CHAPTER III
MATERIALS AND METHODS

3.1 Chemicals

Both of basal culture media, Murashige and Skoog's (MS) and Gamborg B5 (B5) (see appendix) were purchased from GIBCOBRL (Scotland). Plant growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-furfurylaminopurine (kinetin) were purchased from Fluka Laboratories. (+)-Saccharose was purchased from Fisher chemicals. Gelling agent (Bacteriological agar) was purchased from GIBCOBRL (Scotland). All organic solvents used in this experiment (e.g. dichloromethane and ethanol) were HPLC grade. Water was triple deionised and distilled in glass.

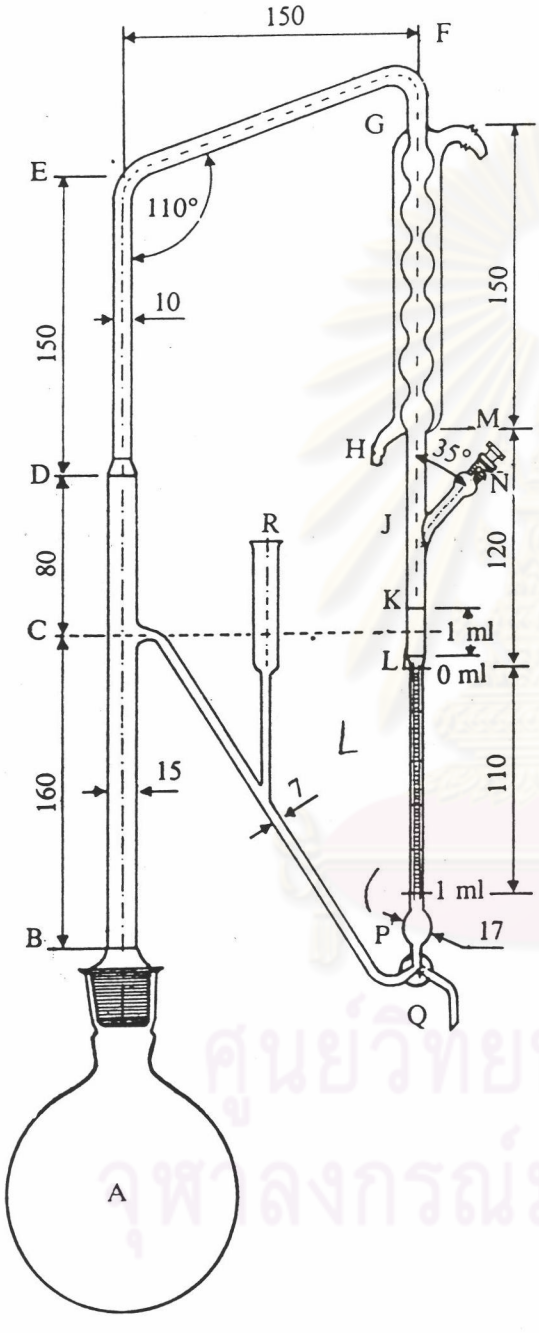
3.2 Plant materials

The leaf explants of *Artemisia dubia* Wall. ex Bess. were collected from plant garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. These explants were identified by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D. of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.3 Hydrodistillation of *Artemisia dubia* Wall. ex Bess. leaves

Essential oil was determined by the method described in the Association of Official Analytical Chemist (method 962.17, AOAC, 1990). The fresh leaves were hydrodistilled in a Clevenger-type apparatus (Fig. 5). Three hundred grams of *Artemisia dubia* Wall. ex Bess. leaves were 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 determination of volatile oil. The content of the flask was distilled until two consecutive reading 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 analysed for its chemical composition by Gas chromatography-Mass spectrometry (GC-MS).



Apparatus

The apparatus (see Figure) is constructed of resistant glass of low coefficient of expansion and has the following dimensions:

(a) a round-bottomed flask (*A*) of suitable capacity with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;

(b) a condenser assembly that closely fits the flask and consists of the following parts fused into one piece:

— a vertical tube (*BD*), 215 to 265 mm long and 14 to 16 mm in internal diameter,

— a bent tube (*DEF*) in which the distances *DE* and *EF* are each 145 to 155 mm long and 9 to 10 mm in internal diameter,

— a bulb-condenser (*GH*), 145 to 155 mm long and 9 to 10 mm in diameter at the restrictions,

— a vented stopper (*M*) and a tube (*N*) with an orifice of diameter about 1 mm that coincides with the vent. The wide end of the tube (*N*) is of ground-glass, having an internal diameter of 10 mm,

— a tube (*HJ*), 75 to 85 mm long and 9 to 10 mm in internal diameter, making a 30° to 40° angle (*HJN*) with the tube (*JN*),

— a graduated tube (*LP*), graduated over 105 to 115 mm to give 1 ml subdivided in 0.01 ml. Above the graduation are two circular marks (*K* and *L*),

— a bulb-shaped swelling (*P*), about 2 ml in capacity,

— a three-way tap (*Q*), and

— a connecting tube (*CQ*), 7 to 8 mm in internal diameter, fitted in the middle with a filling funnel (*R*). The junction (*C*) is at a level 20 mm higher than the uppermost graduation;

(c) a suitable heating device, allowing a fine control; and

(d) a vertical support with horizontal ring covered with insulating material.

Before use, clean the apparatus by successive washings with *acetone*, *water* and *chromic acid cleansing mixture* inverting several times, and rinse with *water*. Drain the apparatus and mount it in a place protected from a draught.

Figure 5 Apparatus for essential oil content determination (Clevenger type apparatus) (Thai Pharmacopoeia, 1987)

3.4 Identification of essential oil obtained from hydrodistillated of *Artemisia dubia* Wall. ex Bess. leaves by using Gas Chromatography-Mass-spectrometry (GC-MS)

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 spectrum was recorded and compared with the terpenes library (Adam, 1995) and NIST mass spectral library (NIST, 1998).

GC-MS condition

Instrument model	Varian Saturn III
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
Carrier gas	Helium (1 ml/min)
Split ratio	100:1
Accelerating voltage	1700 volts
Sample size	1 μl
Solvent	HPLC grade methanol

3.5 Preparation of leaf explants

Leaf explants of *Artemisia dubia* Wall. ex Bess. were surface sterilized by shaking them with surface sterilizing agent (see appendix) for 1 hour, 7% (w/v) and 5% (w/v) hydrogen peroxide for 15 and 7 minutes, respectively using Tween 80 as wetting agent (surfactant), and then washing them with sterilized distilled water for 3 times prior to use in callus initiation.

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3.6 General preparation of semi-solid and liquid media

Murashige and Skoog's medium (MS) and Gamborg B5 medium (B5), were used as basal media throughout this experiment. Both of them have not any plant growth regulators.

Approximately 800 ml of distilled water was measured and poured into a glass beaker. The basal media were slowly added to the distilled water with gentle continuous stirring using a magnetic stirrer. After media completely dissolved, the requirement of sucrose, *L*-ascorbic acid, 2,4-D and kinetin were added to the solution. The pH was adjusted with sodium hydroxide or hydrochloric acid to 5.7-5.8. The solution was made up to 1 L with distilled water. In order to get the semi-solid medium, the agar was added this point. At the final volume, the concentration of sucrose, *L*-ascorbic acid and agar were 3% (w/v), 5 ppm, and 10% (w/v) respectively. The solution was then heated and stirred for dissolving agar completely. The media were poured into suitable container. Semi-solid media were poured into 250-ml screw capped wide mouth bottle, and liquid media were poured into 250-ml Erlenmeyer flasks which were covered with aluminium foil. They were then autoclaved at 121 $^{\circ}$C (15 lb/in^2 or 1 kg/cm^2) for 15 minutes. They were left to cool and stored at room temperature prior to use.

3.7 Initiation and maintenance of callus cultures

The surface sterilized leaf explants of *Artemisia dubia* Wall. ex Bess. were cut into 1x1 cm^2 and placed on semisolid basal media, Murashige and Skoog's (MS) or Gamborg B5 (B5) which containing 2,4-D (1mg/L) and kinetin (0.1 mg/L) for callus initiation. The maintenance of callus cultures was carried out by incubating them at the temperature of 25±2°C under continuous light using cool white fluorescent tubes and then subculture them to the new semi-solid media every 30-45 days, depending on the individual cell growth. When the cultures grew to 3-4 cm^3 or the media showed sign of exhaustion and dehydration, they were selected only good looking or healthy pieces and

aseptically transferred to 250-ml screw capped wide mouth bottles containing 50 ml of new media.

3.8 Initiation and maintenance of cell suspension cultures

After the fourth generation, good looking or healthy pieces of callus were aseptically transferred to liquid media in Erlenmeyer flask and shook. The flasks were kept on an orbital shaker, circulating at 120 rpm with the temperature of 25±2°C under continuous light using cool white fluorescent tubes. The maintenance of suspension cultures were carried out by subculturing them to new fresh liquid media, with a dilution of 1:2, every 15-21 days.

3.9 Methods for improving the production of essential oil constituents in plant cell culture

The cell suspension cultures in MS media were selected for studying methods for improving essential oil constituents, particularly davanone.

3.9.1 Cell immobilisation

Nylon meshes (1x1x1 cm^3), pore size 10 ppi, were washed with distilled water several times and autoclaved (121°C (15 lb/in^2 or 1 kg/cm^2), 15 minutes), to remove any chemical harmful to cell growth. They were transferred to new media and then added suspension cultures to the same media, to enable formation of immobilisation. After 21 days, suspension cultures were successfully immobilised on nylon meshes, and then transferred them to new media prior to use for biotransformation methods.

3.9.2 Precursor feeding

Geranyl acetate was used as precursor in this experiment. Different amount of geranyl acetate (5, 10, 50 and 100 ppm), with the purity being over 98%, were mixed with 70% (v/v) ethanol to get clear solutions, and fed to the

liquid media by passing through pre-autoclaved Mobile Phase Filters (0.45 μM pore size, Whatman).

The immobilised cells, which used for biotransformation methods were transferred to the liquid media containing different amount of precursors (5, 10, 50 and 100 ppm). After cultures were harvest, they were analyzed by using methods described in Section 3.10, 3.11, 3.12 and 3.13, and the yield of davanone were calculated by comparing the area under peak of davanone with total area under peak from integration of GC chromatogram.

3.9.3 Use of adsorbent

Porapak Q (100-120 mesh) (Water) was used as adsorbent in this experiment. It was conditioned at 200 °C overnight, and then packed into glass tubes and washed with dichloromethane, prior to use for collection of essential oil from suspension cultures (Zhu, 2000).

3.9.4 Collected of volatile compounds from *Artemisia dubia* Wall. ex Bess. immobilised cells which had been fed various concentrations of geranyl acetate

Air was passed through *Artemisia dubia* Wall. ex Bess. immobilized cells whilst had been fed various concentrations of geranyl acetate during cell growth via a preautoclaved Mobile Phase Filter (0.45 μM pore size, Whatman) with a Porapak Q adsorbing tube fixed at the outlet to collected volatile oil compounds.

Figure 6 shows the apparatus for collecting volatile compounds in immobilized cells which had been fed various concentrations of geranyl acetate.

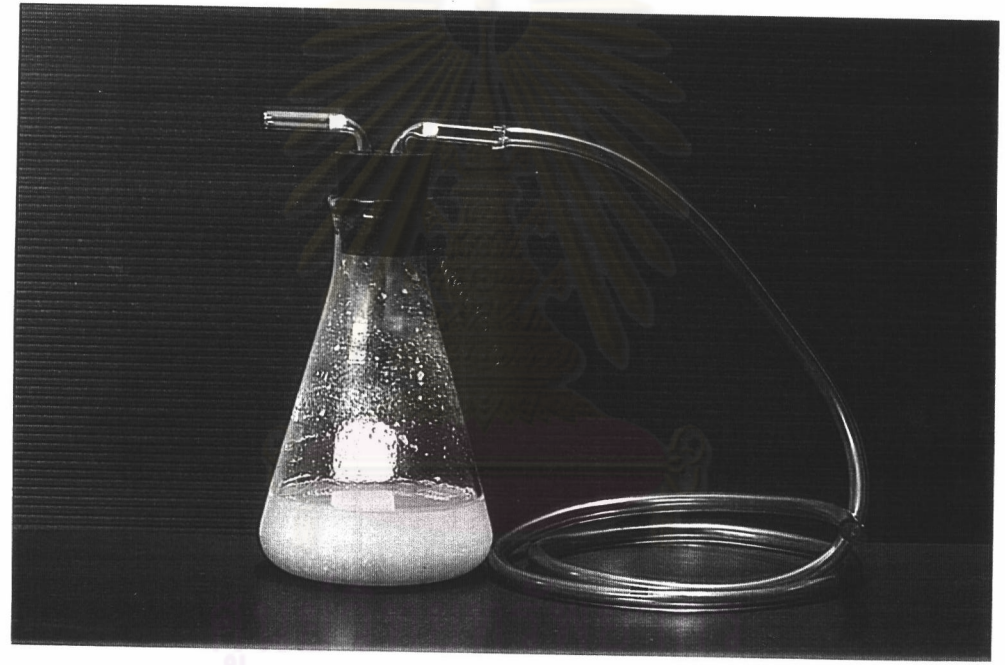

Figure 6 The apparatus for collecting volatile compounds in immobilized cells which had been fed various concentrations of geranyl acetate.

3.10 Extraction of essential oil

The leaves of *Artemisia dubia* Wall. ex Bess. were blended with blender, and 10 g of them were directly extracted with sufficient redistilled dichloromethane. This extract, organic phase were filtered through dichloromethane-moistened Whatman no. 1 filter paper into graduated sample vials, evaporated under nitrogen and concentrated to 100 µl.

Callus cultures were directly extracted by grounding them with a pestle and mortar into fine materials. A known amount of fresh material (10 g) was extracted by using method mentioned above.

Cell suspension cultures and immobilized cells were extracted after separation of the cells and liquid media. After suspensions were filtered, extraction of cells was carried out using the mentioned above.

After using the biotechnological technique, the Porapak Q tubing which used for adsorbing essential oil was then taken and washed with 5 ml of dichloromethane to release the essential oil constituents. These extracts were filtered, then mixed the filtrate with the filtrate from immobilized cells and evaporated by using method mentioned above.

3.11 Analysis of essential oil from dichloromethane extract by using Gas Chromatography (GC)

1 µl of the concentrated extract prepared above was chromatographed by capillary GLC. A Shimadzu Gas Chromatography, fitted with a flame ionization detector (FID), was used for the analysis of essential oil. Nitrogen gas was used as the carrier gas. The column used was OV-1, high performance fused silica linked methyl silicon capillary column; the film thickness was 0.25 µM. The length of the column was 30 m and its internal diameter was 0.22 mm. The results were recorded on the Hewlett Packard 3392A integrator. Normally the operating condition was 50-200 °C at 5 °C/min, and isothermal for 10 minutes. The injector and detector temperature was 250°C. 2-Hydroxy-4-methoxy-benzaldehyde, with the purity of 98%, was used as internal standard for quantitative analysis of davanone and essential oil content.

3.12 Identification of essential oil from dichloromethane extract by using Gas Chromatography-Mass spectrometry (GC-MS)

A Fison instrument series 8000 (GC) fitted with a Fisher instrument (MS) was used for identification of essential oil constituents. Gas chromatography conditions were as follow: helium gas was used as carrier gas and column was DB1 which 30 m long and internal diameter was 0.25 mm, operating condition was 60-280°C at 15°C/min and the injector temperature was 280°C. Mass spectrometer conditions were as follow: ionization potential was 70 eV, analyzer was quadruple and detector was photomultiplier.

Identification was based on sample retention time data and electron impact mass spectra data compare with the NIST library and literatures (Adams, 1995 and Silverstein and Webster, 1998).

3.13 Quantitative analysis of davanone

For quantitative analysis of davanone, the peak area of davanone under Gas chromatography chromatogram in Section 3.11 was calculated by using the internal normalisation method.