CHAPTER III

MATERIALS AND METHODS

1. Sources of Materials

1.1 Plant Materials

The leaves and roots of *A. salviifolium* used in this study were obtained from the plant growing in the open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Tissue and cell cultures of *A. salviifolium* were established from the leaf part of the plant as described in section 3.

1.2 Chemicals and Equipment

Authentic emetine and cephaeline were obtained from Prof. Zenk M.H. of the Institue of Pharmaceutical Biology, University of Munich, Munich, Germany. Organic solvents were all reagent grade or better. Thin layer chromatography plates of silica gel 60 F254 on aluminium sheets were obtained from Merck (Damstadt,Germany).

Chemicals for culture media were all tissue culture grade. Various plant growth regulators were purchased from Gibco Laboratories (New York, USA) and gelling agents were purchased from Difco Laboratories (Detroit Michigan, USA) and Sigma Chemical Co.(St.Louis, Mo, USA). Water was triple deionized.

[Ring-^{3}H]-p-Hydroxyphenylethylamine was pruchased from Du Pont (Wilmington, USA).

Detection of radioactivity was performed by using both Liquid Scintillation Counters Wallac 1409/1411 (Finland) and TLC Linear Analyzer LB 284/285 (Berthold, Germany).

2. Phytochemical Techniques

2.1 Preparation of Crude Extracts for Alkaloid Detection

Crude extracts were prepared from the leaves and roots of *A.salviifolium*. Practically, each plant part (700 g) was blended with 500 ml 95 % ethanol. The resulted crude extract was filtered through cotton wool and evaporated to dryness *in vacuo* to give a dark-brown residue. The residue was then dissolved with 150 ml 1.0 N. HCl. The undissolved residue was separated by filtration using Whatman filter paper No 1. The aqueous acidic solution was then adjusted to about pH 7-8 with 2.5 N NaOH. This solution was extracted three times with chloroform. The combined chloroform extracts were concentrated *in vacuo* and used for alkaloid detection by thin layer chromatography. Toluene : ethyl acetate : diethylamine = 7:2:1 were used as solvent system, followed by spraying with Dragendorff's reagent.

For tissue cultures of *A. salviifolium*, the callus or suspension cultures were refluxed with 95 % ethanol for 2 hr and filterd. The filtrate was concentrated *in vacuo* and used for alkaloid detection by TLC.

2.2 Isolation of Alkaloids from the Leaves of *A. salviifolium*

700 g of fresh *A. salviifolium* leaves were blended with 500 ml 95 % ethanol. After being filtered through cotton wool, the leave residue was further macerated with 500 ml 95 % ethanol for 24 hr and the extract was filtered again. Both filtrates were pooled and concentrated *in vacuo*. The resulted residue was dissolved with 150 ml 1.0 N HCl followed by fitration using Whatman filter paper No 1. The acidic filtrate was adjusted to about pH 7-8 with 2.5 N NaOH. The resulted solution was extracted three times with chloroform. The combined chloroform extracts were concentrated *in vacuo* to obtained crude alkaloid residue. The crude alkaloids were then loaded onto silica gel column chromatography (2.5 cm x 30 cm, dry packing). The column was eluted with benzene : methanol : diethylamine (7:2:1) and 20 ml fractions were collected until no traces of alkaloids could be detected. The fractions were checked for alkaloids by thin layer chromatography, followed by spraying with Dragendorff's reagent. The fractions containing the same alkaloids were pooled and evaporated. Then alkaloid residue was redissolved and streaked on preparative thin layer chromatography plate. This preparative plate was developed three times using the solvent system of toluene : ethyl acetate : diethylamine (7:2:1). By using UV detector(254 nm), the alkaloid residue was divided in three bamds. The major band was eluted with methanol and filtered through plastic syringe with filter holder to remove silica gel. This part of alkaloid extract was crystallized by reduced temperature and let the solvent gradually evaporated. The crystal was washed by cool methanol. The process was repeated until the pure compound was obtained. EIMS spectrum of the purified compound was obtained with Jeol Fx 300 double focusing of Mass spectrometer of the Scientific and Technological Research Equipment Center, Chulalongkorn University.

3. Plant Tissue Culture Techniques

3.1 Preparation of Culture Media

Liquid culture media used in this study included Murashige and Skoog (MS, Murashige and Skoog, 1962), Gamborg (B5, Gamborg *et al.*, 1968), Woody Plant Medium (WPM, Lloyd and Mc Cown, 1980), and Root Medium (RM, Prof. Zenk's recipe). Their composition and preparation methods are described in Appendix. For the preparation of semisolid media, the above nutrient solutions were added with agar to obtain 0.8 % (w/v).

3.2 Preparation of *A. salviifolium* Explants

Young leaves of *A. salviifolium* were surface sterilized by submerging in 70 % ethanol for 1-3 min followed by immersion in 15% Clorox for 15 min and finally 2-3 rinses in sterile distilled water. The surface sterilized leaves were then dissected into small pieces (0.5 x 2.0 cm). The explants were transferred to semisolid nutrient media for callus initiation.

3.3 Establishment of Callus Cultures of *A. salviifolium*

Each medium (MS, B5 and WPM) was supplemented by 30 g/l sucrose and 0.8 % (w/v) agar. It contained various different levels of auxin and cytokinin at the concentration of 0.1, 0.3, 0.5, 1.0, 2.0 mg/l. The growth regulators used in the experiments of callus establishment included NAA, 2,4-D (auxin), Kinetin, BA (cytokinin) and gibberellic acid (GA_3).

The explants of *A. salviifolium* were transferred onto the semisolid media and maintained at 25 ± 2° C under controlled 16 hr photoperiod (2000 lx). The effect of various basal semisolid media and plant growth regulators on the callus induction of these explants were investigated.

After eatablishment, The callus of *A. salviifolium* were subcultured every 3 weeks onto the same medium for proliferation and maintained under the same conditions as described above. The callus formation in each medium was observed periodically and the results were recorded. The appropriate medium was chosen to maintain the callus.

3.4 Establishment of Cell Suspension Cultures

Cell suspension cultures of *A. salviifolium* were initiated from the established callus cultures. The callus were inoculated in 250 ml erlenmeyer flask containing 50 ml of liquid medium with 30 g/l sucrose, and appropriate amount of auxin and cytokinin based on the results obtained from section 3.3. These cultures were kept on a rotary shaker at 100 rpm, 25 ± 2 ° C. The first group of suspension culture were placed under controlled 16 hr photoperiod. The other were kept in the dark. These cell suspensions were subcultured on the same medium every 3 weeks by adding 10 ml of the suspension to 50 ml of fresh medium.

When the suspension cultures had sufficient biomass. The effect of macronutrient (nitrogen, potassium) concentrations on cell growth and emetine alkaloid production was studied. Various nitrogen concentrations used in the experiments were 14.5, 28.3, 43.4, 57.8 mM. Various potassium concentrations were 9.8 , 24.8 mM. After incubated for 1 week, the cultured cells were harvested by vacuum suction, weighed and detected for the alkaloids.

For further study on the secondary metabolite productions, the suspension cultures were supplemented with a) 2,4-D 1 mg/l , NAA 4 mg/l , b) IAA 4 mg/l, NAA 4 mg/l, IBA 8 mg/l. These supplements have been shown to be appropriate for the alkaloid production useful in the cell suspension of *Cephaelis ipecacuanha*.

3.5 Root Induction

The suspension culture of *A. salviifolium* obtained from section 3.4 were transferred in the RM liquid medium for root induction. The cultures were maintained on a rotary shaker at 100 rpm, 25 $\pm$ 2° C in the dark. Auxin (2,4-D, NAA), cytokinin (BA, Ki) and Gibberellin (GA_3) concentrations were added as 0.1, 1.0, 2.0, 3.0 mg/l.

Root from the whole plants were also cleaned up, sterilized and transferred in liquid medium for rooting. Liquid media were changed every 3 weeks.

4. Enzymatic techniques

4.1 Preparation of Enzyme Extracts from the Leaves of *A. salviifolium*

Fresh leaves (20 g) of *A. salviifolium* were quick frozen using liquid nitrogen and ground in a pre-cooled mortar. The resulted leaf powder was added with 20 ml 100 mM tricine-NaOH buffer pH 7.5 in a pre-cooled beaker and allowed to stand with continuous stirring at room temperature for approximately 10 min. Cell debris was removed by filtration through four layers of cheese-cloth, followed by centrifuge at 10000 g for 10 min. The supernatant was finally desalted by passing through a Sephadex G-25 (Pharmacia PD-10 column) to obtain the enzyme extract.

4.2 Detection of Dopamine-Secologanin Condensing Enzyme Activity

The activity of the enzyme responsible for dopamine-secologanin condensation was detected by incubated the enzyme extract with the following incubation mixture : 1.1 mM secologanin, 1.1 mM dopamine, and 120 µl enzyme solution (in 100 mM tricine-NaOH buffer, pH 7.5) in a total volume of 180 µl. The boil control was used as control. The reactions were started by the introduction of enzyme solution. After 60 min of incubation or during a time-course study, the incubation mixture (20 µl) was subjected to TLC (silica gal G) using the solvent system of chloroform : n-propanol : methanol : water (45:15:60:40) (chloroform phase). After the development, the TLC plate was viewed under UV 254 nm and scanned by using TLC-densitometer with the wavelength of 290 nm.

4.3 Time Course Studies on Enzyme Activities

Time course of the reaction-product formation was carried out using the same reaction mixture as described above. After incubation at 30 ° C for 30, 60, 90 and 120 min, each reaction tube was frozen immediately in liquid nitrogen. After thawing, 20 µl from each mixture was removed and subjected to silica gel plates. The same solvent system of chloroform : n-propanol : methanol : water = 45:15:60:40 (chloroform phase) was used for separation of the reaction product. The TLC plate was then scanned with TLC-densitometer using the wavelength of 290 nm.

4.4 Purification and Identification of Enzymatic Product

To identify the enzymatic product, a large scale (18 ml) incubation mixture was prepared. Its conditions were the same as that described above. After 2 hr, the incubation mixture was extracted four times, each time using ethyl acetate 18 ml. The pooled ethyl acetate fraction was concentrated and fractionated on a preparative TLC of silica gel G in chloroform : n-propanol : methanol : water (45:15:60:40) (chloroform phase). The band of product (Rf 0.79) was eluted with absolute ethanol. The product was purified on Waters Nova pak C18 (3.9 x 300 mm), using an isocratic solvent of water-methanol (9:11), at a flow rate 0.5 ml/min and detected by Waters 991 J Photodiode Array detector.

For preparing the compounds of deacetylipecoside and deacetylisoipecoside. A solution containing 14 mg secologanin and 10 mg of 3,4-dihydrophenylethylanine hydrocholride (dopamine) in 500 ul 0.1 M acetic acid - sodium acetate buffer pH 5.0 was allowed to react for 1 hr in darkness at 30 ° C under a nitrogen atmosphere. The reaction mixture was then chromatographed on TLC using the solvent system of

chloroform : n-propanol : methanol : water = 45:15:60:40 (chloroform phasee). The band containing both epimers was eluted with absoluted ethanol. The solution were dried and kept under a nitrogen atmosphere.