CHAPTER III

MATERIALS AND METHODS

The Isolation of ALK from *Anthocephalus chinensis* Leaves

1. Source of plant materials.

The leaves of *A. chinensis* were obtained from Khao Yai National Park. Prachinburi, Thailand.

2. Isolation of ALK (Figure 3.)

The dried coarsely powdered leaves were macerated with 95% ethyl alcohol for 10-15 days and filtered. The marc was remacerated with another portion of ethyl alcohol. The combined filtrate was concentrated under reduced pressure to syrupy mass and then evaporated with water bath till no traces of ethyl alcohol left, mixed with glacial acetic acid then diluted with distilled water to give about 10% acetic acid solution, well shaken and left to stand overnight. The filtered acid extract was made alkaline (pH=8-9) with strong solution of ammonium hydroxide and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield a syrupy crude based.

Crude based was dissolved in a small portion of chloroform, mixed with small amount of aluminium oxide 90 (active neutral for chromatography),let the content air-dried and placed onto the top of dry aluminium oxide column. The ALK was eluted with mixture of chloroform and ethyl alcohol, 8:2, the eluate was collected until no traces of ALK could

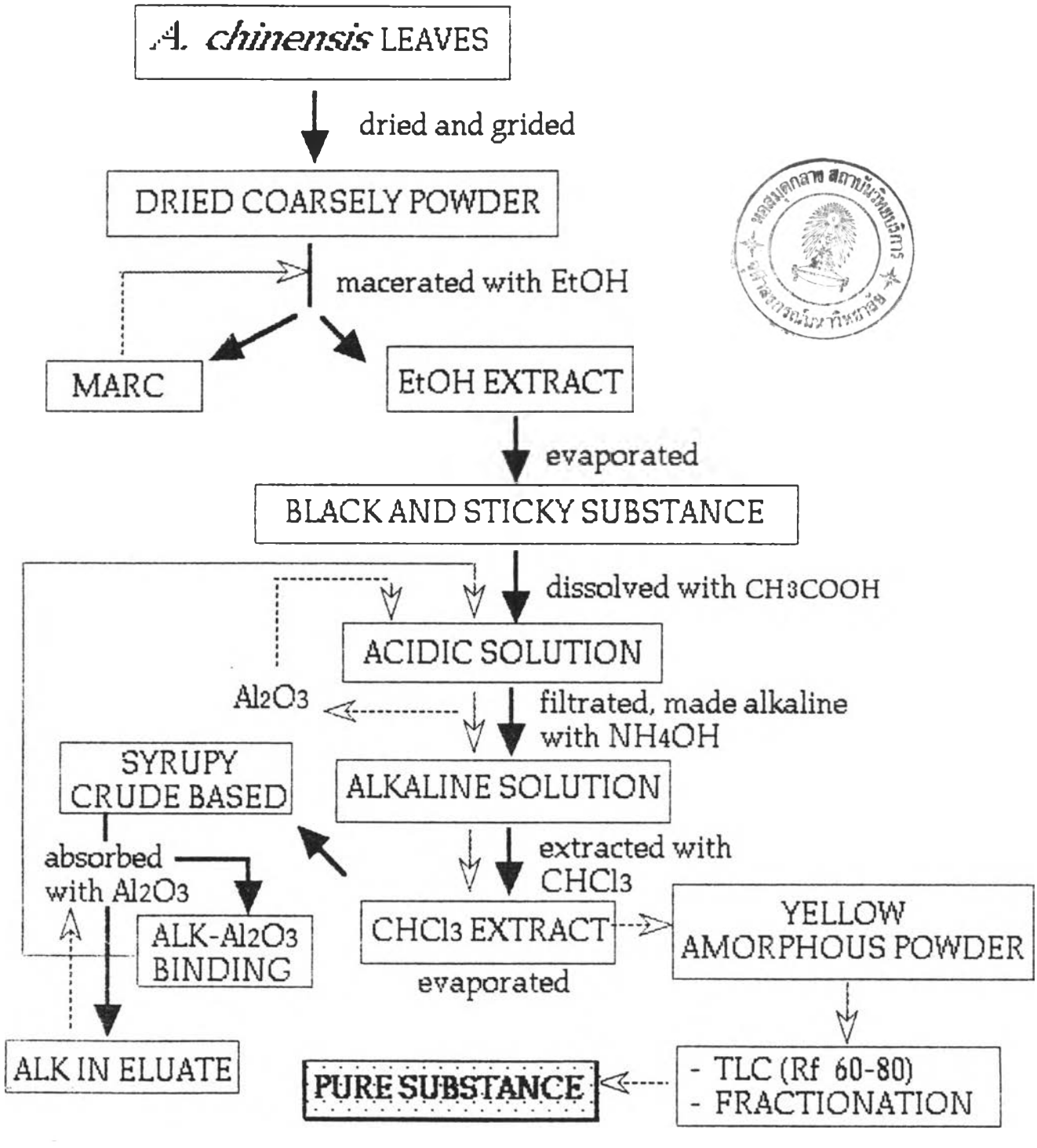

Figure 3. Outline of the extraction and purification of 3α-dihydrocadambine from *Anthocephalus chinensis* leaves. (EtOH, ethyl alcohol; CH_2COOH, acetic acid; NH_4OH, ammonium hydroxide; $CHCl_3$, chloroform; Al_2O_3, aluminium oxide; TLC, thin layer chromatography)

be detected with dragendolf reagent. This eluate was evaporated under reduced pressure and then reextracted with aluminium oxide column chromatography until no traces of ALK could be detected in eluate.

The adsorbent(aluminium oxide) were taken out of the column,air-dried,extracted with 10% glacial acetic acid solution and left to stand overnight. The filtered acid solution was made alkaline (pH=8-9) with strong solution of ammonium hydroxide and extracted with chloroform. The combined chloroform extract was dried over anhydrous sodium sulphate, and concentrated under reduced pressure to dryness yielding light brown amorphous powder.

3. Purification of light brown amorphous powder.

The amorphous powder was washed with diethyl ether to yield a cream coloured amorphous powder, 3α- dihydrocadambine , m.p. 180-181 ° C.

To test the purification of the ALK, 3α - dihydrocadambine, dissolved it in a small portion of chloroform and applied on thin layer chromatography, which was eluted with the mixture of chloroform and ethyl alcohol (8:2). The single band, Rf 60-80, was observed.

At finally, to reconfirm that this cream coloured amorphous powder is 3α - dihydrocadambine, the spectroscopic evidences : Ultraviolet Absorption Spectrum, Infrared Absorption Spectrum and Nuclear Magnetic Resonance (NMR) spectrum of this agent were so examined.

Experimental Animals

The experiments were performed on 60 tree shrews *(Tupaia glis)* of either sex weighing between 100-150 g.

Experimental procedure

1. Preparation of animals.

The animals were anaesthetized with sodium pentobarbital 25 mg/kg intraperitoneally. Supplementary doses (8-10 mg/kg) of sodium pentobarbital were given during experiment to maintain anaesthesia.

Polyethylene tube (PE 50; O.D.= 0.945 mm), filled with heparinized (20 μ/ml) saline were inserted into a femoral artery for recording AP and HR , and into a femoral vein for administration of drugs. Body temperature of the animal was maintained at 37-39 °C by a thermostatically regulated heating pad.

The animals were placed in a stereotaxic apparatus for rat (Narishige SR-6) with the bite - bar set 5.0 mm below the ear bars. When the head of the animal was secured in a stereotaxic headholder, the skin and connective tissue overlying the posterior occipital bone was separated to expose the skull. The brain were exposed through a burr hole in the occipital bone, underlying dura was removed and a small cotton pledget soaked in saline was placed on the exposed cerebellum to prevent drying.

2. Measurement of systemic cardiovascular activity

Systemic arterial blood pressure and HR were obtained through a heparinized saline-filled cannula in femoral artery connected with a

pressure transducer(Elcomatic EM 750 SER NO.250S) and were recorded on universal oscillograph (Harvard apparatus NO.50-9323).

3. 3α- dihydrocadambine administration

Cause of the slightly soluble of the ALK, it was dissolved in 10% polyethylene glycol (PEG) in normal saline solution (NSS) intravenous and in 20% PEG in artificial cerebrospinal fluid(aCSF) by intraventricular administration.

About one hour after completion of surgery or after AP and HR of the animal was then allowed to stabilize for at least 15 min, the experiment was begun.

3.1 Intravenous administration (IV)

The 3α - dihydrocadambine solution at doses 0, 0.8, 1.6, 3.2, 6.4, 16.0 and 24.0 mg/kg in 10% PEG in NSS in volume 0.3 ml were injected at the rate about 1 ml/min into femoral vein. And then, the AP was allowed to recover spontaneously.

3.2 Intraventricular administration (VENT)

After fixing the animal in the stereotaxic apparatus, the skin and tissues overlying the cerebrum were cut. From the distance between the bregma and the stereotaxic zero, the estimated position of the lateral ventricle was assessed. And then , the brain under the landmark was exposed, readily for VENT injection.

The ALK at doses 0, 0.4, 0.8,1.6 and 3.2 mg/kg in 20% PEG aCSF in volume 10 µl. were slowly injected into the lateral cerebral

ventricle through microsyringe. At autopsy , the placement of the microsyringe and ALK distribution were verified by slowly injecting of alcian blue dye through the needle to outline the 3rd and 4th ventricle as well as portions of the lateral ventricle and the spinal canal.

4. Electrical stimulation of fastigial nucleus (FN)

4.1 Preparation of a monopolar stimulating electrode.

Electrodes were made of tungsten wire, O.D.= 150 μm., the tip of tungsten wire was etched by applying 9-15 V AC voltage between microelectrode and carbon rod in 20% sodium hydroxide solution. The tip was sharped by repeated dipping in and out of the etching solution until the tip had a diameter between 1-2 μm. and then washing it with distilled water. The tungsten microelectrode was immersed into the insulating paint, varnish, left it slowly and then placed the coated microelectrode on air dry; repeated this step about 3 times.

The final stage was a removal of insulation from the tip, which done by poking the electrode tip into 0.9% NaCl solution and applying 90 V AC voltage, frequency 50 Hz to the electrode. And then, there was a small bubble given off that means the tip of electrode was exposed between 10-20 μm in length and then the electrode was ready for used.

4.2 FN stimulation

The FN was stimulated cathodally through monopolar microelectrode. The anode was a clip attached to the scalp muscle. The pulses were generated by electronic stimulator SEN-3201 (Nihon-kohden) and passed through an isolator SS-201J (Nihon-kohden). The stimulus

current was monitored on an oscilloscope(Leader LBO-522) by continuously displaying the voltage drop through a 10 KΩ resistor placed in series with the circuit (Figure 4.). The microelectrode was mounted on a stereotaxic micromanipulator and lowered through the cerebellum into the FN, to localize the most sensitive sites from which stimulation could elicited a maximal elevation of AP. The stereotaxic zero was established at the interaural line. The electrode was inserted into the cerebellum at 3.2 ± 1.0 mm caudally , lateral 1.0 ± 0.5 mm from the interaural line and horizontally 5.5 mm from the surface of the dura matter. From this point, the stimulating electrode was moved downward in 0.1 mm steps. At each step, the brain was stimulated. The exploratory stimulus consisted of 0.1 mS in duration, at a frequency of 50 Hz and stimulus current of 0.15 mA.

An active site was defined as one from which electrical stimulation elicited the highest elevation of AP and tachycardia. The electrode was then left in place at that active site.

5. Effect of 3α - dihydrocadambine on FPR

FN stimulation was continued for 30-40 sec. During this phase , the AP allowed to stabilize. At the end of this phase, while stimulation continued, the injection of 3α - dihydrocadambine was begun.

6. Histological techniques.

At the end of the experiment , the site of FN stimulation was marked with an electrolytic lesion by applying direct current 0.2 mA through the stimulating electrode for 30 sec, to deposit iron at the electrode tip. Each animal was then perfused through the left ventricle with heparinized 0.9% sodium chloride and then with 4%

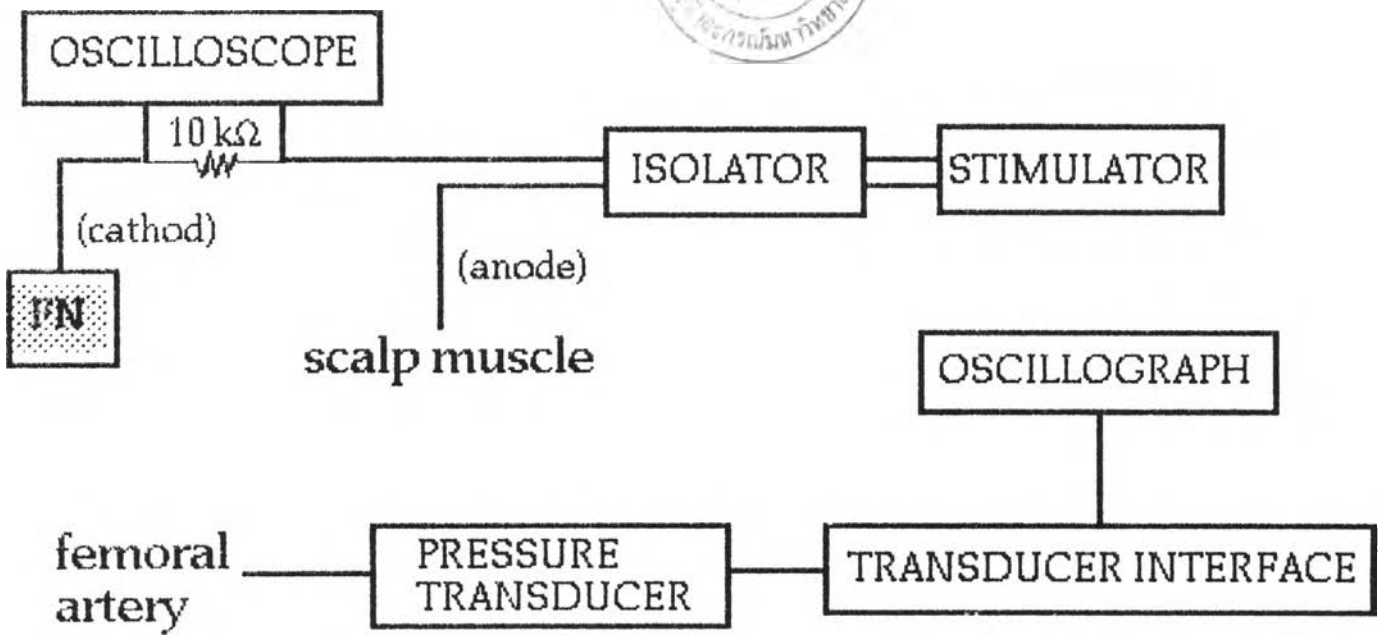

Figure 4. Diagrammatic representative of the experimental set up

paraformaldehyde. After fixing and serial coronal sectioning (100 μm) with a microtome, the brain tissues were stained with cresyl violet,which selectively stained cell bodies of the neurons.

Statistical Analysis

The data were analyzed for statistical significance with either student's paired *t*- test for comparing between control and treatment in each group or student's unpaired *t*-test when comparing between groups difference. In multiple comparisons were evaluated by analysis of varience (ANOVA). Values are expressed as mean ± S.E.M.. A p value < 0.05 was considered to indicate statistical significance.