CHAPTER 3

Experimental

3.1 Instruments and apparatus

1. Analytical balance 3 position (A&D, model GX-800, Japan)
2. Analytical balance 4 position (Mettle Toledo, USA)
3. Electric Autoclave (All American, model 25X, USA)
4. Filter paper No.4 (Whatman, USA)
5. Gas chromatograph-mass spectrometer, Agilent 6850 Series (GC system coupled to 5973 Network mass selective detector, Agilent technology, USA)
6. Heating mantle (Electromentle, UK)
7. Hot air oven (Menmert, Germany)
8. Hotplate Stirrer (Harmony, model HTS-1003, Japan)
9. Incubator shaker (Gallenkamp, England)
10. Micropipette (Thermo Scientific, USA)
11. Mixer UZUSIO (Harmony, model VTX-3000L, Japan)
12. Petri dish (Pyrex, USA)
13. Sonicator (Bandelin, Germany)
14. Spectrophotometer (Thermo scientific, USA)
15. Steam distillation apparatus
16. Test tube (Pyrex, USA)
17. Vacuum rotary evaporator (Buchi, Switzerland)

3.2 Chemicals

1. Acetone, commercial grade (Lab scan, Thailand)
2. Agar
3. Barium chloride 99.99% (Aldrich Chem, USA)
4. Dichloromethane, AR grade (Lab scan, Thailand)
5. Dimethylsulphoxide (DMSO), AR grade (RCI Labscan, Thailand)
6. Distilled water
7. Erythromycin free base bio chemica (Appli Chem, Germany)
8. Ethanol, AR grade (Merck, Germany)
9. Ethanol, commercial grade (Alcoh-AX, Thailand)
10. Ethyl acetate, commercial grade (RCI Lab scan, Thailand)
11. Hexane, AR grade (Lab scan, Thailand)
12. Helium gas 99.99%, HP grade (TIG, Thailand)
13. Methanol, commercial grade (RCI Labscan, Thailand)
14. Sodium sulphate anhydrous (Merck, Germany)
15. Standard normal-alkanes, (C_8-C_{22}) (Fluka, Switzerland)
16. Sulfuric acid (RCI Labscan, Thailand)
17. Tryptic Soy Broth, (TSB) (Criterion, USA)

3.3 Microorganisms

The microorganisms used in this study consisted of two strains of acne inducing bacteria. *Staphylococcus epidermidis* was obtained from the Thailand Institute of Scientific and Technological Research. Moreover, *Staphylococcus aureus* was obtained from the Department of Biology, Faculty of Science, Chiang Mai University. The bacteria were grown and maintained on nutrient agar slants.

Gram-positive bacteria

1. *Staphylococcus aureus* ATCC25923 (*S. aureus*)
2. *Staphylococcus epidermidis* TISTR518 (*S. epidermidis*)

3.4 Plant materials

Six plant materials were collected from northern Thailand (Table 3.1). The taxonomy of plant materials was also identified by Dr. Angkhana Inta, Department of Biology, Faculty of Science, Chiang Mai University.

Table 3.1 Plant materials used in this study

Scientific name	Local name	Collections
C. lansium	ma-fai-jeen (มะไฟจีน)	July 2013, Nan province
C. harmandiana	Pong-fa (โปร่งฟ้า)	July 2013, Nan province
E. communis	e-luen (อีหลืน)	September 2013, Mae Hong Son province
E. stachyodes	harn (ฮาน)	November 2013, Mae Hong Son province
E. griffithii	loom-poom (ลุมปุม)	November 2013, Mae Hong Son province
E. sp.	e-luen-pa (อีหลืนป่า)	November 2013, Chiang Mai province

3.5 Extraction

3.5.1 Extraction of essential oil

The fresh aerial parts of *Elsholtzia* and leave of *Clausena* species were cleaned and chopped into small pieces. A portion of plant materials (100 g) were extracted by steam-distillation for 3-4 hours using steam-distillation apparatus (Figure 3.1). The obtained essential oil was dried over sodium sulphate anhydrous and stored in the refrigerator for further analysis.

Figure 3.1 Steam-distillation apparatus

3.5.2 Solvent extraction

After the completion of steam distillation, the solid residues were collected and air-dried. The dried solid residues (100 g) were re-extracted with 500 ml of hexane, acetone, and ethanol, respectively by sonication for 30 minutes about 3 times. The extracts were filtered and evaporated of the solvent under reduce pressure. The hexane, acetone, and ethanol residual crude extracts were obtained.

Note: Only the solid residue of *C. harmandiana* was re-extracted with hexane, ethyl acetate and methanol, respectively.

3.6 Gas chromatography-mass spectrometry (GC-MS) analysis

The essential oils were analysed by gas chromatography-mass spectrometry (GC-MS) consisting of Agilent 6850 Gas chromatograph equipped with a HP-5MS capillary fused silica column (30 m, 0.25 mm. I.D., 0.25 µm film thickness) coupled with an 5973 Network mass selective detector. The column temperature conditions are shown in Table 3.2. The injector and detector temperature were 250 and 280 ^{o}C, respectively. Helium was used as carrier gas at 1 ml/min. The injection volume of diluted essential oil (1/100 in dichloromethane, v/v) of 1 µl with split ratio 1:100 was used. Electron ionization mass spectra in the range from *m/z* 45 - 450 amu were recorded at 70 eV ionisation energy.

Table 3.2 The temperature programme conditional used

Plant species	Conditions	Rate	Temperature	Time
Clausena species				
	Initial	-	50 ^{o}C	2 min
	Ramp 1	3 ^{o}C/min	130 ^{o}C	0 min
	Ramp 2	5 ^{o}C/min	250 ^{o}C	0 min
	Post run	-	270 ^{o}C	5 min
Elsholtzia species				
	Initial	-	70 ^{o}C	10 min
	Ramp 1	5 ^{o}C/min	200 ^{o}C	0 min
	Ramp 2	10 ^{o}C/min	250 ^{o}C	0 min
	Post run	-	270 ^{o}C	5 min

The identification of each compound was assigned by comparison of their retention index with relative to a standard mixture of n-alkanes (C_8-C_{22}) under the same experimental conditions by comparing with the MS literature data.[21] Mass spectral database (NIST 98 and WILEY7n) was also used for spectral matching. The relative percentage amounts of the separated compounds were calculated from total ion chromatography by a computerized integrator. Retention index was calculated as presented in Equation 3.1.

$$\mathrm{RI}_{(\mathrm{X})} = 100Z + 100\Delta Z\left[\frac{(\mathrm{RT}_{(\mathrm{X})} - \mathrm{RT}_{(\mathrm{Z})})}{(\mathrm{RT}_{(\mathrm{Z+1})} - \mathrm{RT}_{(\mathrm{Z})})}\right] \qquad \text{(Equation 3.1)}$$

Where $RT_{(X)}$ is the retention time of compound X, $RT_{(Z)}$ and $RT_{(Z+1)}$ are retention times of the reference n-alkane hydrocarbons eluting closely before and after chemical compound X. The obtained $RI_{(X)}$ was compared with Adam's reference which is retains the RI values of most components in various essential oils.[21]

3.7 Determination of antibacterial activity

3.7.1 Preparation of essential oils and residual crude extracts

The essential oils and residual crude extracts were diluted in DMSO. Erythromycin was used as an antibacterial standard (positive control) while DMSO was used as negative control.

3.7.2 Preparation of microorganisms [54]

Two standard strains of bacteria as *S. aureus* and *S. epidermidis* were cultured on Tryptic Soy Agar (TSA) plate. At least three well-isolated colonies of the same morphological type from culture plate were picked up and suspended in 50 ml of Tryptic Soy Broth (TSB) contained in flasks. The suspension were cultivated at 37 ^{o}C for 24 hours with shaking incubator at 160 rpm/min. The turbidity of the actively growing culture was adjusted with culture broth to be a turbidity standard at 0.5 McFarland standard.

Note: 0.5 McFarland standard was a barium sulphate ($BaSO_4$) turbidity standard prepared by adding 0.5 mL of 1.175% w/v barium chloride ($BaCl_2$) solution to 99.5 ml of 1% v/v sulfuric acid (H_2SO_4). This turbidity is containing approximately 1.5 x 10^8 CFU/ml for the density of bacteria.

3.7.3 Disc diffusion assay [55]

The essential oils and residual crude extracts were tested against standard *S. aureus* and *S. epidermidis* bacterial strains using the paper disc diffusion method. The bacterial suspensions were adjusted to 0.5 McFarland standard and 100 µl of these were swabbed on the TSA medium using a sterilised cotton bud. Then, sterilised filter paper discs (6 mm diameter, Whatman No.4) were impregnated with 10 µl of the sample at different concentrations and placed on the surface of TSA covered with tested bacterial strain, respectively. DMSO was used as solvent and negative control while erythromycin as positive control. Then, the plates were incubated at 37 ^{o}C for 24 hours. Antibacterial activity was investigated by measuring the zones of inhibition (mm). All tests were carried out for three replications and the results were averaged.

Note: Particularly, the determination of antibacterial activity of essential oils after the paper discs which impregnated with the essential oil placed on the surface of TSA covered with tested bacterial strain. The plates were stored in the refrigerator for 2 hours to diffuse the oil into medium before the incubation.[56]

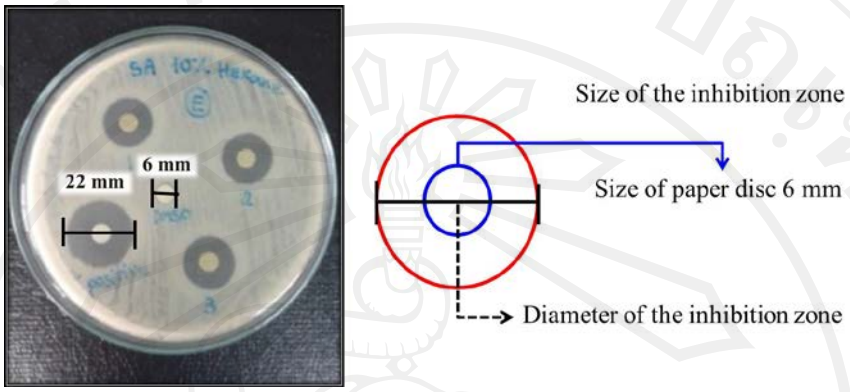

Figure 3.2 Measurement the diameter of the inhibition

3.7.4 Determination of minimum inhibitory concentration (MIC)[57]

The minimum inhibitory concentration (MIC) value of the effective antibacterial solutions were determined. This study, erythromycin, essential oils, and residual crude extracts solutions were prepared by serial two-fold dilution method. This method was performed filling 0.5 ml TSB into the sterilised test tube number 2-12 (one set of MIC experiment consisting of 12 tubes). The amount of 1.0 ml of samples was filled into tube number 1. Then, the amount of 0.5 ml of samples in tube number 1 was transferred to the tube number 2. The mixture in tube number 2 was mixed and transferred to the third tube for 0.5 ml. This was soon until tube number 10 that 0.5 ml of the mixture was left. The amount of 0.5 ml of the adjusted bacterial suspensions was added to every tube except number 11. Therefore, the final concentrations of antimicrobial agents were $1/2^1$, $1/2^2$, $1/2^3$ up to $1/2^{10}$ as shown in Figure 3.3. All experiment was performed in triplicate and the experiment was incubated at 37 ºC for 24 hours. The MIC was determined by looking for the lowest concentration of the antimicrobial agent containing tube that showed no bacteria growth, as shown in Figure 3.4.

Note: The initial concentration of erythromycin was 125 µg/ml. The initial concentration of *E. communis* and *E.* sp. essential oils was 250 µl/ml whereas *E. stachyodes* oil was 100 µl/ml. Moreover, the initial concentration of residual crude extracts solutions were 100 mg/ml.

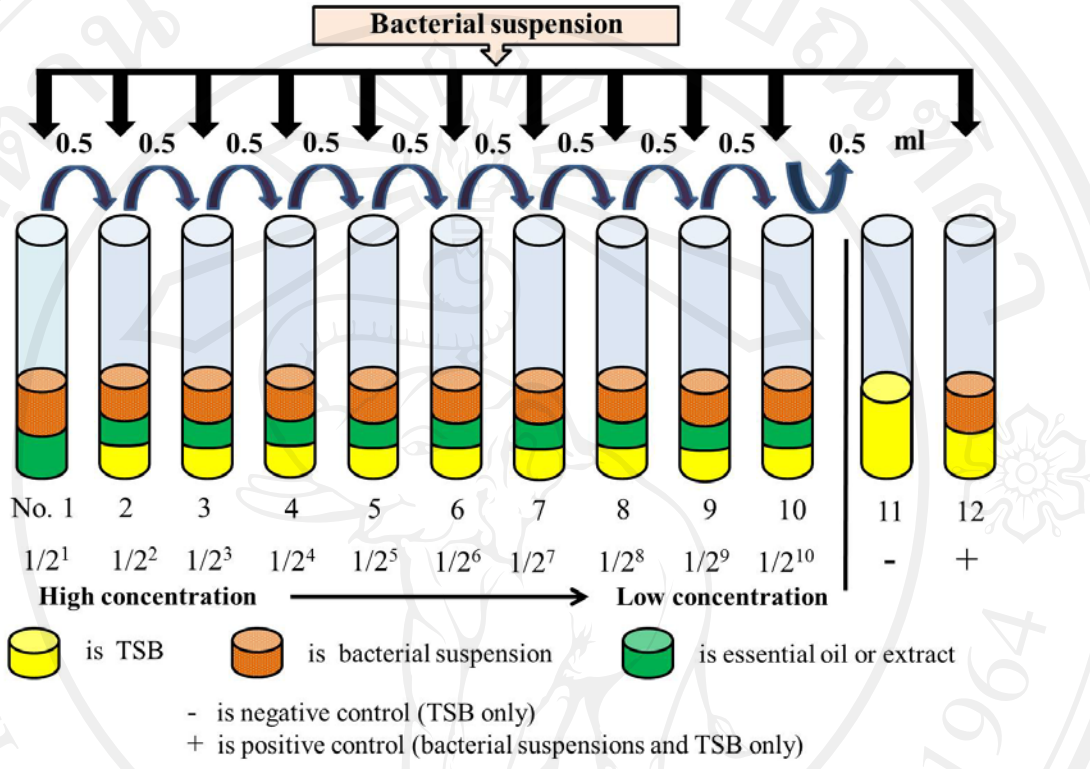

Figure 3.3 Two-fold dilution methods for minimum inhibitory concentration (MIC)

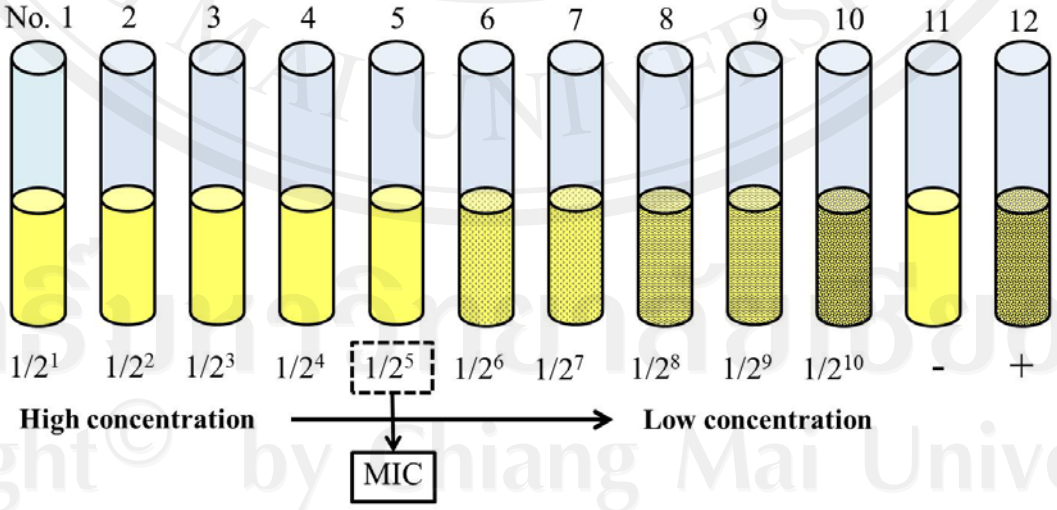

Figure 3.4 Determination of minimum inhibitory concentration (MIC)

3.7.5 Determination of minimum bactericidal concentration (MBC) [57]

After the determination of MIC, the MBC was observed. The tubes, showing no visible of bacterial growth was swabbed on the TSA medium using a sterilised cotton bud (Figure 3.5) and observed for the bactericidal colonies on TSA after incubated at 37 °C for 24 hours. The MBC was determined as the lowest concentration of the sample which is showing less than 0.1% viable bacteria.

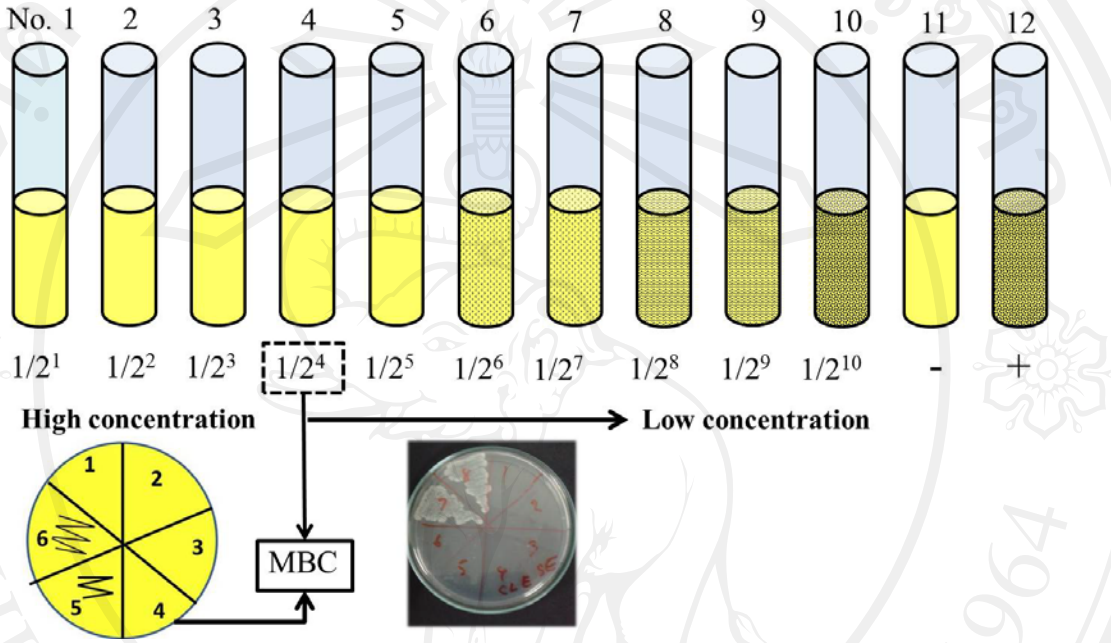

Figure 3.5 Determination of minimum bactericidal concentration (MBC)