CHAPTER III
MATERIALS AND METHODS

3.1 Instruments

All instruments used in this study were listed as following

1. Analytical Balance (Sartorious Model BP110, AG Gottingen Co. Ltd., Germany)
2. Conductivity meter (Cyberscan CON 11, Eutech instruments, Singapore)
3. GC-MS (Hewlett-Packard model 6890 (GC) / HP 5973 MS)
4. High Performance Liquid Chromatography (HPLC) (Hewlett Packard, USA)
5. High pressure homoginizer (Micron LAB40, Homogenizer Systems, Germany)
7. Hot air oven (Thelco. Model 27 GCA. Co., Ltd., USA.)
8. Incubator (National Appliance Co., Model 332, USA.)
9. Micropipette (Eppendorf, USA)
10. Microtiter plate reader (Biorad 680, USA)
11. Multiple-Point Stirrer (Thermo scientific, McQueen Labs, USA)
12. pH meter (Hanna pH 211 microprocessor, USA)
13. Photon correlation spectroscopy (Malvern Zetasizer IV, Malvern Instruments, UK)
14. Refractometer (ATAGO Model 3T, Atago Co. Ltd., Tokyo, Japan)
15. Refrigerator (SANYO, Japan)
16. Sonicator T460/H (Elma Model Transsonic, Germany)
17. Steam distillation / Clevenger apparatus
18. Surface tensiometer (FACE-CBVP-A3, Japan)

19. UV-sepectrometer (Shimazu UV-2450 Japan)
20. Vortex mixer (Model 1291, LAB-LINE Instrument, Inc., USA)
21. Water bath with shaker (Memmert, GMBH Co., Ltd., Germany)
22. Water Filter (Milli-Q®, USA)

3.2 Chemicals

1. Acetone AR (Merck, Germany)
2. Acetonitrile HPLC (LAB-SCAN Analytical Science, Thailand)
3. Acetic acid (Fisher Chemicals, Loughborough, UK)
4. Acetronitrile AR (Merck, Germany)
5. Acetronitrile HPLC (Merck, Germany)
6. Acetylcholinesterase (AChE, specific activity 425.94 U/mg) (Sigma-Aldrich, USA)
7. Acetylthiocholine iodide (ATCI) (Fluka, Steinheim, Garmany)
8. 2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt) (ABTS) (Sigma- Aldrich, USA)
9. n-Butanol AR (Merck, Germany)
10. Butylated hydroxytoluene (BHT) (Sigma-Aldrich, USA)
11. Butyrylcholinesterase (BChE, specific activity 7.4 U/mg) (Sigma-Aldrich, USA)
12. Butyrylthiocholine iodide (BTCI) (Fluka, Steinheim, Garmany)
13. Calcium chloride (Fisher Chemicals, Loughborough, UK)
14. Chloroform AR (Merck, Germany)
15. Clotrimazole (Sigma-Aldrich, USA)

16. Dialysis membrane (MWCO = 12000-14000) (Cellu.Sep T4, USA)
17. Dichloromethane AR (Merck, Germany)
18. Dichloromethane HPLC (Fisher Scientific, UK)
19. Diethyl ether AR (Merck, Germany)
20. Dimethyl sulfoxide (DMSO)
21. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), (Sigma-Aldrich, USA)
22. 2,2-Diphedyl-1-picryl-hydrazyl (DPPH) (Sigma Aldrich, USA)
23. Disodium hydrogen phosphate (Ajax Finechem, New Zealand)
24. 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, USA)
25. Ethanol 95% (BDH Chemicals Ltd, England)
26. Ethanol absolute AR (Merck, Germany)
27. Ferric chloride ($FeCl_3.6H_2O$) (Sigma-Aldrich, USA)
28. Ferrous sulfate ($FeSO_4.7H_2O$) (Sigma-Aldrich, USA)
29. Fetal bovine serum (FBS) (Biochrom, Germany)
30. Ficoll-paque plus, Lymphoprep™ Axis-Shield PoC AS, (Oslo, Norway)
31. n-Hexane AR (Merck, Germany)
32. Hexan-1-ol (Asia Pacific Specialty Chemical Limited ABN, Australia)
33. Human blood, Human serum (Volunteers in Chiangmai, Thailand)
34. Hydrochloric acid (BDH Chemicals Ltd, England)
35. 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) (Fluka, Switzerland)
36. L-dopa (Fluka Chemical, Japan)
37. Methanol AR (Merck, Germany)

38. Methanol HPLC (Merck, Germany)
39. n-Octanol (Merck, Germany)
40. Octylphenol-polyethylene glycolether (Triton X-114) (Acros Organics, New Jersy, USA)
41. n-Pentanol (Merck, Germany)
42. Phosphoric acid (Merck, Germany)
43. Polyethylene glycol (PEG) 200 / PEG 300 / PEG 400 / PEG 600 (Fluka, Switzerland)
44. Polyoxyethylene (10) oleyl ether (Brij 97) (Steinheim, Garmany)
45. Polyethylene glycol sorbitan trioleate (Tween 85) (Acros Organics, New Jersy, USA)
46. Potassium persulfate (BDH Chemicals Ltd, England)
47. Propylene glycol (Fluka, Switzerland)
48. iso-Propanol (Merck, Germany)
49. n-Propanol (Merck, Germany)
50. RPMI 1640, GIBCO™ Invitrogen (Grand Island, NY, USA)
51. Sesame oil (Siam Sesame Co., LTD, Mae Hong Son, Thailand)
52. Sesamin (Phytolab GmbH, Germany)
53. Sodium acetate (Fluka, Switzerland)
54. Sodium chloride (Fisher Chemicals, Loughborough, UK)
55. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) (Sigma, USA)
56. Tween 20 / Tween 80 (Namsiang Co., Ltd. (Bangkok, Thailand)
57. Tyrosinase enzyme (Fluka Chemical, Japan)
58. Trypan blue GIBCO™ Invitrogen (Grand Island, NY, USA)

59. α-Tocopherol (Fluka Chemie GmbH, Switzerland)

60. γ-Tocopherol (Phytolab GmbH, Germany)

61. Other glassware, such as beakers, stirrers

3.3 Plants materials

Plants used in this study as shown in Table 3.1 were collected from the northern part of Thailand. Most of them are edible plants. The criteria for plant selection was that the plants should provide some odor to show that they have essential oil existing. Except for sesame oil which is a fixed oil model used in this study. It was selected because of its belonging advantage activities such as antioxidant [121-123], anti-inflammatory [124, 125], analgesic [126, 127], antimicrobial [128, 129] activities.

All plants samples were collected during June to November, 2008.

Table 3.1 Plants for oil extraction used in this research.

No.	Scientific name	Local name	Family
1	*Apium graveolens* Linn.	คื่นไช่	Apiaceae
2	*Anethum graveolens* Linn.	ผักชีลาว	Apiaceae
3	*Centella asiatica* Urban.	บัวบก	Apiaceae
4	*Coriandrum sativum* Linn.	ผักชี	Apiaceae
5	*Eryngium foetidum* Linn.	ผักชีฝรั่ง	Apiaceae
6	*Polyscias fruticosa* Harms.	เล็บครุฑ	Araliaceae

Table 3.1 (continued)

No.	Scientific name	Local name	Family
7	*Eupatorium odoratum* Linn.	สาบเสือ	Asteraceae
8	*Spilanthes acmella* Murr.	ผักคราดหัวแหวน	Asteraceae
9	*Cymbopogon citratus* Stapf.	ตะไคร้	Gramineae
10	*Coleus amboinicus* Lour.	เนียมหูเสือ	Lamiaceae
11	*Melissa officinalis* Linn.	สะระแหน่	Lamiaceae
12	*Ocimum basilicum* Linn.	โหระพา	Lamiaceae
13	*Ocimum canum* Sims.	แมงลัก	Lamiaceae
14	*Ocimum gratissimum* Linn.	ยี่หร่า	Lamiaceae
15	*Ocimum sanctum* Linn.	กะเพรา	Lamiaceae
16	*Cinnamomum bejolghota* Sweet.	อบเชย	Lauraceae
17	*Piper sarmentosum* Roxb.	ชะพลู	Piperaceae
18	*Polygonum odoratum* Lour.	ผักแพรว	Polygonaceae
19	*Citrus aurantifolia* Swing.	มะนาว	Rutaceae
20	*Citrus maxima* (Burm.) Merr.	ส้มโอ	Rutaceae
21	*Houttuynia cordata* Thunb.	พลูคาว	Saururaceae
22	*Boesenbergia pandurata* (Roxb.)	กระชาย	Zingiberaceae
23	*Curcuma longa* Linn.	ขมิ้นชัน	Zingiberaceae
25	*Curcuma zedoaria* (Berg) Rosc.	ขมิ้นอ้อย	Zingiberaceae

Table 3.1 (continued)

No.	Scientific name	Local name	Family
26	*Sesamum indicum* Linn.	งา	Pedaliaceae
27	*Zingiber cassumunar* Roxb.	ไพล	Zingiberaceae
28	*Zingiber officinale* Roscoe.	ขิง	Zingiberaceae

3.4 Extraction of essential oil

In the process of essential oil extraction, different parts of the plants were use as shown in Table 3.2. The plant materials were washed with clean water. Next, they were cut into small pieces. Each sample was put in the 1-liter round glass flask, as shown in Figure 3.1A and subjected to a hydro-distillation apparatus for 3 hr using a Clevenger type apparatus to collect the oil as shown in Figure 3.1B. The essential oils were dried over anhydrous sodium sulfate and kept in light protected containers at 4ºC until further experiments.

In case of sesame oil which is the fixed oil used in this study, the oil was obtained from Siam Sesame Ltd. Company. The nominated process of extraction is cold-press.

Table 3.2 The part of the plant used in extraction of oil.

No.	Scientific name	Local name	Part of Plants
1	*Apium graveolens* Linn	คื่นไช่	Whole Plant
2	*Anethum graveolens* Linn	ผักชีลาว	Whole Plant
3	*Centella asiatica* Urban	บัวบก	Whole Plant
4	*Coriandrum sativum* Linn	ผักชี	Whole Plant
5	*Eryngium foetidum* Linn	ผักชีฝรั่ง	Whole Plant
6	*Polyscias fruticosa* Harms	เล็บครุฑ	Leaf
7	*Eupatorium odoratum* Linn	สาบเสือ	Whole Plant
8	*Spilanthes acmella* Murr	ผักคราดหัวแหวน	Whole Plant
9	*Cymbopogon citratus* Stapf	ตะไคร้	Stem
10	*Coleus amboinicus* Lour.	เนียมหูเสือ	Leaf
11	*Melissa officinalis* Linn.	สะระแหน่	Leaf
12	*Ocimum basilicum* Linn.	โหระพา	Leaf
13	*Ocimum canum* Sims.	แมงลัก	Stem and leaf
14	*Ocimum gratissimum* Linn.	ยี่หร่า	Leaf
15	*Ocimum sanctum* Linn.	กะเพรา	Leaf
16	*Cinnamomum bejolghota* Sweet.	อบเชย	Leaf
17	*Sesamum indicum* Linn.	งา	Seed
18	*Piper sarmentosum* Roxb.	ชะพลู	Leaf

Table 3.2 (continued)

No.	Scientific name	Local name	Part of Plants
19	*Polygonum odoratum* Lour.	ผักแพรว	Stem and leaf
20	*Citrus aurantifolia* Swing.	มะนาว	Leaf
21	*Citrus aurantifolia* Swing.	มะนาว	Peel
22	*Citrus maxima* (Burm.) Merr.	ส้มโอ	Leaf
23	*Citrus maxima* (Burm.) Merr.	ส้มโอ	Peel
24	*Houttuynia cordata* Thunb.	พลูคาว	Leaf
25	*Boesenbergia pandurata* Roxb.	กระชาย	Rhizome
26	*Curcuma longa* Linn.	ขมิ้นชัน	Rhizome
27	*Curcuma zedoaria* (Berg.) Rosc.	ขมิ้นอ้อย	Rhizome
28	*Zingiber cassumunar* Roxb.	ไพล	Rhizome
29	*Zingiber officinale* Roscoe.	ขิง	Rhizome

(A) **(B)**

Figure 3.1 Essential oil hydrodistillation

(A) Hydrodistillation apparatus

(B) Cleventure apparatus

3.5 Study of outer appearance and density of the oils

The oil samples were observed by visualization for their appearance and color. The density of the oils was measured by using the following micropipette method.

1) The weight of the oil at a volume of 300 microliter sucked by micropipette was measured.

2) The density of the oil was calculated from the following equation.

$$D = \frac{M}{V}$$

D means the density of the oil (g/mL)

M means the weight of the oil (g)

V means the volume of the oil (mL)

The experiment was done in triplicate and the average value of D was calculated.

3.6 Study of essential oil components by gas chromatography/mass spectrometer (GC-MS)

The GC-MS analysis was performed on an Agilent 6890 gas chromatograph coupled to electron impact (EI, 70 eV) using an HP 5973 mass selective detector fitted with a fused silica capillary column (HP-5MS) supplied by HP, Palo Alto, CA, USA (30.0 m × 250 mm, *i.d.* 0.25 mm film thickness). The analytical conditions were; carrier gas: helium (ca. 1.0 mL/min), injector temperature: 260°C, oven temperature: 3 min isothermal at 100°C (No peaks before 100°C after first injection), then at 3ºC/min to 188°C and then at 20°C/min to 280°C (3 min isothermal), and detector temperature: 280°C. The programmed-temperature Kováts retention indices (RI) were obtained by GC-MS analysis of an aliquot of the volatile oil spiked with an *n*-alkane mixture containing each homologue from *n*-C11 to *n*-C27. Identification of the compounds was based on a comparison of their mass spectra database (WILEY&NIST) and spectroscopic data. The percentage amount of each component was calculated based on the total area of all peaks obtained from the oil. The data obtained were used as a standard for further batches of the oil.

3.7 Study of the biological activities of the oils

In this study, two activities of the oils which support skin health, i.e. antioxidation and antityrosinase activities were investigated. The study on antioxidant activity was performed by using two standard methods, including ABTS and FRAP. The inhibitory effect on tyrosinase was investigated by inhibiting the enzymatic activity of tyrosinase, which is an enzyme that plays an important role in the occurrence of pigment (melanin) formation in the skin.

3.7.1 Determination of antioxidant activity

The antioxidant activity of oil was determined by 2 methods, including FRAP and ABTS methods.

3.7.1.1 Ferric reducing antioxidant power

The ferric reducing/antioxidant power (FRAP) assay was carried out according to the method described by Okonogi et al [130]. This assay measures the reducing properties of antioxidants based on the reduction of ferric ion. Therefore, ferrous sulfate ($FeSO_4$) was used for calibration. Briefly, a freshly prepared FRAP solution contained 50 mL of 0.3 M acetate buffer (pH 3.6) plus 5 mL of 10 mM TPTZ solution in 40 mM HCl (previously prepared) and 5 mL of 20 mM ferric chloride. After mixing 180 µL of FRAP solution with 20 µL of each sample, the ferric reducing ability was measured using a 96-well microplate reader at the end of 5 min at an absorbance of 595 nm. The results were reported as equivalent capacity (EC) indicating the ability to reduce ferric ions, expressed as mM $FeSO_4$ equivalents per mg of the oil. Each experiment was done in triplicate.

3.7.1.2 ABTS

A free radical scavenging activity test or ABTS assay was performed according to the method described by Tachakittirungrod et al [131]. with some modifications. An ABTS free radical solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulphate ($K_2S_2O_8$) in a ratio of two to three. After incubation in the dark for 16 hr, the ABTS free radical solution was then diluted with 20-fold ethanol to obtain absorbance of 0.7 ± 0.1 units at 750 nm. The ABTS free radical solution (180 μL) was mixed with 20 μL of each sample. The disappearance of ABTS free radicals was determined by measuring the decrease in absorbance at 750 nm at the end of 5 min using a 96-well microplate reader. Solutions of Trolox with known concentrations were used to construct a calibration curve. Each sample was performed at various concentrations to obtain sufficient data for plotting % inhibition *versus* concentration. The results were expressed in terms of Trolox equivalent antioxidant activity (TEAC).

3.7.2 Determination of tyrosinase inhibition activity

Tyrosinase inhibition activity was determined using the modified dopachrome method [132] with some modification as below and shown in Figure 3.2.

Preparation of tyrosinase enzyme solution: The mushroom tyrosinase enzyme solution 1000 units/ml was prepared by dissolved the enzyme in 1/15 M phosphate buffer (pH 6.8) and kept in -20°C. Then 1 mL of the stock enzyme solution was diluted with 4 mL of 1/15 M phosphate buffer (pH 6.8). The final concentration of working mushroom tyrosinase solution in the experiment is 100 unit/mL.

Preparation of sample: Mixture of Tween 20 and 1/15 M phosphate buffer (pH 6.8) at a ratio of 3:1 was prepared. The volatile oil (100 µL) was dissolved in the mixture (2.9 mL) to the final volume of 3 mL.

Preparation of L-DOPA solution: L-DOPA substrate solution was prepared by dissolving 0.004 g of L-3,4-dihydroxyphenylalanine in 1/15 M phosphate buffer (pH 6.8) and adjusted to 10 mL by this buffer to have a final concentration of 2 µM.

Antityrosinase activity test: The assay was conducted in a 96-well microtiter plate using a spectrophotometer to measure an absorbance at 492 nm. Each well contained 40 µL of the sample solution with 80 µL of 1/15 M phosphate buffer (pH 6.8), 40 µL of tyrosinase enzyme solution and 40 µL of L-DOPA (2 µM). Each sample was accompanied by blank that had all the components except L-DOPA solution. Results were compared with a control consisting of 1/15 M phosphate buffer (pH 6.8) in place of sample.

Calculation of the tyrosinase inhibition: The percentage tyrosinase inhibition was calculated by using the following equation

$$Tyrosinase\ inhibition\ (\%) = \frac{\Delta A_{Blank} - \Delta A_{Sample}}{\Delta A_{Blank}} \times 100$$

When ΔA_{Blank} is the difference absorbance of wells containing tyrosinase enzyme with L-DOPA and L-DOPA only. All blank wells contained no oil sample.

ΔA_{Sample} is the difference absorbance of wells containing tyrosinase enzyme with L-DOPA and L-DOPA only. It is noted that all sample wells contained the oil sample.

a) Blank with tyrosinase enzyme

40 µl Tyrosinase-PBS
40 µl Tween-PBS
40 µl L-DOPA in PBS
80 µl PBS pH 6.8

b) Blank without tyrosinase enzyme

40 µl PBS pH 6.8
40 µl Tween-PBS
40 µl L-DOPA in PBS
80 µl PBS pH 6.8

c) Sample with tyrosinase enzyme

40 µl Tyrosinase-PBS
40 µl Sample in Tween-PBS
40 µl L-DOPA in PBS
80 µl PBS pH 6.8

d) Sample without tyrosinase enzyme

40 µl PBS pH 6.8
40 µl Sample in Tween-PBS
40 µl L-DOPA in PBS
80 µl PBS pH 6.8

Figure 3.2 Tyrosinase inhibitory determination

3.8 Solubility study

In this study the solvents commonly used in pharmaceutical field were studied. Nine solvents with different polarity values, as shown in Table 3.3, were investigated for solubilizing power of the selected oil in this study. The details of solubility test were as follows.

Exactly volume of each solvent was gradually added into the test tube containing the known volume of the tested oil then mixed well by using a vortex mixer (as shown in Figure 3.3). More solvent was added and mixed in the same manner until all of the oil was completely miscible. In case of extremely high amount of solvent was used, a beaker is used instead of a test tube and the multipoint stirrer was used for mixing instead of a vortex mixer (as shown in Figure3.4). The solvent was added until the oil could dissolve or miscible completely when observed by the naked eyes. Minimum volume of solvent to completely dissolve 1 mL oil was recorded.

Solubility property of the oil in each solvent was expressed as a part of the minimum solvent used to dissolve one part of the oil. The low value of solvent part indicates that the solvent can dissolve the oil well. On the other hand, the high value indicates that the solvent can hardly dissolve the oil.

Table 3.3 Polarity, dielectric constant and solubility parameter of the solvents used in this experiment [133].

Solvent	Polarity index	Dielectric constant	Solubility parameter
Water	9	80	23.4
Propylene glycol		32.1	14.0
Dimethyl sulfoxide	7.2		13.4
Isopropyl myristate			8.02
Methanol	5.1	33	
PEG 400		32.4	
Ethanol	5.2	25	
Hexane	0		7.28
Mineral oil			7.09

Figure 3.3 Vortex mixer

Figure 3.4 Multipoint stirrer

3.9 Refractive index and surface tension of oil

Refractive index was measured by Precision Abbe Refractometer (Atago/3T, Japan). The distilled water was used to calibrate the scale. At 20ºC, turned the measurement knob to get the refractive index scale to indicate approximately 1.3330 and the Brix percent scale to indicate approximately 0.00%. The temperature was controlled by circulating constant temperature bath. 2-3 drops of essential oil was

3.10 Cytotoxicity test

The cytotoxicity of oil samples on normal human cells using peripheral blood mononuclear cells (PBMCs) was determined using a colorimetric technique and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PBMCs were plated in 96-well plates to obtain a cell concentration of 1×10^5 cells/well. Serial dilutions of oils were added to the wells. The wells were incubated in a 37°C, 5% CO_2 and 90% humidity incubator for 48 hr. After the corresponding time, 15 µL of MTT at 5 mg/mL was added into each well in the 96-well plate and further incubated for 4 hr in a 37°C, 5% CO_2 and 90% humidity incubator. One hundred and seventy microlitres of medium with MTT was removed from every well and 100 µL DMSO was added to each well to extract and solubilize the formazan crystals by incubating for 20 min in a 37°C, 5% CO_2 incubator. Finally, the plate was read at 540 nm using an ELISA Reader. The percentage of cell viability was calculated by the following formula.

$$\% \text{ Cell viability} = 100 \times \frac{(Ds - Dc)}{Dc}$$

where Ds is the OD of sample and Dc is the OD of control.

3.11 The study of phase diagram

In this study, the phase diagram composted of water, oil and surfactant system (surfactant and cosurfactant mixture) was constructed. Sesame oil and lemongrass oil were selected to use in this study

3.11.1 Preliminary study for phase diagram construction

In this study lemongrass was used as an oil model. Tween 20 and Tween 80 were used as principle surfactants, and ethanol was used as a cosurfactant. For the formulation surfactant and cosurfactant with four different ratios of 1:1, 1:2, 1:3 and 1:9 were firstly mix by vortex mixer. Then the oils were added and mixed well. After that the exact amount of water was added. The amounts of all substances were selected to mix with the oil. Each system was mixed well by using vortex mixer. Outer appearance of each system was recorded at 30 min after mixing. The weight ratio of the mixture was shown in Table 3.4 to Table 3.11.

Table 3.4 The amount of substances used in a system of Tween 20: ethanol = 1:1

System	Formula	Weight (g)			
		Tween20-ethanol	Oil	Water	Total
1	T(1)E(1)-1	2.0138	0.0478	0.0000	2.0616
2	T(1)E(1)-2	2.0016	0.0471	0.0485	2.0972
3	T(1)E(1)-3	2.0066	0.0437	0.0873	2.1376
4	T(1)E(1)-4	2.0137	0.0443	0.5279	2.5859
5	T(1)E(1)-5	2.0111	0.0468	1.0191	3.077
6	T(1)E(1)-6	2.0108	0.0433	2.0178	4.0719
7	T(1)E(1)-7	2.0061	0.0504	3.0264	5.0829
8	T(1)E(1)-8	2.0004	0.0434	4.0013	6.0451
9	T(1)E(1)-9	2.0325	0.0539	5.0391	7.1255
10	T(1)E(1)-10	2.0072	0.0430	6.005	8.0552
11	T(1)E(1)-11	2.0185	0.0409	7.0064	9.0658
12	T(1)E(1)-12	2.0164	0.0433	8.0116	10.0713
13	T(1)E(1)-13	2.0051	0.0439	0.0011	2.0501

Table 3.5 The amount of substances used in a system of Tween 20: ethanol = 2:1

System	Formula	Weight (g)			
		Tween20-ethanol	Oil	Water	Total
1	T(2)E(1)-1	2.0023	0.0437	0.0000	2.0460
2	T(2)E(1)-2	1.9974	0.0429	0.0384	2.0787
3	T(2)E(1)-3	1.9975	0.0425	0.1050	2.1450
4	T(2)E(1)-4	1.9990	0.0462	0.5104	2.5556
5	T(2)E(1)-5	1.9972	0.0478	1.0206	3.0656
6	T(2)E(1) -6	2.0030	0.0428	1.5142	3.5600
7	T(2)E(1)-7	2.0097	0.0417	2.0096	4.0610
8	T(2)E(1)-8	1.9986	0.0464	3.0088	5.0538
9	T(2)E(1)-9	2.0078	0.0448	4.0387	6.0913
10	T(2)E(1)-10	2.0005	0.0478	5.0268	7.0751
11	T(2)E(1)-11	2.0088	0.0477	6.0173	8.0738
12	T(2)E(1)-12	2.0084	0.0471	7.0076	9.0631
13	T(2)E(1)-13	2.0081	0.0442	8.0006	10.0529
14	T(2)E(1)-14	2.0119	0.0455	9.0300	11.0874

Table 3.6 The amount of substances used in a system of Tween 20: ethanol = 3:1

System	Formula	Weight (g)			
		Tween20-ethanol	Oil	Water	Total
1	T(3)E(1)-1	2.0099	0.0403	0.0000	2.0502
2	T(3)E(1)-2	2.0088	0.0444	0.0401	2.0933
3	T(3)E(1)-3	2.0156	0.0471	0.5299	2.5926
4	T(3)E(1)-4	2.0106	0.0459	1.0696	3.1261
5	T(3)E(1)-5	2.0107	0.0486	2.0051	4.0644
6	T(3)E(1)-6	2.0044	0.0451	3.0003	5.0498
7	T(3)E(1)-7	2.0110	0.0491	4.0107	6.0708
8	T(3)E(1)-8	2.0125	0.0429	5.0005	7.0559
9	T(3)E(1)-9	2.0068	0.0439	6.0051	8.0558
10	T(3)E(1)-10	2.0063	0.0439	7.0035	9.0537
11	T(3)E(1)-11	2.0030	0.0454	8.0098	10.0582
12	T(3)E(1)-12	2.0083	0.0452	9.0175	11.0710

Table 3.7 The amount of substances used in a system of Tween 20: ethanol = 9:1

System	Formula	Weight (g)			
		Tween20-ethanol	Oil	Water	Total
1	T(9)E(1)-1	2.0123	0.0488	0.0000	2.0611
2	T(9)E(1)-2	2.0095	0.0467	0.0369	2.0931
3	T(9)E(1)-3	2.0063	0.0454	0.0912	2.1429
4	T(9)E(1)-4	2.0153	0.0464	0.5246	2.5863
5	T(9)E(1)-5	2.0091	0.0451	1.0237	3.0779
6	T(9)E(1)-6	2.0054	0.045	2.0075	4.0579
7	T(9)E(1)-7	2.0133	0.0441	3.0083	5.0657
8	T(9)E(1)-8	2.0184	0.0445	4.007	6.0699
9	T(9)E(1)-9	2.0200	0.0477	5.0133	7.0810
10	T(9)E(1)-10	2.0090	0.0500	6.0049	8.0639
11	T(9)E(1)-11	2.0112	0.0432	7.0089	9.0633
12	T(9)E(1)-12	3.9823	0.0411	8.0056	12.029
13	T(9)E(1)-13	2.0146	0.0448	9.0089	11.0683

Table 3.8 The amount of substances used in a system of Tween 80: ethanol = 1:1

System	Formula	Weight (g)			
		Tween80-ethanol	Oil	Water	Total
1	T80(1)E(1)-1	2.0094	0.0468	0.0000	2.0562
2	T80(1)E(1)-2	2.0103	0.0806	0.0000	2.0909
3	T80(1)E(1)-3	2.0062	0.0754	0.0695	2.1511
4	T80(1)E(1)-4	2.0108	0.0511	0.0419	2.1038
5	T80(1)E(1)-5	2.0032	0.0752	0.0679	2.1463
6	T80(1)E(1)-6	2.0036	0.0575	0.0690	2.1301
7	T80(1)E(1)-7	2.0121	0.0881	0.5040	2.6042
8	T80(1)E(1)-8	2.0134	0.0688	0.1000	2.1822
9	T80(1)E(1)-9	2.0012	0.0765	1.0053	3.0830
10	T80(1)E(1)-10	2.0115	0.0820	2.0074	4.1009
11	T80(1)E(1)-11	2.0048	0.0790	5.0078	7.0916
12	T80(1)E(1)-12	2.0081	0.064	7.0292	9.1013
13	T80(1)E(1)-13	2.0075	0.0553	0.1486	2.2114
14	T80(1)E(1)-14	2.0095	0.0779	0.5464	2.6338

Table 3.9 The amount of substances used in a system of Tween 80: ethanol = 2:1

System	Formula	Weight (g)			
		Tween80-ethanol	Oil	Water	Total
1	T80(2)E(1)-1	2.0071	0.0574	0.0000	2.0645
2	T80(2)E(1)-2	2.0023	0.0847	0.0000	2.0870
3	T80(2)E(1)-3	2.0091	0.0738	0.0544	2.1373
4	T80(2)E(1)-4	2.0102	0.0705	0.0829	2.1636
5	T80(2)E(1)-5	2.0050	0.0780	0.1250	2.2080
6	T80(2)E(1)-6	2.0068	0.0875	0.1060	2.2003
7	T80(2)E(1)-7	2.0075	0.0854	0.5026	2.5955
8	T80(2)E(1)-8	3.0128	0.0819	0.1056	3.2003
9	T80(2)E(1)-9	2.0231	0.0869	1.0075	3.1175
10	T80(2)E(1)-10	2.0040	0.0887	2.0138	4.1065
11	T80(2)E(1)-11	2.0072	0.0841	5.0224	7.1137
12	T80(2)E(1)-12	2.0098	0.0888	7.0107	9.1093
13	T80(2)E(1)-13	2.0028	0.0909	0.0827	2.1764

Table 3.10 The amount of substances used in a system of Tween 80: ethanol = 3:1

System	Formula	Weight (g)			
		Tween80-ethanol	Oil	Water	Total
1	T80(3)E(1)-1	2.0106	0.0985	0.0000	2.1091
2	T80(3)E(1)-2	2.0034	0.0933	0.0506	2.1473
3	T80(3)E(1)-3	2.0172	0.0900	0.1068	2.2140
4	T80(3)E(1)-4	2.0180	0.0938	0.0751	2.1869
5	T80(3)E(1)-5	2.0139	0.0977	0.0618	2.1734
6	T80(3)E(1)-6	2.0065	0.0918	0.1126	2.2109
7	T80(3)E(1)-7	2.0063	0.0965	0.5128	2.6156
8	T80(3)E(1)-8	2.0060	0.0959	1.0085	3.1104
9	T80(3)E(1)-9	2.0109	0.0962	2.0503	4.1574
10	T80(3)E(1)-10	2.0020	0.0992	5.0028	7.1040
11	T80(3)E(1)-11	2.0136	0.0992	7.0104	9.1232

Table 3.11 The amount of substances used in a system of Tween 80: ethanol = 9:1

System	Formula	Weight (g)			
		Tween80-ethanol	Oil	Water	Total
1	T80(9)E(1)-1	2.0058	0.1589	0.0000	2.1647
2	T80(9)E(1)-2	2.0045	0.1735	0.0000	2.178
3	T80(9)E(1)-3	2.0084	0.1802	0.0565	2.2451
4	T80(9)E(1)-4	2.0023	0.1881	0.0344	2.2248
5	T80(9)E(1)-5	2.0066	0.1607	0.0509	2.2182
6	T80(9)E(1)-6	2.0075	0.1635	0.0854	2.2564
7	T80(9)E(1)-7	2.0052	0.1648	0.5058	2.6758
8	T80(9)E(1)-8	2.0088	0.1658	0.1153	2.2899
9	T80(9)E(1)-9	2.0052	0.1644	1.0013	3.1709
10	T80(9)E(1)-10	2.0144	0.1641	2.0068	4.1853
11	T80(9)E(1)-11	2.0166	0.1656	5.0021	7.1843
12	T80(9)E(1)-12	2.0125	0.1629	7.0019	9.1773
13	T80(9)E(1)-13	2.0031	0.1614	0.5044	2.6689
14	T80(2)E(1)-14	2.0112	0.1666	0.7522	2.9300

3.11.2 Psedoternary phase diagram construction

The pseudo-ternary phase diagram of the oil was constructed using a water titration method [134]. Each surfactant was firstly mixed with a cosurfactant at various weight ratio to obtain a surfactant mixture or a so-called surfactant system. For each phase diagram, the weight ratios of the oil and the surfactant system were varied as ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. These mixtures were titrated with water, under moderate agitation at ambient temperature. Outer appearance such as turbidity, color, clarity and gel formation or separation of the mixtures was observed. The phase boundary was determined by observing the changes of the sample appearance going from transparent to turbid. The experiment was done in triplicate. The pseudo-ternary phase diagram was drawn by SigmaPlot® for Windows version 10.0. The samples were classified as microemulsions when they appeared as a clear liquid. In this experiment, various factors as the followings were studied.

1) **Type of oil**

   Two selected oils; lemongrass oil and sesame oil, were used.

2) **Type of surfactant and cosurfactant**

   The nonionic surfactants used in this study were; Tween 20, Tween 85, Brij 97 and Triton X114. Ethanol, propanol, butanol, pentanol, hexanol and octanol were used as cosurfactants

3) **Electrolytes**

   In this study, monovalent electrolyte such as sodium chloride and divalent electrolyte such as calcium chloride were used.

4) pH

The aqueous phase was adjusted to pH of 4.0, 6.0 and 8.0 by using phosphate buffer solution with respective pH instead of water.

3.12 Microemulsion base and drug loaded microemulsion preparation

The appropriate compositions for the o/w microemulsion formulation of lemongrass oil and sesame oil were selected from the phase diagrams. Two microemulsion formulations containing 10% of each oil were prepared. The characteristic of these microemulsions was evaluated in comparison with the other formulations containing the ingredient as shown in Table 3.12 for sesame oil and lemongrass oil respectively.

All 10 formulations were divided into 2 groups. One was incorporated with clotrimazole to obtain 1% of drug in the formulation. Another group was used as base formulation without the drug. All systems were evaluated for their characteristics as follows.

1) Physical properties: clarity, turbidity, separation and gel like properties
2) Size and size distribution of droplet by using photon correlation spectroscopy (PCS)
3) Conductivity by using conductivity meter

Table 3.12 Microemulsion systems used in this study

System	Name	Quantity (% w/w)				Technique of preparation
		Oil	Surfactant	Co-surfactant	Water	
		(Lemongrass oil = LM)				
1	LM-1	10	10	0	80	Conventional
2	LM-2	10	33	0	57	Microemulsion
3	LM-3	10	33	17	40	Microemulsion
4	LM-4	10	40	0	50	Microemulsion
5	LM-5	10	40	20	30	Microemulsion
		(Sesame oil = SE)				
1	SE-1	10	10	0	80	Conventional
2	SE-2	10	33	0	57	Microemulsion
3	SE-3	10	33	17	40	Microemulsion
4	SE-4	10	40	0	50	Microemulsion
5	SE-5	10	40	20	30	Microemulsion

3.13 Drug release study

Formulations without phase separation after standing for 10 hr after preparation were selected to use in this study. Defungal®, a 1% clotrimazole o/w cream product was used as a positive control. The determination of the amount of drug release was done by HPLC. The performances of drug release study are as followings detail.

1) Four grams of formulations containing 1% clotrimazole was weighed, then put into the dialysis bag. The bag was tightly closed.

2) The dialysis bag was immersed into 100 ml of 50% ethanol (receiving medium) at 37 °C with stirring at 50 rpm.
3) Sample of 1 mL was collected every 10, 30, 30, 45, 90, 120 min and every hour until 10 hr. The medium was adjusted to volume with fresh 50% ethanol
4) The concentration of released drug in the different samples was measured by high performance liquid chromatography (HPLC) at following conditions.

   Column: Zobrax SB-C18, Agilent, USA, 4.6 mm ID x 250 mm (5 µm)

   Mobile phase: MeOH:K_2HPO_4=85:15

   Flow Rate: 1.0 mL/min

   Injector: 20 µL

   Detection: UV-Vis 230 nm
5) The amount of drug released was calculated by compared with the standard curve.

3.14 Stability study of the microemulsions

The microemulsions with and without drug (1% clotrimazole) keeping at room temperature (around 30 ℃), 4 ℃ and 45 ℃ for 5 months were studied. The microemulsion was also stored in the fluctuated temperature cycle, known as heating and cooling cycle. The cycle of fluctuated temp used in this study was at cool temperatures (approximately at 4℃) for 24 hr and then, at high temperature (approximately 45℃) for 24 hr. The samples were subjected to this fluctuated condition for 6 cycles. The results were compared with the conventional emulsions

kept in the same conditions. The formulations before and after storing at the above conditions were characterized by the following subjects.

1) Phase separation observed by the naked eye
2) Drug precipitation observed by the naked eye
3) Color changing of microemulsion system
4) Chemical drug stability in the formulations determined by HPLC