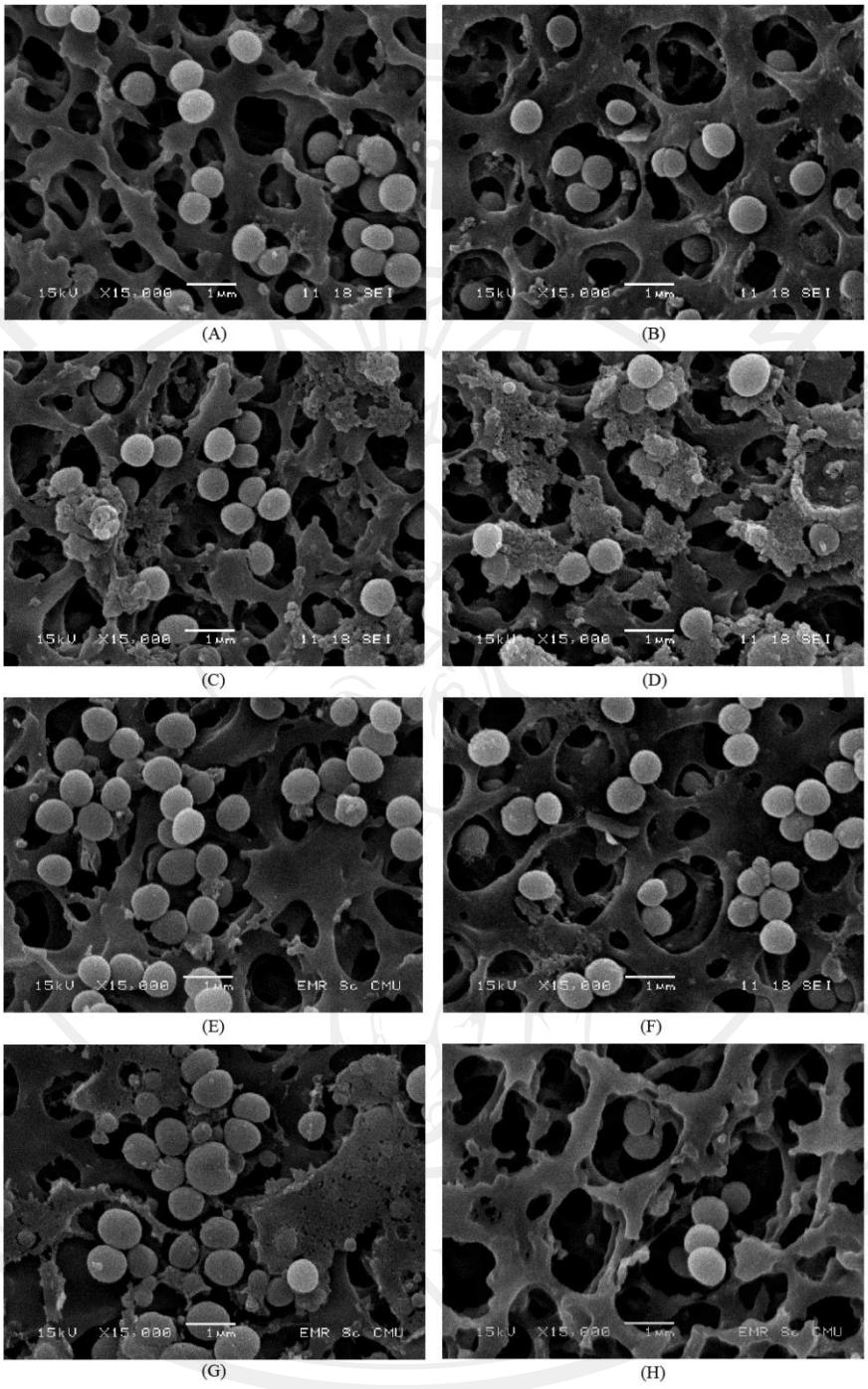


Figure 3.27 Scanning electron micrograph of MRSA 64 treated with ethanolic extract of *C. fenestratum* (A-D) and *S. venosa* (E-H) at 37 °C for 24 hours from different concentration; untreated control (A, E), 1MIC (B, F), 2MIC (C, G) and 4MIC (D, H)

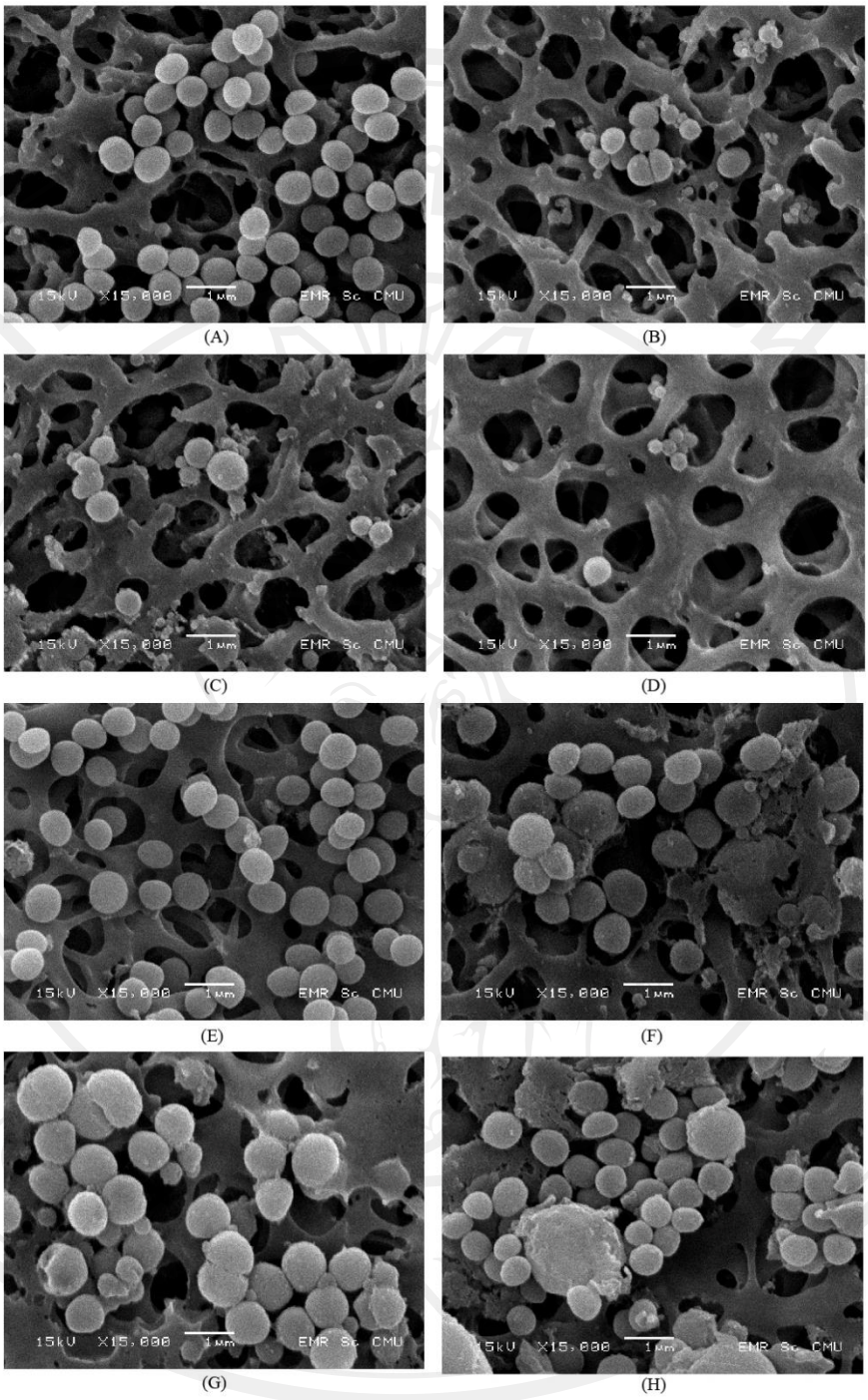


Figure 3.28 Scanning electron micrograph of MRSA 80 treated with ethanolic extract of *C. fenestratum* (A-D) and *S. venosa* (E-H) at 37 °C for 24 hours from different concentration; untreated control (A, E), 1MIC (B, F), 2MIC (C, G) and 4MIC (D, H)

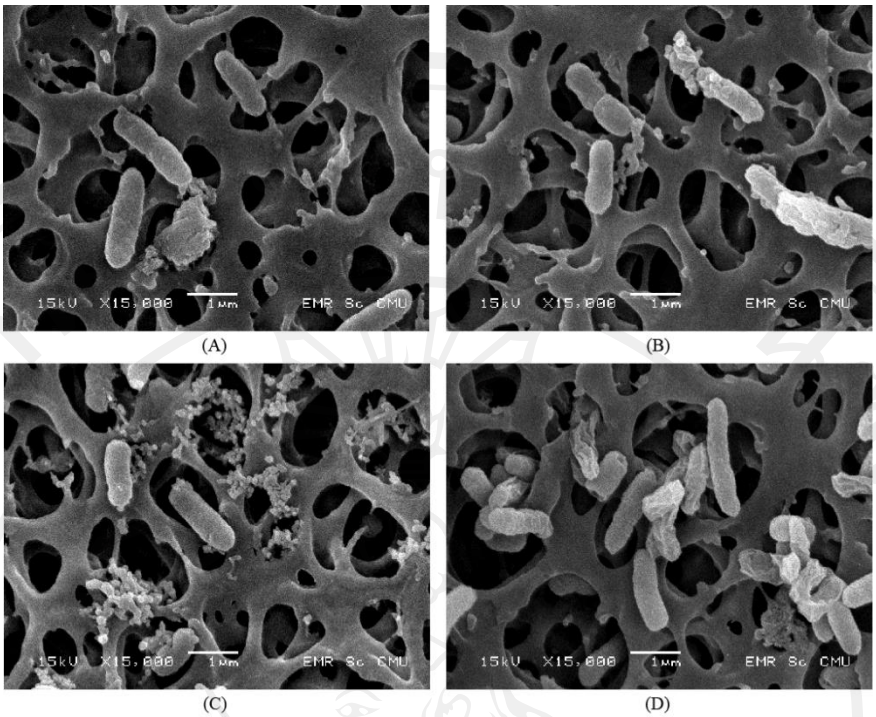


Figure 3.29 Scanning electron micrograph of *E. coli* O157:H7 treated with ethanolic extract of *S. venosa* at 37 °C for 24 hours from different concentration; untreated control (A), 1MIC (B), 2 MIC (C) and 4MIC (D)

3.3.6 Effect of *C. fenestratum* and *S. venosa* extracts on gene expression in *S. aureus* and MRSA

For many years, the emergences of MRSA were increasing dramatically. The increase in development resistance to new antibacterial agents leaded to dramatic changes in epidemiology and disease incidence. Numerous virulence factors produced by *S. aureus*, played a significant role in the pathogenesis of infection such as α-toxin, enterotoxins, toxic shock syndrome toxin 1 (TSST1), and cell wall-associated proteins. Therefore, there was an urgent need to develop new antimicrobial agents used for the prevention and treatment virulence factor that produced by these bacteria (Cheung *et al.*, 2004). In the present study, the effect of *C. fenestratum* and *S. venosa* extracts on the expression of α-toxin gene (*hla*), methicillin resistance gene (*mecA, mecR1*, *mecI*) and nucleaseA gene (*nucA*) were demonstrated. Quantitative real time PCR (qPCR) analysis was used to quantify mRNA levels of the investigated genes in *S. aureus* and MRSA cultures after treatment with some plant extracts. The gene expression level was normalized with *fabD*, which encoded malonyl CoA-acyl carrier protein transacylase. The

previous study showed that *fabD* was stably expressed in cultures grown in the presence of ethidium or berberine. Therefore, this housekeeping gene was suitable for use as an internal control to analyze the expression in MRSA (Theis *et al.*, 2007).

The transcriptional level of *hla* mRNA level in normal strain of *S. aureus* compared with resistant strains after treatment with 4 fold of minimal inhibitory concentration (MIC) of the ethanolic extracts of *C. fenestratum* and *S. venosa* was shown in Figure 3.30. The result showed that both *C. fenestratum* and *S. venosa* extracts decreased significantly in the expression levels of *hla* gene in *S. aureus* by 12.76 and 7.38 folds, respectively. Moreover, only *S. venosa* extract decreased *hla* expression level in MRSA 80 by 3.60 fold. By contrast, the ethanolic extract of *C. fenestratum* extract increased *hla* expression level of MRSA 80 by 1.20 fold but the induction was not statistical significance when compared to untreated control.

Recent studies were investigated the effect of antibiotic on the expression of some virulence factors in some microorganisms. Some antibiotics displayed an anti-virulence activity against toxin producing bacteria such as clindamycin and linezolid. These antibiotics are protein synthesis-suppressing antibiotics and recommended for the treatment of *S. aureus* produced toxic syndromes using concentrations below the MIC (Herbert *et al*., 2001; Bernardo *et al*., 2004). Previous reports revealed that subinhibitory concentrations of antibiotics can modulate the expression of virulence factors in *S. aureus* and thus may impact the outcome of severe staphylococcal infections. Otto *et al*. (2013) had studied the effect of antibiotic including clindamycin, daptomycin, linezolid, tigecycline and vancomycin on the expression of *pvl*, *hla* and *spa* which encoded Panton–Valentine leucocidin (PVL), α- hemolysin and protein A in community acquired methicillin resistant *S. aureus* (CA-MRSA) isolates, respectively. Their result showed that clindamycin and linezolid dramatically reduced mRNA levels of *pvl* and *spa* in all tested strains. Tigecycline also decreased the *pvl* and *spa* mRNA levels in some strains, whereas daptomycin and vancomycin had no significant effect. For *hla* mRNA transcription level, it was found that only clindamycin consistent suppressed mRNA expression. Linezolid, tigecycline and daptomycin could decrease mRNA expression, which depended upon tested bacteria, and vancomycin had no relevant effect. Therefore, it was indicated that the effect of sub MIC on the expression

of resistant gene was depended on the antibiotics and virulence factor. Additionally, there were numerous researchers studied the effect of natural product on the expression of toxin associated gene. Qiu *et al.*, 2010 found that the sub MIC of costal oil affected the expression of secreted virulence factor including α-toxin, staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin 1 (TSST-1) and accessory gene regulator A by decreasing the expression level. Qiu *et al*. (2011) found that perilla oil also decreased the expression level of α-toxin, SEA, SEB and TSST-1. Moreover, Leng *et al.* (2011) reported that allicin, which was an active compound from garlic could reduce the production of α-toxin in both mRNA and protein levels. On the other hand, β-lactam antibiotics such as methicillin had proven to be unfavorable because sub-inhibitory concentrations leaded to an increase in α-toxin expression (Ohlsen *et al.*, 1998).

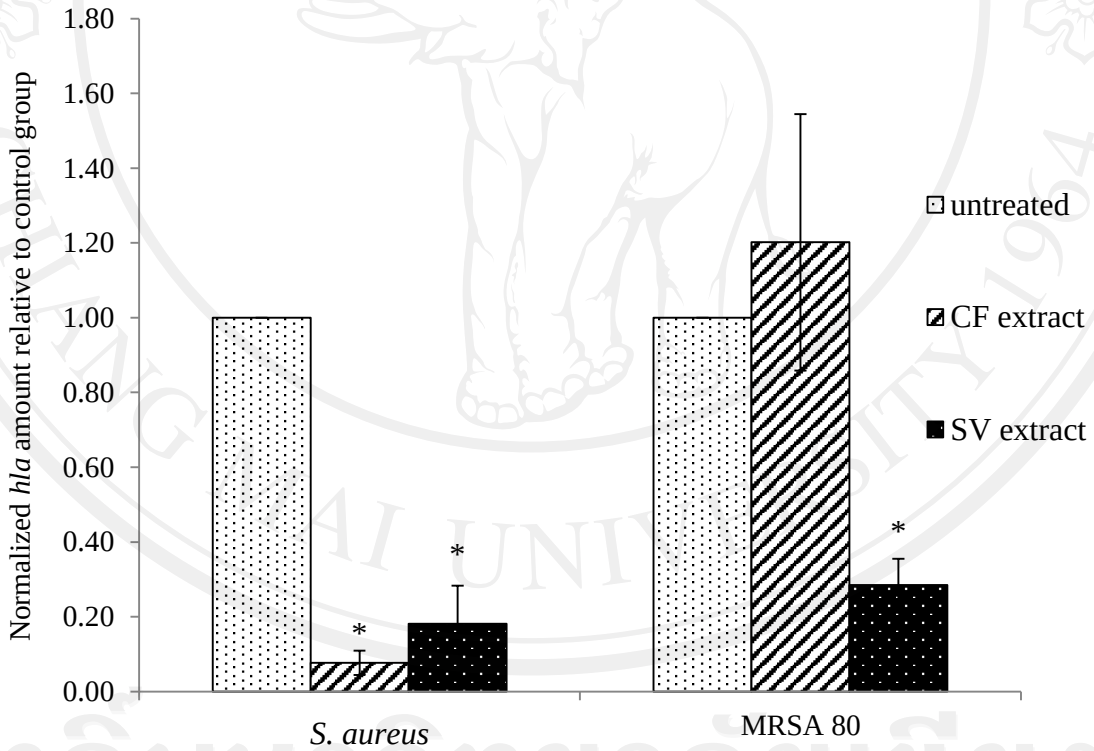


Figure 3.30 Effect of ethanolic extracts of *C. fenestratum* (CF) and *S. venosa* (SV) on the transcriptionof α- toxin gene (*hla*) in *S. aureus* and MRSA 80. Data are expressed as the means ± SD for three independent experiments.

Moreover, the emergence of MRSA strains were also depended on *mecA* gene which transcribed to penicillin binding protein 2a (PBP-2a) and resulted in the low affinity of β-lactam antibiotic. Expression of PBP-2a was controlled by two regulator genes on *mec* DNA including *mecI* and *mecR1*, located upstream of *mecA*, which encoded *mecA* repressor protein and signal transducer protein, respectively (Kobayashi *et al.,* 1998) (Figure 3.31). For the expression of the gene involved in β-lactam antibiotic, the ethanolic extracts of *C. fenestratum* and *S. venosa* induced a significant decrease in *mecR1* mRNA level by 1.97 and 1.79 fold (Figure 3.32). Moreover, both *C. fenestratum* and *S. venosa* extracts increased *mecI* mRNA transcription level by 2.22 and 2.07 fold but the induction was not statistical different. Therefore, from our result indicated that both *C. fenestratum* and *S. venosa* extracts could reduce significantly the expression of *mecR1*, which was a signal transducer gene. From the present study, it was hypothesized that these plants might affect the expression of *mecA* resistant gene in MRSA. Nevertheless, the result showed that *C. fenestratum* extract increased *mecA* transcription level by 7.52 fold while *S. venosa* extract could reduce to *mecA* expression level by 5.42 fold but the reduction was not statistical significance.

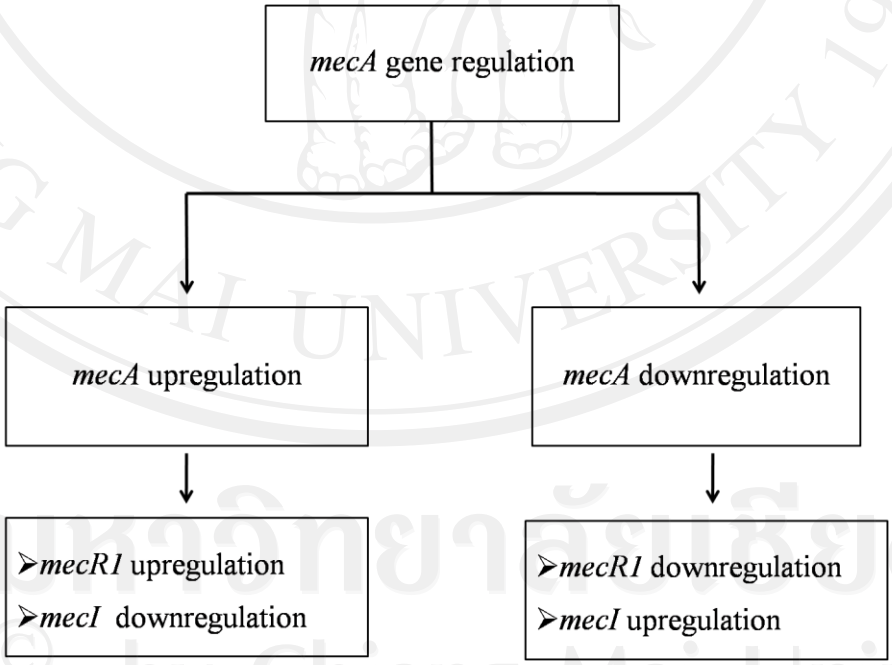


Figure 3.31 Schematic diagram of *mecA* expression regulation controlled by regulatory gene *mecR1* (transducer gene) and *mecI* (repressor gene)

Lee *et al*. (2007) also studied the antibacterial activity and the inhibition of resistant genes including *mecA, mecI, mecR1* and *femA* using reverse transcriptase polymerase chain reaction (RT-PCR) after treatment by *Saliva miltiorrhiza* extract. They found that the hexane fraction gave the highest levels of antimicrobial activity against *S. aureus* and MRSA. Moreover, hexane and chloroform fraction also exhibited the expression of *mecA, mecR1* and *femA* by dose dependent manner. Interestingly, this extract could induce or had no effect on the expression level of resistant gene when using at low concentration and completely inhibited when increase the concentration of extract. In 2009, Lee *et al*. also studied in another plant and they found that the chloroform fraction of *Glycyrrhiza uralensis* showed antimicrobial activity by 2.5 times higher than penicillin. Furthermore, chloroform fraction also correlated with methicillin resistant gene expression by inhibition of *mecA, mecR1* and *femA* expression but did not inhibit *mecI* expression. Some researchers reported that although *mecR1* and *mecI* largely control the expression of *mecA*, other additional genes may also regulate the expression of *mecA*. Previous study found that the expression of resistant gene in some MRSA strains were regulated by homologous the regulatory gene for *blaZ*. These gene, *mecI* and *mecR1*, which regulated *mecA* were similar to that regulation of *blaZ* by genes *blaI* and *blaR1*, thus this gene might be regulated *mecA* expression in MRSA (Lowy, 2003). From our finding, it was concluded that the ethanolic extract of *C. fenestratum* and *S. venosa* had the antibacterial activity against MRSA by decreasing *mecR1* mRNA expression level and also reduced *mecI* mRNA expression. Moreover, the ethanolic extract of *S. venosa* did not show significant reduction of *mecA* transcription level and only *C. fenestratum* extract could increase *mecA* resistant gene level. Therefore, it was suggested that these extracts might inhibit the growth of MRSA by inhibition of RNA and protein synthesis by interfering to other metabolic pathways that involved *mec* gene expression.

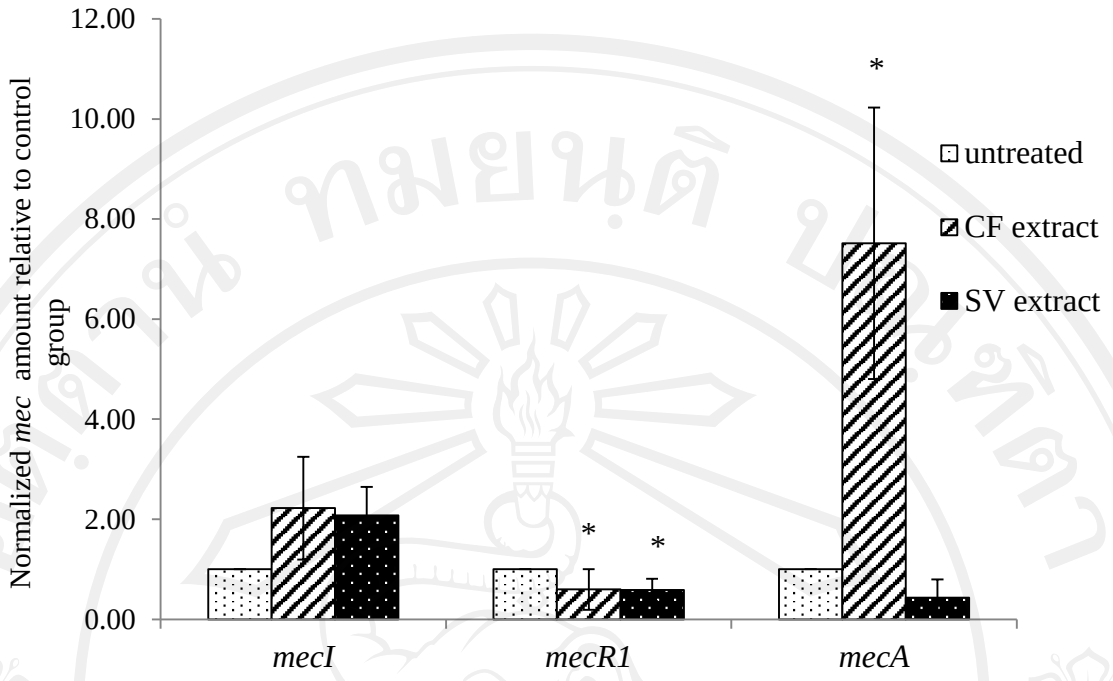


Figure 3.32 Effect of ethanolic extracts of *C. fenestratum* (CF) and *S. venosa* (SV) on the transcription of *mecI*, *mecR1* and *mecA* of MRSA 80. Data are expressed as the means ± SD for three independent experiments.

Furthermore, biofilm is one of the virulence factor that bacterial produced for protection against antibiotics due to the persistent of chronic infection (Yarwood *et al.* 2004). *S. aureus* secreted enzymes include thermostable nuclease (*nuc*), which was highly conserved among clinical isolates and had been used as a marker for direct detection of *S. aureus* in blood cultures. Furthermore, biofilm is one of the virulence factor that bacterial produced for protection against antibiotics. Consequently, the discovery of new anti-staphylococcal agents that could prevent adherence or biofilm formation remained an important challenge to the sciencetific community and would be of great use (Lagace-Wiens *et al.,* 2007). Nuc enzyme could inhibit the biofilm development, thus it was hypothesized that the induction of *nuc* gene might be affect biofilm development in bacteria. In this study, *C. fenestratum* and *S. venosa* extracts could affect *nucA* gene by significant increase the expression level by 2.21 and 2.40 folds, respectively (Figure 3.33). In addition, both *C. fenestratum* and *S. venosa* extracts also could increase significantly in *nucA* transcription level by 7.57 and 3.59

folds in MRSA. Therefore, it concluded that both *C. fenestratum* and *S. aureus* extracts could increase *nucA* expression in MRSA.

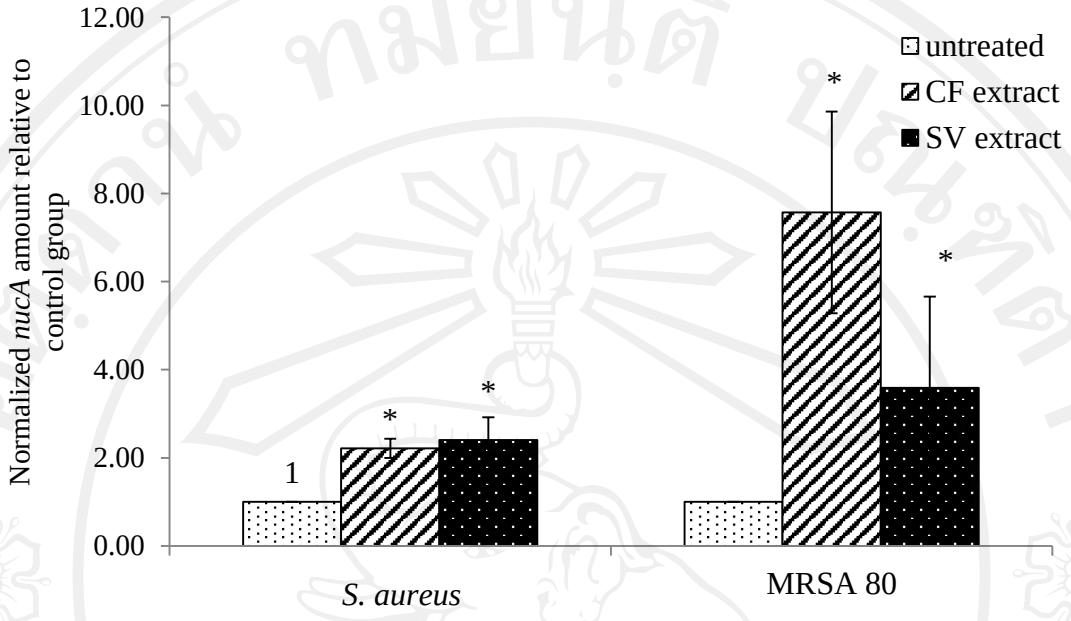


Figure 3.33 Effect of ethanolic extracts of *C. fenestratum* (CF) and *S. venosa* (SV) on the transcription of nucleaseA gene (*nuc*A) in *S. aureus* and MRSA 80. Data are expressed as the means ± SD for three independent experiments.

3.3.7 Effect of *C. fenestratum* and *S. venosa* extracts on PBP2a expression in MRSA

Penicillin binding proteins (PBPs) are a group of membrane bound enzymes, which catalyze carboxy peptidase or transpeptidase reactions in bacterial peptidoglycan synthesis. Peptidogycan is the major component of bacterial cell walls, which is essential to growth, cell division and maintaining the cellular structure in bacteria. Therefore, the inhibition of PBPs leads to abnormality in cell wall structure such as elongation, lesions, loss of selective permeability, and eventually cell death and lysis. PBPs are targets of β-lactam antibiotics. These antibiotics are structural analogs of the natural PBP substrate and inhibit transpeptidation domain of PBPs site thus interfering with the cross-linking reaction and, a loss of cell wall integrity (Stapleton and Taylor, 2002). Penicillin-binding protein 2a (PBP2a) has a low affinity to β-lactam antibiotic found on methicillin resistant strain. Therefore, the development and characterization

of new substances interfering with the activity of PBP2a proteins would be useful for methicillin resistance control.

In the present study, the effects of ethanolic extracts of *C. fenestratum* and *S. venosa* were determined on the synthesis of PBP2a proteins. Fifty microliter of total protein was loaded into 10% SDS-PAGE, then transfer to membrane and specified detect using horseradish peroxidase-conjugated IgG against anti-PBP2a. Protein from untreated bacteria was served as a positive control. The western blot results were shown in Figure 3.34-3.35 and the percentage inhibition of PBP2a protein was shown in Figure 3.36. The approximately PBP2a protein size was 76 kDa. The quantification of an image analysis system showed the intensity of PBP2a protein band after treatment with different concentration *C. fenestratum* extracts (0.031, 0.313, 3.13 mg/ml) were reduced by 0, 22.5 and 100 %, respectively. Moreover, the protein band intensity was also reduced after treatment with *S. venosa* extract at the concentration of 0.0039, 0.039 and 0.39 mg/ml by 6.75, 24.97 and 59.62 %, respectively. In the present study, it was found the multiple bands observed in Western blotting that might be occurred from other isoform of penicillin binding protein. Bacterial usually express several isoform of PBPs, each form had a different molecular weight. In *S. aureus*, four PBPs including PBP1 (85kDa), PBP2 (81 kDa), PBP3 (75 kDa) and PBP4 (45 kDa) had been identified and the extra PBP2a (76 kDa) also found in resistant strains (Labischinski, 1992).

Previous research found that the extended-spectrum cephalosporin, ceftobiprole, was developed specifically to bind to PBP2a of MRSA. Davies *et al.* (2007) determined the PBPs affinity of ceftobiprole in *S. aureus.* It was found that ceftobiprole had good affinity for PBP1, PBP2, PBP3, and PBP4 in a methicillin-susceptible *S. aureus* (MSSA) isolates and for PBP2a in an MRSA isolates. In 2007, Lee *et al.* reported that the hexane fractions of *Saliva miltiorrhiza* at 10 µg/ml concentration inhibited the expression levels of the resistant protein, PBP2a. Moreover, the combination of oxacillin and thioridazine could reduce the transcription of *mecA* gene and also reduced the protein level of PBP2a (Klitgaard *et al.*, 2008). On the other hand, some compounds could not inhibit the expression of PBP2a. Epigallocatechin gallate, which is an active compound in tea, had a good bactericidal activity against MRSA and methicillin-susceptible *S. aureus* (MSSA). However, this compound did not suppress

either PBP2a mRNA expression or PBP2a production, as confirmed by reverse transcription-PCR and a semiquantitative PBP2a latex agglutination assay (Zhao *et al.*, 2001). Some researcher found that sesquiterpene farnesol could not suppress the expression of PBP2a but significant reduced β-lactamase secretion (Kuroda *et al.*, 2006). Therefore, it was concluded that the ethanolic extracts of *C. fenestratum* and *S. venosa* inhibited the expression of the resistant protein, PBP2a. When the PBP2a level is lowered or the protein is inactive, the MRSA is not protected against plant extracts and the MRSA was killed. These results revealed that both plant extracts may be proved to be a valuable choice for studied target towards the development of antimicrobial agents.

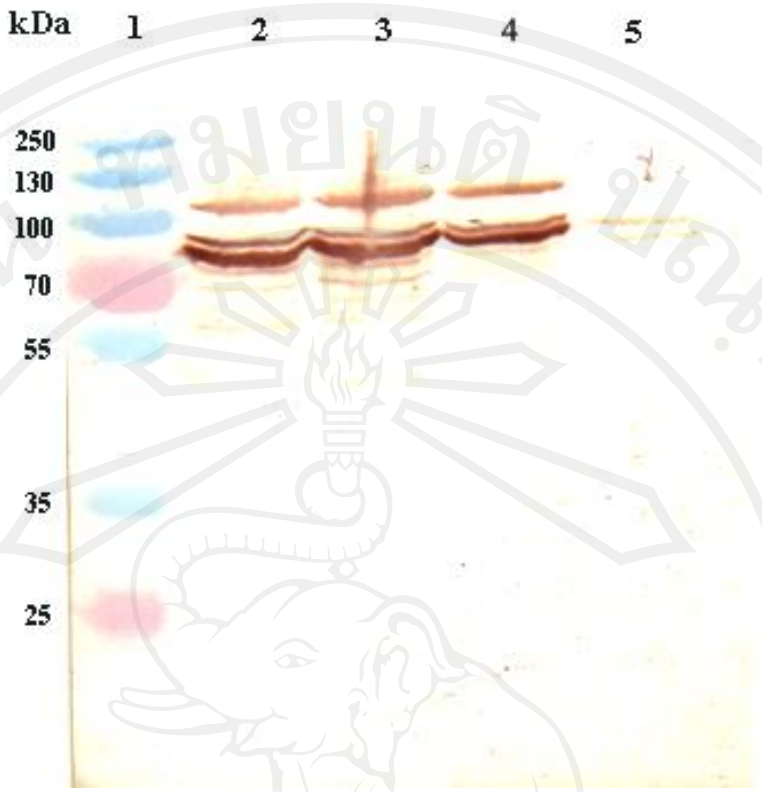


Figure 3.34 Effect of *C. fenestratum* extract on PBP2a expression in MRSA 80 detected by Western blotting analysis, PBP2a protein control (lane 2), PBP2a in the presence of *C. fenestratum* extract at concentration of 0.001 MIC (0.031 mg/ml) (lane 3), PBP2a in the presence of *C. fenestratum* extract at concentration of 0.01 MIC (0.313 mg/ml0 (lane 4) and PBP2a in the presence of *C. fenestratum* extract at concentration of 0.1 MIC (3.13 mg/ml) (lane 5) after detection by horseradish peroxidase-conjugated IgG against anti-PBP2a.

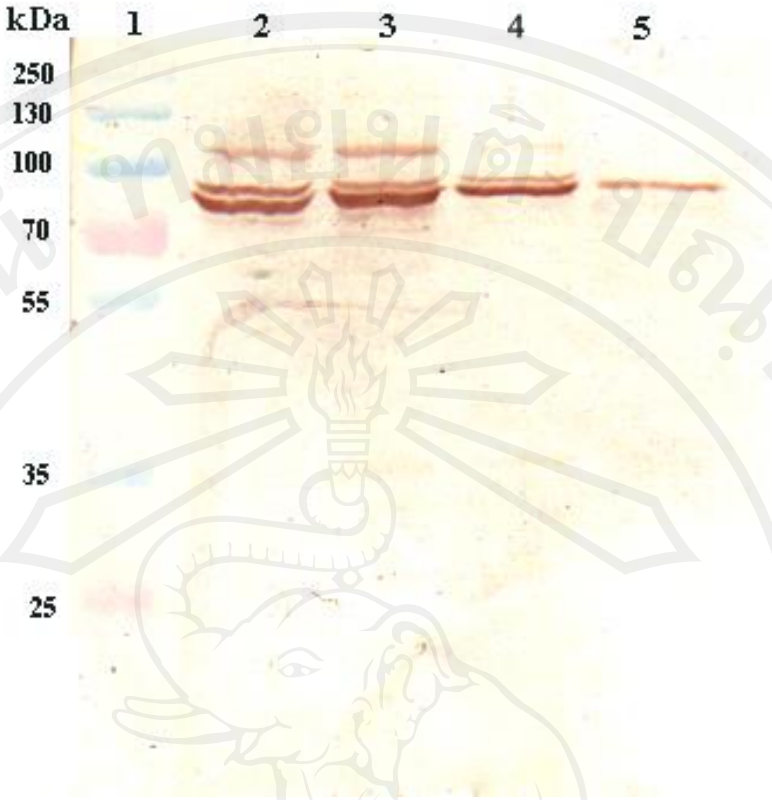


Figure 3.35 Effect of *S. venosa* extract on PBP2a expression in MRSA 80 detected by Western blotting analysis, PBP2a protein control (lane 2), PBP2a in the presence of *S. venosa* extract at concentration of 0.001 MIC (0.0039 mg/ml) (lane 3), PBP2a in the presence of *S. venosa* extract at concentration of 0.01 MIC (0.039 mg/ml) (lane 4) and PBP2a in the presence of *S. venosa* extract at concentration of 0.1 MIC (0.39 mg/ml) (lane 5) after detection by horseradish peroxidase-conjugated IgG against anti-PBP2a.

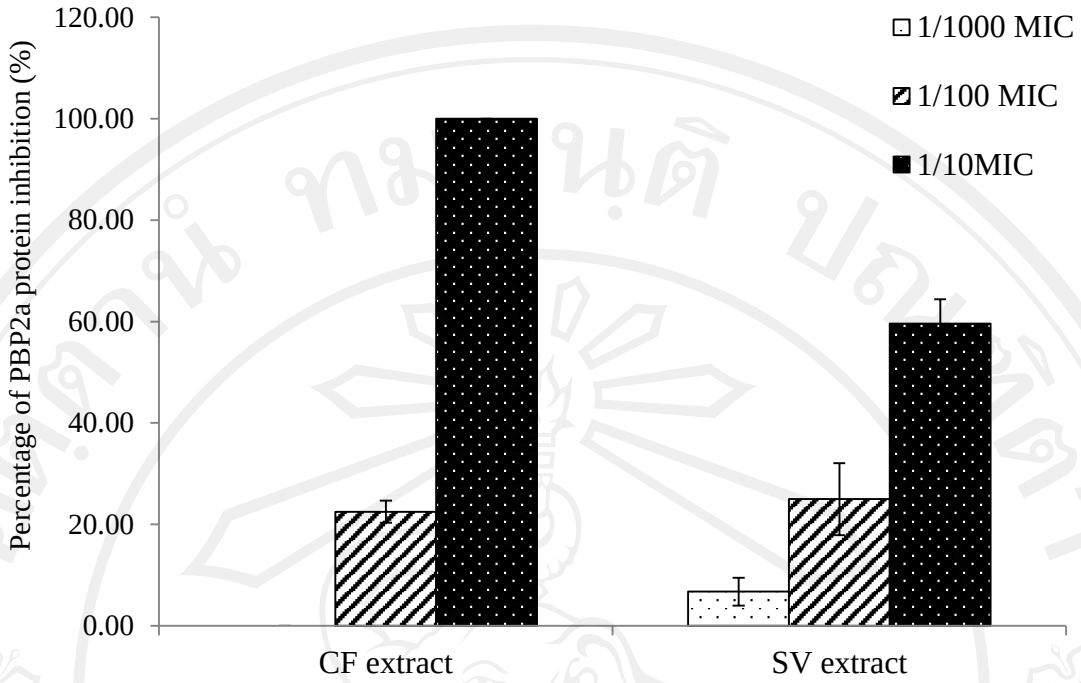


Figure 3.36 Percentage of PBP2a protein inhibition of MRSA 80 after treatment with *C. fenestratum* extract (CF) at concentration of 0.001 MIC (0.031 mg/ml), 0.01 MIC (0.313 mg/ml), 0.1 MIC (3.13 mg/ml) and *S. venosa* extract (SV) at concentration of 0.001 MIC (0.0039 mg/ml), 0.01 MIC (0.039 mg/ml) and 0.1 MIC (0.39 mg/ml).

3.3.8 Plant Isolation and their antibacterial activity

From preliminary study, two medicinal plants; *C. fenestratum* and *S. venosa* which showed the highest antibacterial activity, were selected for partition purification using partition technique and column chromatography. Then, an effective fraction was further screened for phytochemical including alkaloids, flavonoids, coumarins, saponins, cardiac glycosides, antraquinone glycosides, tannins and phenolics.

1) Isolation of bioactive fractions of *C. fenestratum*

The methanolic extract of *C. fenestratum* was fractionated by partition technique using *n*-hexane, chloroform, *n*-butanol and water as solvents to obtain four fractions (Figure 3.37).

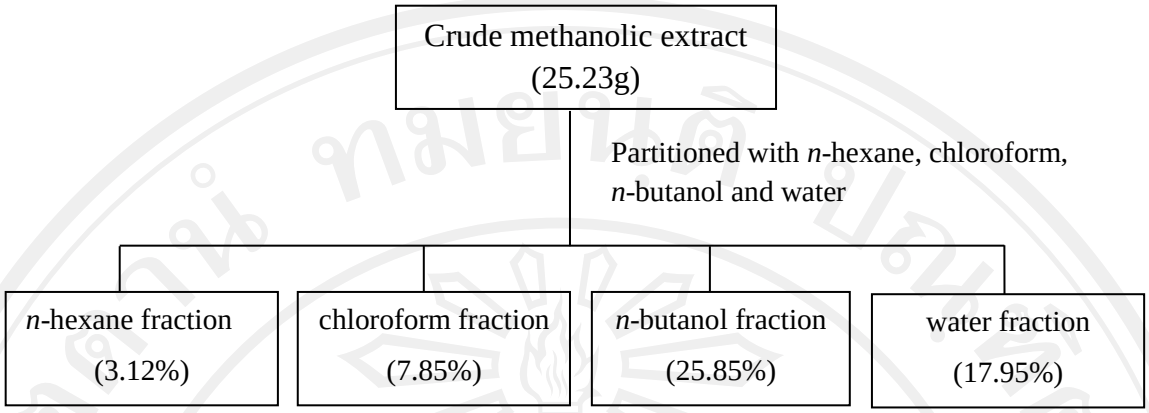


Figure 3.37 Schematic diagram shows the isolation procedure of *C. fenestratum* by partition technique.

After that, each fraction was further determined activities on antibacterial activity using agar disc diffusion and broth dilution methods. The result of preliminary screening was shown in Table 3.21-3.22 and Figure 3.38-3.39. It was found that chloroform, *n*-butanol and aqueous fractions showed great antibacterial activity against all Gram positive bacteria. The chloroform fraction could inhibit the growth of tested bacteria with inhibition zone ranging between 13.7-31.7 mm. The *n*-butanol fraction gave the inhibition zone ranging between 11.0-31.7 mm. Moreover, the aqueous fraction also had high antibacterial activity with inhibition zone ranging between 10.7-30.3 mm. MIC and MBC of chloroform, *n*-butanol and aqueous fractions was ranging between 0.06-125 mg/ml. The *n*-hexane fraction gave the lowest antibacterial activity against *S. aureus*, *St. pyogenes*, *P. acnes*, MRSA isolate number 64 and 67 with inhibition zone ranging between 8.0-22.7 mm. MIC and MBC of *n*-hexane fraction was ranging between 31.3-125 mg/ml.

Table 3.21 Effect of *C. fenestratum* fractions (500 mg/ml) on growth of pathogenic bacteria by agar disc diffusion method

Bacterial strains	**Zone of inhibition (mm) *± SD**			
	***n*-hexane fraction**	**chloroform fraction**	***n*-butanol fraction**	**aqueous fraction**
E. coli O157:H7	0	0	0	0
Ps. aeruginosa	0	0	0	0
S. aureus	8.0 ± 0.0	20.7 ± 1.5	21.0 ± 2.6	20.7 ± 2.1
S. epidermidis	0	19.3 ± 0.6	19.0 ± 1.7	18.0 ± 1.0
St. pyogenes	11.0 ± 1.0	31.7 ± 1.2	31.7 ± 4.9	30.3 ± 0.6
P. acnes	22.7 ± 1.5	29.7 ± 0.6*	27.3 ± 0.6	29.3 ± 0.6*
MRSA64	7.3 ± 0.6	13.7 ± 0.6*	12.0 ± 1.0	9.3 ± 0.6
MRSA65	0	14.0 ± 1.0*	11.0 ± 0.0	10.7 ± 0.6
MRSA66	0	18.0 ± 1.0	19.7 ± 0.6	18.0 ± 1.0
MRSA67	9.3 ± 0.6	21.7 ± 0.6	21.7 ± 0.6	20.7 ± 0.6

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using post hoc Duncan's test. (*) represent the significant difference values within each row ($P<0.05$)

Table 3.22 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *C. fenestratum* fractions against pathogenic bacteria using broth dilution method

Bacterial strains	MIC and MBC (mg/ml)							
	***n*-hexane fraction**		**chloroform fraction**		***n* -butanol fraction**		**aqueous fraction**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E. coli o157:H7	-	-	-	-	-	-	-	-
Ps. aeruginosa	-	-	-	-	-	-	-	-
S. aureus	31.3	62.5	0.49	0.98	1.9	3.9	31.3	62.5
S. epidermidis	-	-	0.49	0.98	3.9	7.8	31.3	62.5
St. pyogenes	62.5	125	0.06	0.06	0.06	0.06	0.06	0.06
P. acnes	62.5	125	0.06	0.06	0.06	0.06	0.06	0.12
MRSA64	62.5	125	31.3	62.5	62.5	125	62.5	125
MRSA65	-	-	15.6	31.3	31.3	62.5	62.5	125
MRSA66	-	-	31.3	62.5	31.3	62.5	62.5	125
MRSA67	62.5	125	62.5	125	62.5	125	62.5	125

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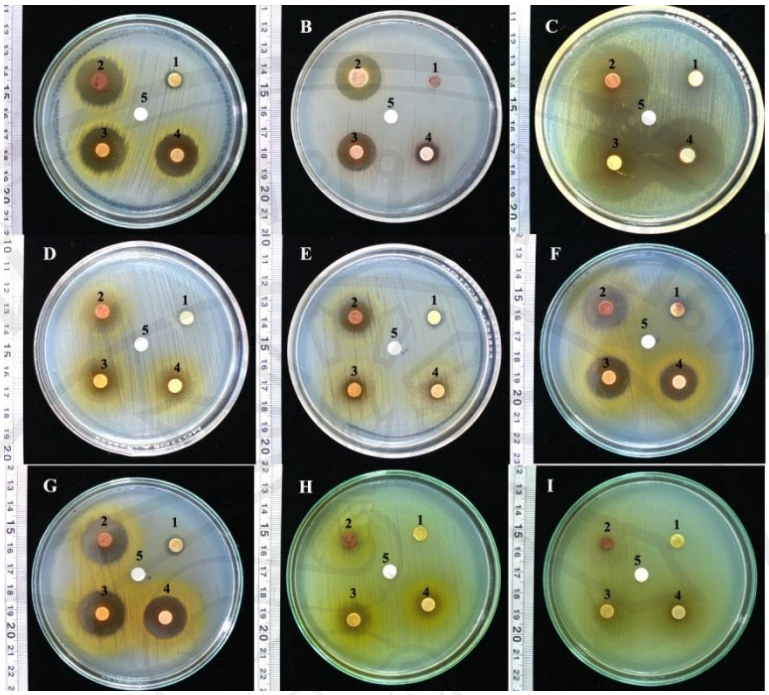

Figure 3.38 The effect of four fractions of *C. fenestratum* including *n*-hexane (1), chloroform (2), *n* - butanol (3) and aqueous (4) fractions on the growth of *S. aureus* (A), *S. epidermidis* (B), *St. pyogenes* (C), MRSA 64 (D), MRSA 65 (E), MRSA 66 (F) and MRSA 67 (G), *E. coli* O157: H7 (H) and *Ps. aeruginosa* (I) using agar disc diffusion method.

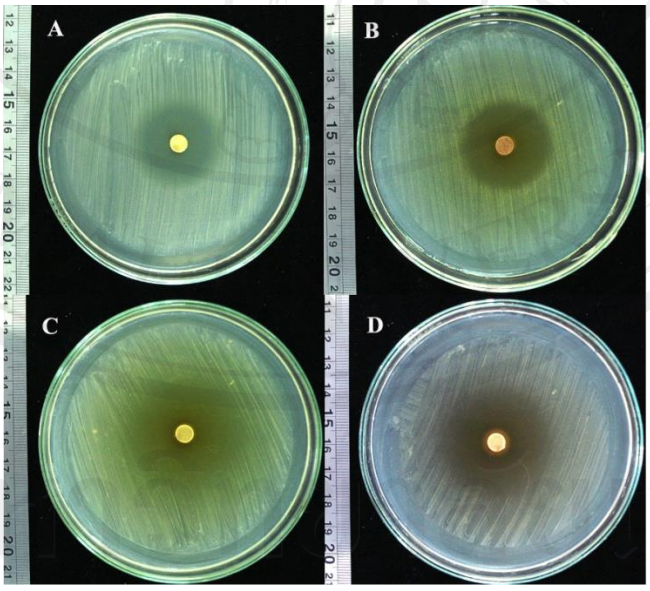

Figure 3.39The effect of partial purified fraction of *C. fenestratum*; *n*-hexane (A), chloroform (B), *n*-butanol (C) and aqueous (D) fraction on the growth of *P. acnes* by agar disc diffusion method.

Moreover, the crude methanolic extract of *C. fenestratum* was fractioned by column chromatography using dichloromethane, dichloromethane: ethanol and ethanol as a mobile phase to obtain three major fractions including CF01, CF02 and CF03 (Figure 3.40). For percentage yield recovery, the CF03 fraction showed the highest percentage yield of 79.22 % followed by CF01 and CF02 fraction with 10.52% and 5.62%, respectively.

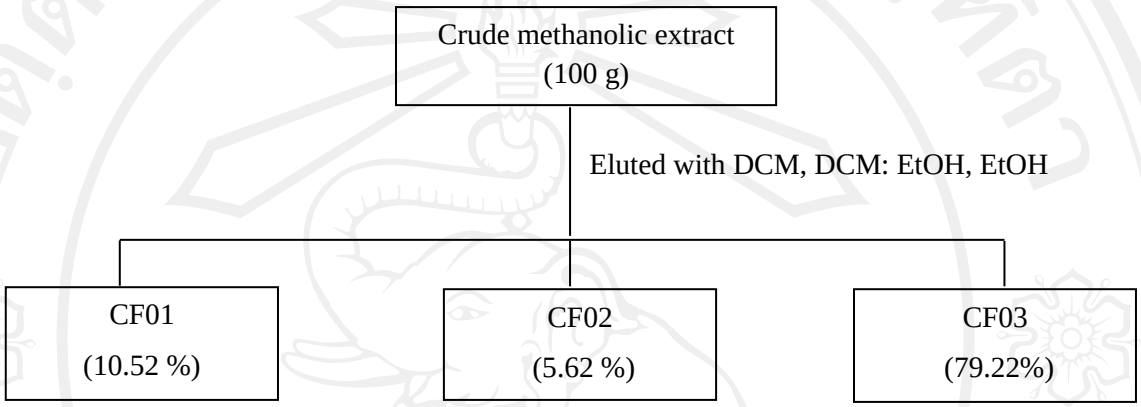

Figure 3.40 Schematic diagram shows the isolation procedure of *C. fenestratum* by column chromatography.

Thin layer chromatography (TLC) of each fraction was conducted comparative to berberine. The result showed that all three fractions gave similar TLC pattern with R_f values of 0.61 and 0.72 but showed a minor different in each fraction with R_f value lower than 0.61 in fraction CF02 and CF03. Berberine, active constituent found in *C. fenestratum*, gave the R_f value of 0.72. Therefore, all fractions consisted of berberine. After that, each fraction was further determined antibacterial activity using agar disc diffusion method. The result of preliminary screening indicated that the CF01 fraction had the highest antibacterial activity against almost tested bacterial species with inhibition zone ranging from 14.7-50.5 mm and the highest inhibitory was found on *P. acnes*. Moreover, CF02 and CF03 fractions also showed high antibacterial bacterial activity with inhibition zone ranging from 14.7-50.0 and 15.0-50.0 mm, respectively (Table 3.23-3.24 and Figure 3.41).

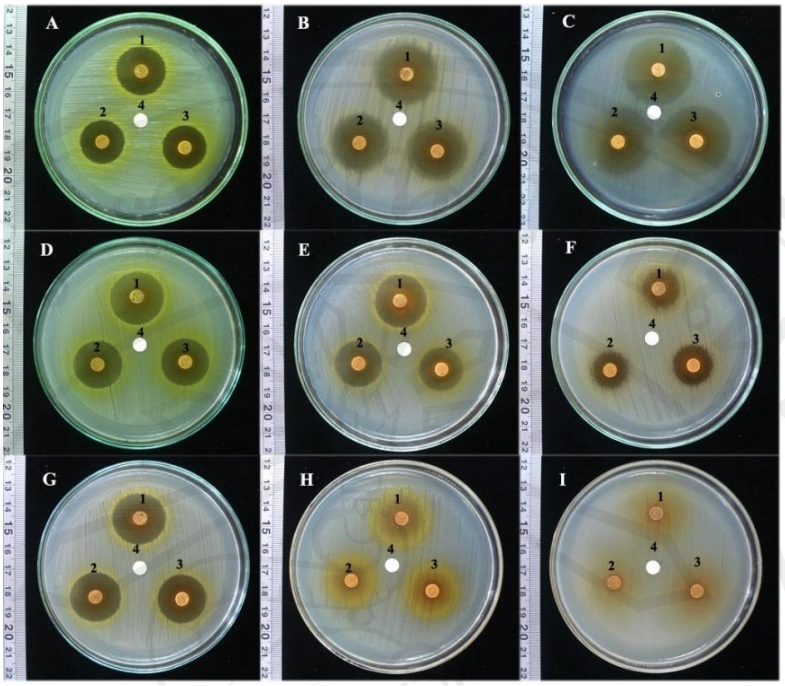

Figure 3.41The effect of four fractions of *C. fenestratum* including CF01 (1), CF02 (2) and CF03 (3) fractions on the growth of *S. aureus* (A), *S. epidermidis* (B), *St. pyogenes* (C), MRSA 64 (D), MRSA 65 (E), MRSA 66 (F) and MRSA 67 (G), *E. coli* (H) and *Ps. aeruginosa* (I) using agar disc diffusion method

Table 3.23 Effect of *C. fenestratum* fractions (500 mg/ml) on growth of pathogenic bacteria by agar disc diffusion method

Fraction	Zone of inhibition (mm)					
	Bacterial strains					
	***E. coli* O157: H7**	***Ps. aeruginosa***	***S. aureus***	***S. epidermidis***	***St. pyogenes***	***P. acnes***
CF01	0	0	25.7 ± 0.6*	21.7 ± 0.6*	31.5 ± 0.0*	50.5 ± 0.0*
CF02	0	0	22.0 ± 1.0	20.0 ± 0.0	30.5 ± 0.0	50.0 ± 0.6
CF03	0	0	22.7 ± 0.6	20.0 ± 0.1	29.0 ± 0.6	50.0 ± 0.0

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using post hoc Duncan's test. (*) represent the significant difference values within each column ($P<0.05$)

Table 3.24 Effect of *C. fenestratum* fractions (500 mg/ml) on growth of MRSA by agar disc diffusion method

Fractions	Zone of inhibition (mm)									
	Bacterial strains									
	MRSA32	**MRSA43**	**MRSA50**	**MRSA64**	**MRSA65**	**MRSA66**	**MRSA67**	**MRSA72**	**MRSA 80**	**MRSA82**
CF01	16.3 ± 1.5	15.7 ± 0.6*	14.7 ± 1.2	25.0± 1.0*	23.3± 1.2*	15.0 ± 0.0	23.3 ± 1.2	17.0 ± 0.0	23.7± 0.6*	23.0 ± 0.0*
CF02	14.7 ± 1.5	14.7 ± 0.6	14.7 ± 1.3	22.7 ± 1.2	20.3± 1.2	15.3 ± 0.6	21.7 ± 1.5	16.3 ± 0.6	21.0 ± 1.0	20.3 ± 0.6
CF03	15.0 ± 1.0	15.0 ± 0.0	15.3 ± 0.6	22.0 ± 1.0	19.7± 0.6	16.3 ± 0.6*	21.0 ± 1.0	16.3 ± 0.7	20.0 ± 0.0	20.7 ± 0.6

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using post hoc Duncan's test. (*) represent the significant difference values within each column ($P<0.05$)

After that, the CF01 fraction which had the highest antibacterial activity was further investigated for phytochemical constituents (Table 3.25). Alkaloids and phenolics were found as major active compounds in this fraction. Moreover, the ethanolic extract of *C. fenestratum* was analyzed volatile components by Gas chromatography/ Mass spectrometer (GC/MS). From GC chromatogram, five main compounds were found. The major components are berberine (34.13%, RT=34.43), quinic acid (24.47%, RT=20.59), methoxy phenol (2.78%, RT=17.45), 1,2-benzenediol (2.57%, RT=15.22) and 1,4-benzenediol (1.32%, RT=16.30) (Figure 3.42). Therefore, it was indicated that berberine was the major alkaloids found as a major constituent in *C. fenestratum* extracts.

Previous research found that berberine, well-known alkaloids was a major active constituent in *C. fenestratum* (Rojsanga *et al.*, 2006). Moreover, berberine possesses a wide of biochemical and pharmacological activities. Rojsanga *et al.* (2007) reported that berberine, a natural isoquinoline alkaloid, could induce cell growth arrest, apoptosis, NAG-1, and ATF3 in human colorectal cancer cells. Moreover, Tungpradit *et al.* (2010) indicated that berberine was the major active compound in *C. fenestratum* and also had anticancer activity by inducing apoptosis in HL-60 leukemia cells. Previous research showed the aqueous and methanolic extract of *C. fenestratum* gave antimicrobial activity against wide range of tested bacterial species including *B. cereus*, *Enterococcus* sp., *E. coli*, *Ps. aeruginosa*, *Sal. typhi* and *S. aureus* (Nair *et al.*, 2005). Moreover, the stem of *C. fenestratum* contained other phytochemical like alkaloids, flavonoids, tannins and steroids. The aqueous and methanolic extract could inhibit the growth of *E. coli, Serratia marcesens, S. aureus, B. cereus, Clostridium. perfringens* and *Sal. typhi* when determined by well diffusion method (Kalpana *et al.*, 2013). In addition, the various extract of *C. fenestratum* including pretoleum ether, benzene, chloroform, methanol and ethanol also had antioxidant activity when investigated by reducing power activity method (Anitha *et al.*, 2013).

Table 3.25 Primary chemical screening test of CF01 fraction of *C. fenestratum*

Test	Results
1. Alkaloids	
Dragendroff's reagent	+
Wagner' reagent	+
Hager's reagent	+
Mayer's regent	+
2. Flavonoids	-
3. Cumarins	-
4. Saponins	-
5. Cardiac glycosides	
Liebermann-Burchard's test (steroidal nucleus)	+
Keller-Kiliani's test (deoxy sugar)	-
6. Antraquinone glycosides	-
7. Tannins	
1% gelatin test	-
1% $FeCl_3$ test	-
Formaldehyde – HCl test	-
Vanillin – HCl test	-
$CaOH_2$ solution test	-
Lead acetate test	-
8. Phenolics	+

+ Positive result - Negative result

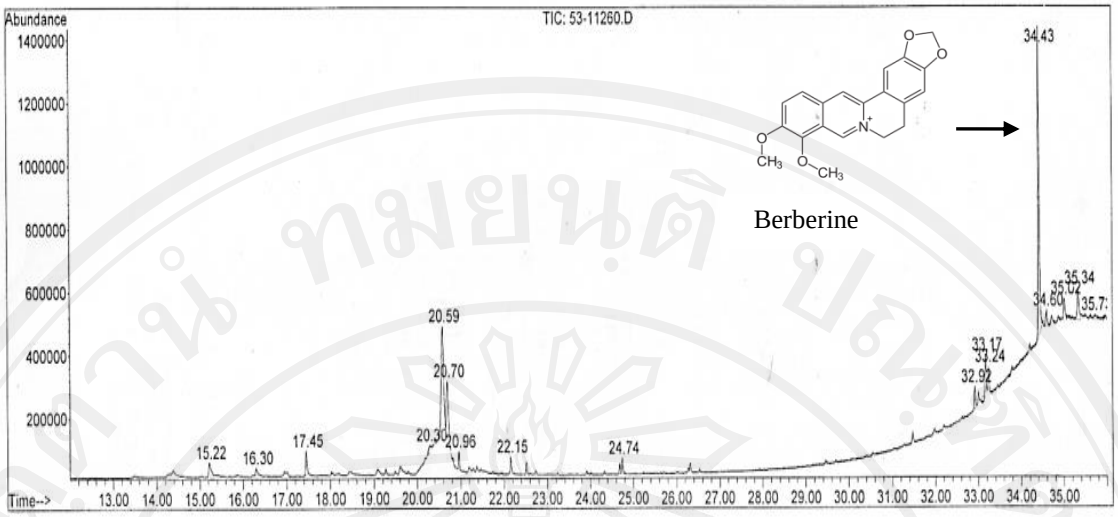

Figure 3.42 GC chromatograms of ethanolic extracts of *C. fenestratum*

2) Isolation of bioactive fractions of *S. venosa*

The crude methanolic extract of *S. venosa* was separated by partition technique to obtain four fractions including *n*-hexane, chloroform, *n*-butanol and aqueous fractions (Figure 3.43). For percentage yield recovery, the *n*-butanol fraction had the highest percentage yield with 19.41 % followed by chloroform, aqueous and *n*-hexane fractions with 19.18, 18.75 and 10.50 %, respectively.

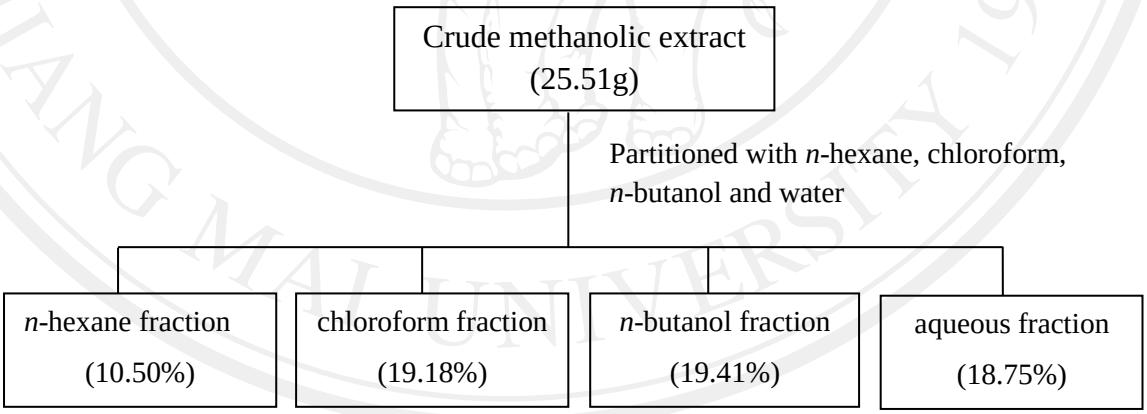

Figure 3.43 Schematic diagram shows the isolation procedure of *Stephania venosa* by partition technique

After that, each fraction was further determined antibacterial activity using agar disc diffusion and broth dilution methods. The result of preliminary screening was shown in Table 3.26-3.27, Figure 3.44-3.45). It was found that the chloroform fraction showed the highest antibacterial activity against *E. coli* O157: H7, *S. aureus*,

S. epidermidis, *St. pyogenes*, *P. acnes*, MRSA isolate number 64, 65, 66 and 67 with inhibition zone ranging between 11.0-38.0 mm. MIC and MBC value of chloroform fraction was ranging between 0.06-62.5 mg/ml. Moreover, the *n*-butanol fraction also had high antibacterial activity against *E. coli* O157: H7, *S. aureus*, *S. epidermidis*, *St. pyogenes*, *P. acnes*, MRSA isolate number 64, 65, 66 and 67 with high inhibition zone ranging between 10.3-33.7 mm. MIC and MBC values of *n*-butanol fraction were ranging between 3.9-62.5 mg/ml. The aqueous fraction had low antibacterial activity against *E. coli* O157: H7, *S. epidermidis*, MRSA isolate number 64 and 67 with inhibition zone 9.3, 10.0, 10.0 and 9.3 mm. MIC and MBC value of aqueous fraction was ranging between 1.3-125 mg/ml. On the contrary, the *n*-hexane fraction had the lowest antibacterial activity against only *St. pyogenes* and *P. acnes* with inhibition zone 8.33 and 8.00 mm. MIC and MBC values of *n*-hexane fraction were 31.3 and 62.5 mg/ml.

Table 3.26 Effect of *S. venosa* fractions (500 mg/ml) on growth of pathogenic bacteria by agar disc diffusion method

Bacterial strains	**Zone of inhibition (mm) ± SD**			
	***n*-hexane fraction**	**chloroform fraction**	***n*-butanol fraction**	**aqueous fraction**
E.coli O157:H7	0	11.0 ± 0.6	10.3 ± 0.6	9.3 ± 0.6
Ps.aeruginosa	0	0	0	0
S. aureus	0	22.0 ± 1.0*	15.7 ± 1.2	0
S. epidermidis	0	22.0 ± 1.7*	16.0 ± 1.7	10.0 ± 1.0
St. pyogenes	8.33 ± 0.6	25.3 ± 0.6*	20.0 ± 1.0	0
P .acnes	8.00 ±0.0	38.0 ± 1.0*	33.7 ± 0.6	0
MRSA64	0	20.7 ± 0.6*	15.0 ± 1.0	10.0 ± 1.0
MRSA65	0	21.7 ± 1.2*	15.7 ± 1.5	0
MRSA66	0	22.7 ± 0.6*	19.0 ± 1.0	9.3 ± 2.1
MRSA67	0	21.0 ± 1.0*	17.7 ± 1.2	0

Data in table are given as mean ± standard deviation (SD) of triplicate experiments. Statistical comparison between groups applied using post hoc Duncan's test. (*) represent the significant difference values within each row ($P<0.05$)

Table 3.27 Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *S. venosa* fractions against pathogenic bacteria by broth dilution method

Bacterial strains	MIC and MBC (mg/ml)							
	***n*-hexane fraction**		**chloroform fraction**		***n*-butanol fraction**		**aqueous fraction**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
E.coli O157:H7	-	-	31.3	62.5	31.3	62.5	31.3	62.5
Ps.aeruginosa	-	-	-	-	-	-	-	-
S. aureus	-	-	3.9	7.8	15.6	31.3	-	-
S.epidermidis	-	-	3.9	7.8	3.9	15.6	31.3	62.5
St. pyogenes	31.3	62.5	0.06	0.06	3.9	7.8	-	-
P.acnes	31.3	62.5	0.06	0.12	3.9	7.8	-	-
MRSA64	-	-	15.6	31.3	62.5	31.3	62.5	125
MRSA65	-	-	15.6	31.3	62.5	125	-	-
MRSA66	-	-	15.6	31.3	31.3	62.5	62.5	125
MRSA67	-	-	31.3	62.5	31.3	62.5	-	-

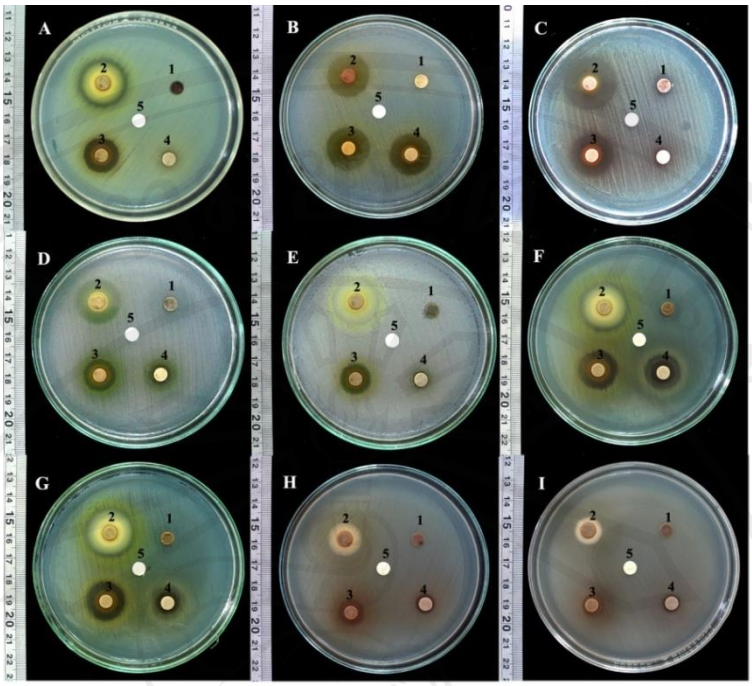

Figure 3.44 The effect of four fractions of *S. venosa* including *n*-hexane (1), chloroform (2), *n*-butanol (3) and aqueous (4) fraction on the growth of *S. aureus* (A), *S. epidermidis* (B), *St. pyogenes* (C), MRSA 64 (D), MRSA 65 (E), MRSA 66 (F) and MRSA 67 (G), *E. coli* O157: H7 (H) and *Ps. aeruginosa* (I) by agar disc diffusion method

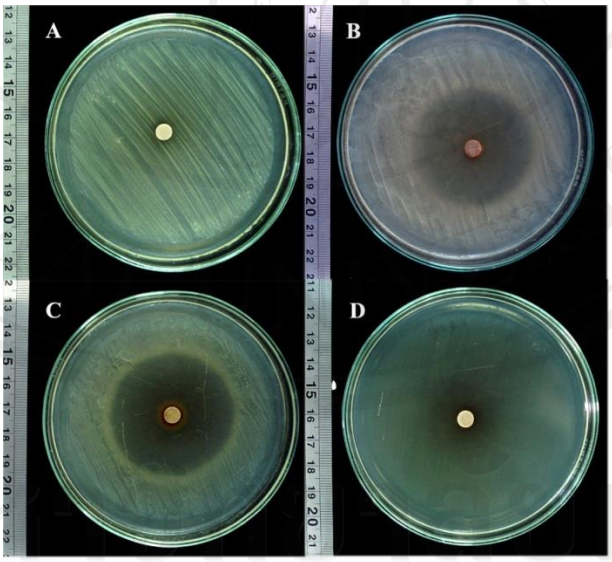

Figure 3.45 The effect of partial purified fraction of *S. venosa*; *n*-hexane (A), chloroform (B), *n*-butanol (C) and aqueous (D) fraction on the growth of *P. acnes* by agar disc diffusion method

Thin layer chromatography (TLC) of each fraction was conducted and the details of each fraction in terms of number of spots on TLC, R_f value and the appearance of partial purified fraction were exhibited in Table 3.28 and Figure 3.46. From the phytochemical screening, it was found that alkaloids and phenolics were major constituents in active fractions of *S. venosa* (Table 3.29).

Table 3.28 R_f values of the partial purified fraction of *S. venosa* extract

Fractions	**UV wavelength**	**Mean R_f values**	**Appearances**
n-hexane	UV254	0.80, 0.30, 0.15	light brown, light yellow, dark brown
	UV366	0.80, 0.63, 0.30	blue, blue, green
chloroform	UV254	0.80, 0.33, 0.13	light brown, dark yellow, dark brown
	UV366	0.80, 0.38, 0.30	blue, green, blue
n-butanol	UV254	0.25	light yellow
	UV366	0.25	green
aqueous	UV254	-	-
	UV366	-	-

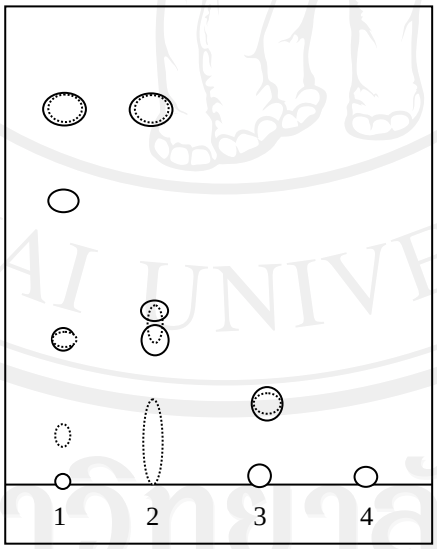

Figure 3.46 TLC chromatograms of partial purified fractions of *S. venosa*, *n*-hexane fraction (1), chloroform fraction (2), *n*-butanol fraction (3) and aqueous fraction (4). TLC analysis was determined using chloroform as mobile phase and observed under UV light at 254 nm (----) and 366 nm (—)

S. venosa is a traditional folk medicine used for cancer treatment and this plant known to be rich of alkaloid such as isoquinoline alkaloids. There are many published reports confirmed that the active constituents in genus *Stephania* was alkaloid compounds. Furthermore, more than 200 alkaloids have been isolated from this genus together with flavonoids, lignans, steroids, terpenoids and coumarins (Semwal *et al.,* 2010). *Stephania* had been reported a potential sources of biologically active compounds. Previous reports demonstrated that the crude extract from *S. venosa* was able to inhibit tumor cell proliferation in breast cancer cell lines (Moongkarndi *et al.,* 2004). Moreover, aporphine from *S. venosa* tuber also had anticancer activity according to antiproliferation and apoptotic activity on an ovarian cancer cell (SKOV3) (Montrirattigri *et al.,* 2008). Five alkaloids, namely 4, 5-dioxodehydrocreba nine, dehydrocrebanine, crebanine, oxostephanine, and thailandine were identified as major active constituents in the tuber and leaves of *S. venosa*. Thailandine showed the strongest activity against lung carcinoma cells (A549) and also had strongest activity against *Plasmodium falciparum*, K1 strain *Mycobacterium tuberculosis* H (37) Ra, *B. subtilis*, *Entrococcus faecilis*, *St. pneumonia*, *St. mutans*, *St. milleri* and *S. aureus*. Moreover, oxostephanine exhibited very strong activity against breast cancer (BC), acute lymphoblastic leukemia cells (MOLT-3) and also inhibited antiviral activity against herpes simplex virus type 1 while dehydrocrebanine demonstrated strong activity against promyelocytic leukemia cells (HL-60) (Makarasen *et al*. 2011). Furthermore, the ethanolic extract of *S. venosa* could inhibit the growth of *B. cereus*, *E. coli* and *S. aureus* (Wutithamawech *et al.,* 2014).

Table 3.29 Primary chemical screening test of chloroform fraction of *S. venosa*

Test	Result
1. Alkaloids	
Dragendroff's reagent	+
Wagner' reagent	+
Hager's reagent	+
Mayer's regent	+
2. Flavonoids	+
3. Cumarins	-
4. Saponins	-
5. Cardiac glycosides	
Liebermann-Burchard's test (steroidal nucleus)	+
Keller-Kikiani's test (deoxy sugar)	-
6. Antraquinone glycosides	-
7. Tannins	
1% gelatin test	-
1% $FeCl_3$ test	-
Formaldehyde -HCl test	-
Vanillin-HCl test	-
$CaOH_2$ solution test	-
Lead acetate test	-
8. Phenolics	+

(+) = Positive result (-) = Negative result