

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 2. Authentication was achieved through comparison with herbarium specimen at the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand.

Table 2 Investigating locations of collected plants and harvest time.

Number	Name	Place	Harvest time
1	<i>Cinnamomum camphora</i> Th. Fries. (leaves)	Bangkok	July 1997
2	<i>Cinnamomum camphora</i> Th. Fries. (leaves)	Rayong	January 1998
3	<i>Cinnamomum iners</i> Bl. (leaves)	Bangkok	November 1998
4	<i>Cinnamomum porrectum</i> Kosterm (leaves)	Phatthalung	May 1998
5	<i>Cinnamomum porrectum</i> Kosterm (bark)	Phatthalung	May 1998
6	<i>Litsea cubeba</i> Pers (leaves)	Chiang Mai	October, 1997
7	<i>Litsea glutinosa</i> C.B. Robinson. (fruits)	Ratchaburi	July 1997
8	<i>Litsea petiolata</i> Hook. f. (leaves)	Krabi	April 1998
9	<i>Litsea petiolata</i> Hook. f. (bark)	Krabi	June 1998
10	<i>Cinnamomum</i> sp.1 (leaves)	Rayong	April 1998
11	<i>Cinnamomum</i> sp.2 (leaves)	Rayong	April 1998
12	<i>Cinnamomum</i> sp.3 (leaves)	Khon Kaen	October 1998
13	<i>Cinnamomum</i> sp.4 (leaves)	Khon Kaen	October 1998

Table 2 (continued)

Number	Name	Place	Harvest time
14	<i>Cinnamomum</i> sp.5 (leaves)	Bangkok	November 1998
15	<i>Cinnamomum</i> sp.6 (leaves)	Bangkok	November 1998
16	<i>Cinnamomum</i> sp.7 (leaves)	Bangkok	November 1998
17	<i>Cinnamomum</i> sp.8 (leaves)	Bangkok	November 1998
18	<i>Cinnamomum</i> sp.9 (leaves)	Bangkok	December 1997
19	<i>Laurus nobilis</i> L. (leaves)	Chiang Mai	November 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 Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC, method 962.17) (Helrich, 1990). One hundred and fifty grams of each sample were put into a 1000 ml round bottom flask. The tridistilled water were 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 reading taken at one hour interval showed no change in oil content. 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.

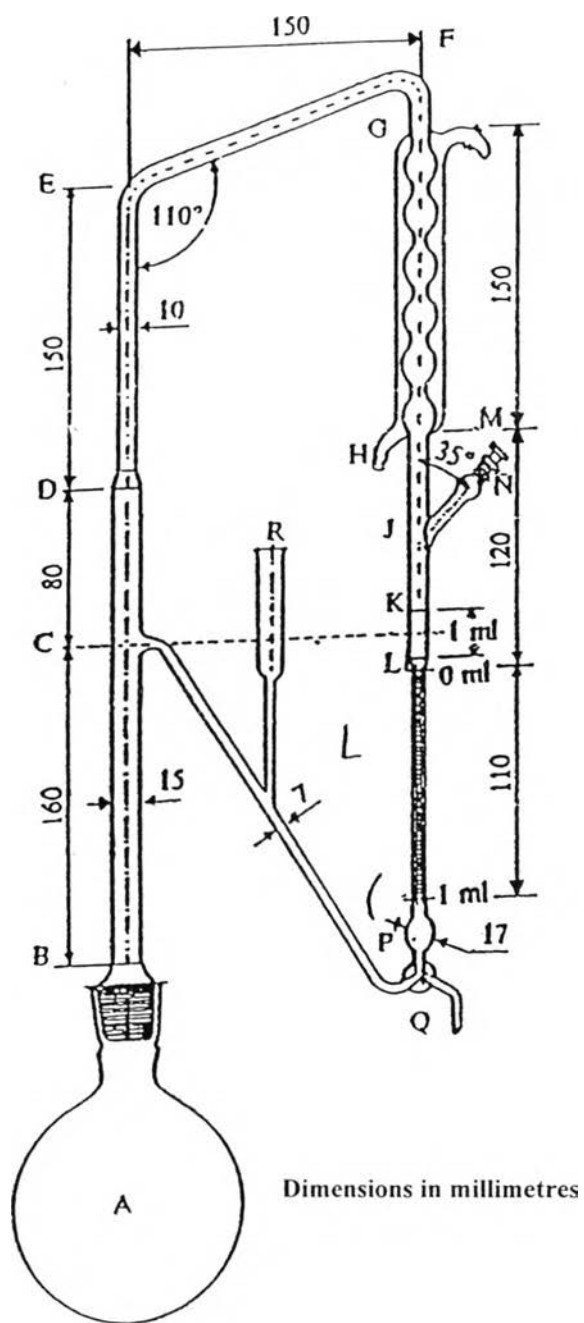


Figure 1 Apparatus for essential oil content determination

3.2.2 Gas chromatography-mass spectrometry

For the identification of the composition of essential oils, a gas chromatography-coupled with a mass spectrometry (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 program (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 (95% dimethyl 5% diphenyl polysiloxane) (J&W). film thickness 0.25 μ m
Column programming	60-240°C rate 3.3°C/min
Injector temperature	180°C
Helium carrier gas	1 ml/min
Split ratio	100:1
Accelerating voltage	1700 volts
Sample size	1 μ l
Solvent	methanol (HPLC grade)

3.3 Determination of antimicrobial activities of essential oils

3.3.1 Agar diffusion assay (Barry, 1991).

3.3.1.1 Preparation of sample

The essential oils were diluted to a final concentration of 10% in 0.1% sterile Tween 80.

3.3.1.2 Preparation of the inoculum

The bacterial strains used were as follows :

- *Staphylococcus aureus* ATCC29213
- *Enterococcus faecalis* ATCC29212
- *Bacillus subtilis* ATCC6633
- *Escherichia coli* ATCC25922
- *Pseudomonas aeruginosa* ATCC27853

Preparations of bacterial inocula were done according to the standard method. 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 broth to match a 0.5 turbidity standard of McFarland No 1.

The fungal strains used were as follows :

- *Candida albicans* ATCC10231
- *Microsporium gypseum* (clinical isolate)

Candida albicans ATCC10231 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 McFarland No 1.

Spores of *Microsporium 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 McFarland No 1

3.3.1.3 Preparation of test plates

- For testing bacteria :

Mueller Hinton agar (MHA) was melted and allowed to cool at 45 - 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.

3.3.1.4 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 degree.

3.3.1.5 Assay procedure

A 50 μ l of each 10% oil sample or diluent (0.1% sterile Tween 80) was delivered to each hole (6 mm diameter) in the inoculated medium. This was done in triplicate. After maintaining at room temperature 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).

3.3.2 Determination of minimal inhibitory concentration (MIC).

Determination of the MIC of essential oil was done by the broth microdilution test (Barry, 1991 ; Woods and Washington, 1995 ; Espinel-Ingroff and Pfaller, 1995).

3.3.2.1 Preparation of the inoculum

- For testing bacteria :

The inoculum was prepared as described in section 3.3.1.2. The turbidity of the 0.5 McFarland turbidity standard No 1 provides approximately 1×10^8 CFU(colony forming unit)/ml. The inoculum was further diluted to 1:100 in Mueller Hinton broth.

- For testing fungi :

The inoculum was prepared as described in section 3.3.1.2. This produced a fungal suspension containing 1×10^6 to 5×10^6 organisms per ml. The inoculum was further diluted to 1:100 in Sabouraud dextrose broth.

3.3.2.2 Preparation of the essential oil dilutions (Mann and Markham, 1998).

- For testing bacteria :

The oil samples were mixed with equal volume of 0.5% tween 80 and diluted with MHB containing 0.1% agar in a two-fold dilution to give the concentrations ranging from 10% to 0.039% v/v

- For testing fungi :

Sabouraud dextrose broth (SDB) was used instead of Mueller Hinton broth (MHB) and prepared as above.

3.3.2.3 Assay procedure

A 50 μ l volume of each concentration of the essential oil was dispensed to the corresponding well of sterile multiwell microdilution plate (96-Flat-shaped wells). Another 50 μ l volume of diluted inoculum was added into each well. After incubating the tray at 37°C for 24 h, the lowest concentration of oil sample that showed growth inhibition was considered as the MIC. This determination was done in duplicate. The corresponding concentrations of oil in diluent were used as turbidity control. Inhibitory effect of oil was examined by measurement of culture turbidity in each well using microplate reader (Bio-Rad, model 450).