# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Plant materials

Sources of plant samples and commercial-grade essential oils used in this study are shown in Table 2.1.

**Table 2.1** Sources of essential oils used in this study

| No | Plant material | Plant part | Brand name |
|---|---|---|---|
| | **Geraniaceae** | | |
| 1 | *Pelargonium graveolens* : Geranium | Pure essential oil | Lemongrass House's Shop |
| | **Gramineae** | | |
| 2 | *Cymbopogon citratus* Stapf. : Lemongrass | Aerial part | - |
| 3 | *Cymbopogon nardus* Rendle. : Citronella grass | Pure essential oil | The Body Shop |
| | **Lamiaceae** | | |
| 4 | *Mentha cordifolia* Opiz. : Kitchen mint | Pure essential oil | Lemongrass House's Shop |
| 5 | *Ocimum gratissimum* Linn. : Yira | Leaves | - |
| 6 | *Ocimum sanctum* Linn. : Holy basil | Leaves | - |
| 7 | *Rosemarinus officinalis* : Rosemary | Pure essential oil | The Body Shop |
| 8 | *Hyptis suaveolens* Poit. : Maeng lak kha | Leaves | - |
| | **Lauraceae** | | |
| 9 | *Cinnamomum bejolghota* Sweet. : Cinnamon | Pure essential oil | Lemongrass House's Shop |

**Table 2.1** (continued)

| No | Plant material | Plant part | Brand name |
|---|---|---|---|
| 10 | *Litsea cubeba* Pers. : Litsea | Pure essential oil | Lemongrass House's Shop |
| | **Myrtaceae** | | |
| 11 | *Eucalyptus citriodora* Hook. : Yukhalip | Pure essential oil | Lemongrass House's Shop |
| 12 | *Eugenia caryophyllus* Bullock & Harrison.. : Clove | Pure essential oil | Lemongrass House's Shop |
| | **Oleaceae** | | |
| 13 | *Jasminum officinalis* : Jasmine | Pure essential oil | Lemongrass House's Shop |
| | **Piperaceae** | | |
| 14 | *Piper chaba* Hunt.: Java long pepper | Fruits | - |
| 15 | *Piper sarmentosum* Roxb. : Cha phlu | Leaves | - |
| | **Rutaceae** | | |
| 16 | *Citrus hystrix* DC. : Bergamot | Leaves | - |
| 17 | *Citrus reticulata* Blanco. : Tangerine | Pure essential oil | Lemongrass House's Shop |
| | **Scrophulariaceae** | | |
| 18 | *Limnophila aromatica* Merr. : Phak khayaeng | Aerial part | - |
| | **Zingiberaceae** | | |
| 19 | *Alpinia galanga* Sw. : Khaa lek | Rhizomes | - |
| 20 | *Curcuma aromatica* Salisb. : Waan naang kham | Rhizomes | - |
| 21 | *Curcuma domestica* Valeton. : Turmeric | Rhizomes | - |
| 22 | *Kaempferia galanga* Linn. : Proh hom | Rhizomes | - |
| 23 | *Boesenbergia pandurata* Holtt. : Kra chaai | Rhizomes | - |
| 24 | *Zingiber cassumunar* Roxb. : Phai | Rhizomes | - |
| 25 | *Zingiber officinale Roseoe.* : Ginger | Rhizomes | - |

Note : Lemongrass House's Shop, Bangkok; The Body Shop, Bangkok

## 2.2 General procedures for hydrodistillation

Essential oil was hydrodistillated by the method described in the Textbook of Practical Organic Chemistry (Dean-stark distillation) (Vogel, 1980). Each sample was finely chopped and put into a 1000 mL round bottom flask. The deionized water was added into the flask to about half full. The flask was connected to the Dean-stark apparatus for hydrodistillation of the essential oil (Fig. 2.1). The hydrodistillation was carried out for approximately four hours or until no oil come out with the distillate. After cooling, the distillate was extracted by diethyl ether twice and was concentrated by rotary evaporator. The derived oil volume was measured, calculated as gram of oil per one hundred grams of sample. The essential oil obtained was then collected and stored in the dark at 4°C to avoid the oxidation reaction until being tested for antifungal activity.

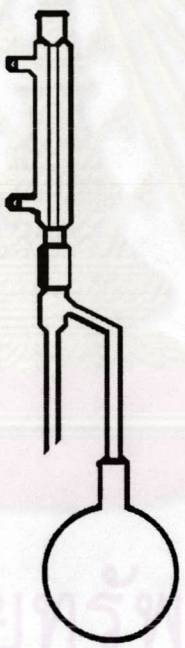


**Fig. 2.1** Dean-stark apparatus

## 2.3 GC-MS analysis

The GC-MS analysis was performed on GC star 3400 Cx and MS Saturn 4D (Varian). The essential oil mixture was subjected to the GC-MS analysis using DB-5 column. The temperature program was increased from 60 °C to 250 °C with a rate of 20 °C per minute. The injection temperature was 250 °C and the transferred line was

220 °C. The components of essential oil from *Limnophila aromatica* Merr. were determined by comparing the mass spectra of oil components and mass spectra from NIST data library.

## 2.4 Chemicals

Merck's TLC (aluminium sheet, silica gel 60 F254 pre-coated 20x20 cm., layer thickness 0.2 mm.) was used for the bioautographic assay. All solvents were purified by distillation, except diethyl ether, which was analytical grade.

## 2.5 Preliminary screening for antifungal activity

### 2.5.1 Fungal cultures

*Fusarium oxysporum* 43-68, *Alternaria* sp. 43-89 and *Phytophthora* sp. 572 were kindly supplied by the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand. All cultures were subcultured every month and maintained at 4°C on PDA slants.

### 2.5.2 Bioassays

The pure essential oil was diluted to 1000 ppm before determining the antifungal activity, focusing on mycelial growth and spore germination inhibition.

*2.5.2.1 Mycelial growth inhibition assay*

The bioassay was conducted using the agar medium assay (Virginia *et al.* 2001). The tested essential oils were applied in melting modified potato dextrose agar at the appropriated concentrations. The tested medium was vigorously stirred until the tested essential oil formed an emulsion. The fungi were grown on PDA at 26°C and agar discs (0.5 cm in diameter) covered with mycelium were served as inoculum. The discs were taken from the margin of 7 day-old colony for *F. oxysporum* and *Phytophthora* sp. and 14 day-old colony for *Alternaria* sp. and placed face-down on the tested medium. The plates were incubated at room temperature. After 7 and 14 days after inoculation, colony diameter was measured and calculated for percent inhibition. The modified PDA was used as control. All treatments were replicated four times.

$$\text{Percentage inhibition} = \frac{(C-T) \times 100}{C}$$

C : colony diameter of control plate

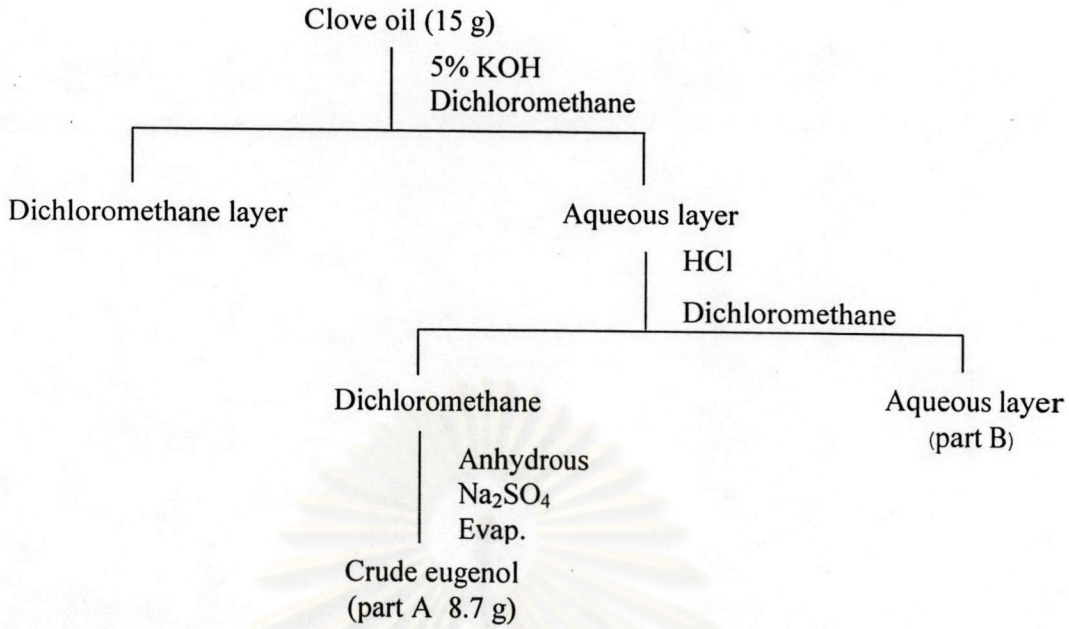
T : colony diameter of treated plate

*2.5.2.2 Conidial germination inhibition assay*

Each essential oil was tested for the conidial germination inhibition activity. The fungal spores were washed from the surface of agar plates with sterile 0.5 % (v/v) Tween 80 in distilled water. The spore suspension was diluted with potato dextrose broth to the concentration of approximately 1.0 x $10^3$ spore/mL. The inocula were stored at 4 ° C for further used. Mixed solution containing 1000 ppm of each essential oil in 1 mL of spore suspension was manually stirred for fifteen minutes before spreading on PDA. Spreaded plates were incubated for 36 hours and spore germination was counted as colony forming unit (CFU) and calculated percent germination. Spreaded plates of spore suspension were used as control. All treatments were replicated four time.

## 2.6 Isolation of eugenol from clove oil

Eugenol was separated from the clove oil by following the method described in Techniques and Experiments for Organic Chemistry (Fessenden, 1983). The clove oil (15 g) was dissolved in 5% aqueous KOH. The mixture was extracted with dichloromethane twice. The upper layer containing potassium salt of eugenol was neutralized with dilute aqueous HCl and then extracted with dichloromethane twice. The eugenol-dichloromethane solution was dried over anhydrous $Na_2SO_4$. The dichloromethane was removed by rotary evaporator to yield crude eugenol (8.7 g). The general procedure for eugenol separation from clove oil is summarized in Scheme 2.1.




**Scheme 2.1** The general procedure for eugenol separation from clove oil

## 2.7 Evaluation of antifungal activity of potential essential oil

The potential essential oils from the preliminary screening were evaluated based on the results of mycelial growth and sporulation inhibition at various concentrations. Each potential essential oil was prepared for 1, 10, 100 and 1000 ppm in melting modified PDA. The mycelial growth inhibition assay described above and calculated for percentage inhibition and calculated $IC_{50}$ using Probit Analysis Program (SPSS Inc.). To collect conidia for sporulation, petri dishes from each treatment were rinsed with 10 mL sterile distilled water. The surface was scrapped with a glass rod. The number of spore was determined by haemocytometer and converted to spores per square centimeter of plate culture (Dimitra, Basil and Moschos, 2000).

## 2.8 Antifungal tests by bioautographic assay

### 2.8.1 Spore suspension preparation

*F. oxysporum* and *Alternaria* sp. were grown on PDA slants for 10 days at 26 °C. Spores were harvested by adding steriled distilled water supplement with Tween 80 (0.5 %, v/v) filtering through steriled cotton wool. The number of conidia was estimated by haemocytometer.

### 2.8.2 Bioautographic assay

Fungal growth Inhibition on thin layer chromatographic (TLC) plate was evaluated by bioautographic assay modified from the method described by Beom (Beom, Surk and Byung, 2000). Each essential oil was spotted on silica gel TLC plate and developed with hexane-ethyl acetate (85:15 v/v). After air-drying to remove the solvent, the TLC plate was sprayed with spore suspension. After incubation in water agar for 3 days at 28 °C, the plate was stained with 1% (v/v) lacto-phenol in 5% (v/v) acetic acid for 5 minutes and then destained with 5% (v/v) acetic acid for 10 minutes. The $R_f$ value of antifungal active substance was calculated based on the bands visualized under UV light and vanillin-sulfuric acid reaction. All treatment replicated three times.

## 2.9 Application on post harvest control

### 2.9.1 Fruit preparation

The unriped bananas were obtained from orchard grower in Chainat district. Fruits were washed with tap water, air dried and stored at 10-14 °C before use.

### 2.9.2 Inoculation

Conidia of *F. oxysporium* 43-68 were recovered from 7 day-old culture and adjusted to $1.0 \times 10^6$ conidia/mL. Fruits were inoculated with 50 µL of conidial suspension on the hand of the fruit and incubated at room temperature for 24 hours.

### 2.9.3 Treatment of inoculated fruits

The inoculated fruits were dipped with 1000 ppm of each selected essential oil for 15 minutes (5 replicates per treatment). Treated fruits were packed in a paper box and allowed fruits ripening. After the ripening period, the infection area was observed and the infection area was calculated based on tissue necrosis per total area.