CHAPTER III

MATERIALS AND METHODS

3.1 Plant Materials

The plant materials were collected from various locations in Thailand and at different periods of time as shown in Table 1. Authentication was achieved through comparison with herbarium specimens at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agricuiture and Coorperative, Thailand.

Table 2 Cultivating locations of collected plants and harvesting times.

Number	Name	Place	Harvesting times
1	*Coleus amboinicus* Lour.	Faculty of Pharmaceutical Science Chulalongkorn University, Bangkok	December 1996
2	*Hyptis suaveolens* Poit	Pak Thong Chai Nakorn Ratchasima,	December 1996
3	*Mentha arvensis* L. var *piperascens* Malinvaud	Chiang Mai	October 1997
4	*Mentha cordifolia* Opiz	Nakornprathom	April 1997
5	*Ocimum basilicum* L.	Pakkred Nonthaburi	August 1996
6	*Ocimum canum* Sims.	Pakkred Nonthaburi	August 1996
7	*Ocimum gratissimum* L.	Bangkok	June 1997
8	*Ocimum sanctum* L.	Pakkred Nonthaburi	September 1996
9	*Perilla frutescens* Britt.	Chiang Mai	May 1997
10	*Pogostemon cablin* Benth	Faculty of Pharmaceutical Sciences Chulalongkorn University	April 1997

3.2 Essential oil content and composition

3.2.1 Essential oil content determination

Essential oil was determined by the method described in the Association of Official Analytical Chemists (method 962.17, AOAC, 1990). One hundred and fifty grams of each sample was put into a 1000 ml round bottom flask. The tridistilled water was added into the flask to about half full. The flask was connected to the apparatus for the determination of volatile oil (Fig. 1). The content of the flask was distilled until two consecutive readings taken at one hour interval showed no change in oil content (about four hours). After cooling, the oil volume was measured, calculated and expressed as millilitre of the oil per one hundred grams of sample. The essential oil obtained was then collected and stored at 4°C until being analyzed for its chemical composition by GC-MS.

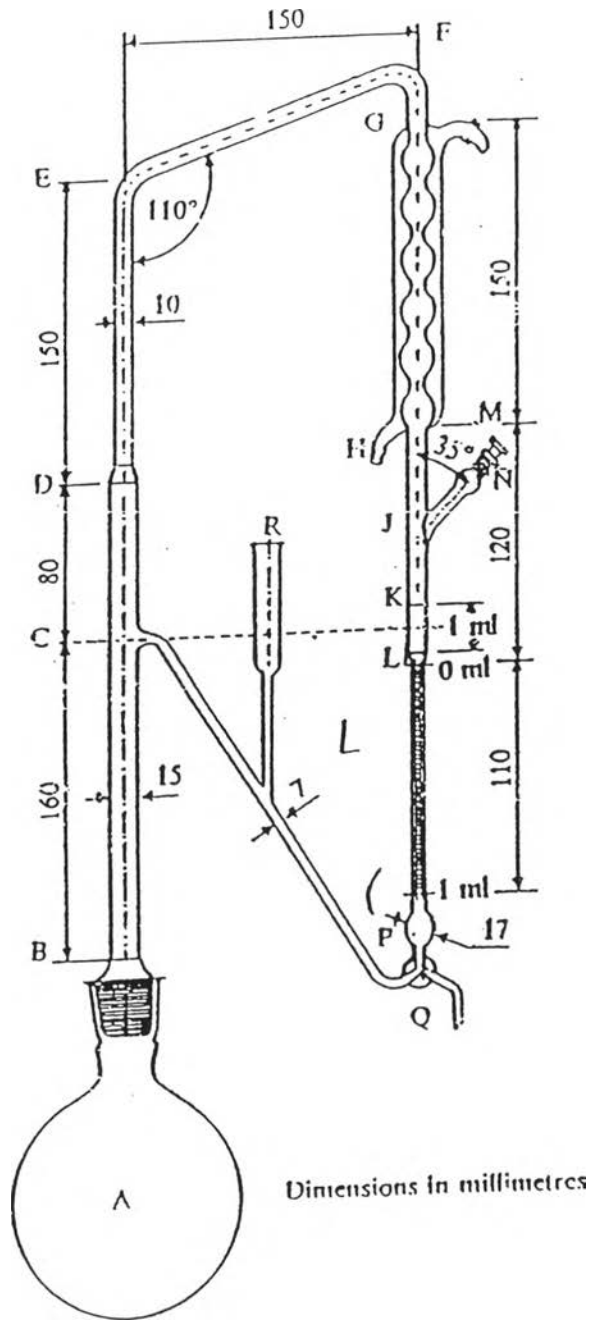

Figure 1 Apparatus for volatile oil content determination.

3.2.2 Gas chromatography-mass spectrometry

For identification of the composition of essential oil, a gas chromatography coupled with a mass spectrometer (GC-MS) was used . The essential oil was diluted to 1:100 in methanol before being injected into GC-MS system. The condition of GC-MS was described below. The spectra were recorded and compared with the terpene library (Adam,1995).

GC-MS Condition

Instrument model	**Varian Saturn 3**
Column	fused silica capillary column (30 m x 0.25 mm.i.d.) coated with DB-5 (J&W) film thickness 0.25 μm.
Column programming	60-240°C rate 3°C/min
Injector temperature	240°C
Helium carrier gas	1 ml/min
Split ratio	100:1
Accelerating voltage	1700 volts
Sample size	1 μl
Solvent	HPLC grade methanol

3.3 Scanning electron microscopic examination (Weakly,1972)

The leaf was cut into small pieces (3x5 mm^2). Then, they were fixed in a primary fixative (2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2) at room temperature for 1 h or kept in refrigerator overnight. After that they were washed 3 times with buffer, 10-15 min in each and were treated with secondary fixative,1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2, for 1-2 h. The specimens were dehydrated through an ascending series of ethanol 30%, 50%, 70%, 90%, and absolute ethanol. Each dehydration step took 10-15 min. Finally they were dehydrated in the

critical point dryer. the specimen was fixed to a stub and coated with a thin film of gold by Sputter coater.

The glandular trichomes responsible for the essential oil accumulation were examined in a Jeol JSM-5410LV scanning electron microscope.

3.4 Determination of antimicrobial activities of essential oils

3.4.1 Agar diffusion assay

The preliminary study of antimicrobial activities of essential oil was done by using the agar diffusion method (Edwin *et al.*, 1985; Lorian, 1991)

3.4.1.1 Preparation of sample

Each essential oil was diluted to a final concentration of 10% in 0.1% sterile Tween 80.

3.4.1.2 Preparation of the inoculum

The bacterial strains used were as follows :

- *Staphylococcus aureus* ATCC 29213
- *Enterococcus faecalis* ATCC 29212
- *Bacillus subtilis* ATCC 6633
- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853

Preparations of bacterial inocula were done according to the standard method (Lorian, 1991). Each bacterial strain was cultured overnight on Trypticase Soy Agar (TSA) plate at 37°C. Four well isolated colonies of the overnight grown culture were inoculated into a 5-ml Trypticase Soy Broth (TSB) and incubated at 37°C for 2-3 h. The turbidity of inoculum was adjusted with sterile normal saline solution to match a 0.5 turbidity standard of Mc Farland No 1.

The fungal strains used in this study were as follows :

- *Candida albicans* ATCC 10231
- *Microsporum gypseum* (clinical isolate)

Candida albicans ATCC 10231 was grown on Sabouraud Dextrose Agar (SDA) slant at 30°C for 24 h. The inoculum was prepared by suspending the culture in sterile normal saline solution and turbidity of the inoculum was adjusted to match a 0.5 turbidity standard of Mc. Farland No 1.

Spores of *Microsporum gypseum* grown on SDA slant at 30°C for 4 days were washed from the slant culture with sterile 0.05% Tween 80. The turbidity of the spore suspension was adjusted to match 0.5 turbidity standard of Mc. Farland No 1.

3.4.1.3 Preparation of test plates

- For testing bacteria :

Mueller Hinton Agar (MHA) was melted and allowed to cool at 45°C - 50°C in a water bath. Then 25 ml of the melted agar medium was dispensed into sterile glass petri dishes, with internal diameters of 9 cm, to yield a uniform depth of 4 mm. The agar was allowed to harden on a flat level surface. The plates were dried for 1 h at 37°C

- For testing fungi :

Sabouraud Dextrose Agar (SDA) was used and prepared as described above.

3.4.1.4 Inoculation of agar plates

A sterile cotton swab was dipped in each inoculum and the excess was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The entire surfaces of the MHA plate and the SDA plate for testing bacteria and fungi, respectively, were inoculated by streaking with the swab for 3 times and each time the plate was rotated 60 degrees.

3.4.1.5 Assay procedure

A 50 µl of each 10% oil sample or diluent (0.1% sterile Tween 80) was delivered to each hole (6 mm dia.) in the inoculated medium. This was done in triplicate. After maintaining at room termperature for 1 h, the bacterial and fungal plates were incubated at 37°C overnight and 30°C for 48-72 h, respectively. The oil samples showing inhibition zone were examined further for their minimal inhibitory concentrations (MIC).

3.4.2 Determination of Minimal Inhibitory Concentration (MIC).

Determination of MIC of essential oil was done by the broth microdilution test (Lorian, 1991).

3.4.2.1 Preparation of the inoculum

The inoculum was prepared as described above in 4.1.2. The turbidity of the 0.5 McFarland turbidity standard No 1. provides approximatedly 1 x 10^8 CFU/ml. The inoculum was further diluted 1:200 in Mueller Hinton broth.

3.4.2.2 Preparation of the essential oil dilutions

Each oil sample was mixed with equal volume of dimethyl sulfoxide (DMSO) and diluted with Mueller-Hinton broth (MHB) in a two fold dilution to give the concentrations ranging from 10% to 0.039% v/v.

3.4.2.3 Assay procedure

A 100 µl of each concentration was dispensed to the corresponding well of sterile multiwell microdilution plate (96-Flat-shaped wells). A 100 µl of diluted inoculum was added into each well. After incubating the tray at 37°C for 24 h, the lowest concentration of the oil sample that showed growth inhibition was considered as the MIC. This was done in duplicate. The corresponding concentrations of DMSO were also tested for their antibacterial activities. Inhibitory effect of DMSO was examined by measurement of culture turbidity in each well using microplate reader (Bio-Rad, model 450).