CHAPTER II

EXPERIMENTAL

2.1 Plant material

The plant material used in this study was obtained commercially . It had been collected in Kanchanaburi province in February 1994 . A voucher specimen (BKF 81621) has been deposited with the herbarium of the Royal Forest Department , Bangkok , Thailand. The roots were air - dried and minced mechanically . Minced roots (13 kg) were extracted by percolation with organic solvents : hexane , dichloro-methane , ethyl acetate and methanol.

2.2 General Procedure

2.2.1 Physical Separations. :

The crude extract was separated into fractions by column chromatography [19] on Merck silica gel 230-400 mesh . The column was eluted with an increasing gradient of dichloromethane in hexane , then methanol in dichloromethane .

Some fractions was reseparated by preparative TLC or by chromatotron [20] on Merck silica gel 60 PF_{254} , using a Harrison Research Chromatotron model 7924 T equipped with a solvent pump . The thickness of absorbent layer was 1 mm.

For analysis of each fraction , analytical TLC plates with silica gel precoated on aluminum sheet (Merck kieselgel 60F-254) was used . A fraction was concentrated to a small volume and checked by TLC . The fractions which had the same components were combined .

2.2.2 Spectroscopic studies .

IR spectra were obtained using a Fourier transform - Infrared Spectrophotometer (FT-IR.) , Perkin - Elmer Model 1760 x. The spectra of solids were obtained by incorporating the sample into a KBr pellet.

^{1}H , ^{13}C NMR and 2D - NMR spectra were obtained by using Bruker FT Nuclear Magnetic Resonance Spectrometer Model AC-F 200 and Joel FT Nuclear Magnetic Resonance Spectrometer model JNM-A 500 . All experiments were performed in deuterated solvents : chloroform-d 99.9% ($CDCl_3$) and dimethylsulfoxide-d_6 99.5% (CD_3SOCD_3).

Mass spectrometry (MS) was carried out on a Fison Instrument Model Trio 2000 at 70 ev.

Gas chromatography was performed with a Shimadzu Gas Chromatograph GC - 7AG.

The melting points were obtained on a Fisher - John apparatus and are uncorrected .

2.3 Chemical reaction test

Alkaloid test : the small amount of the crude extract , 1 g. , tested with many reactions and reagents ; Maeyer's , Valser's , Wagner's , Dragendorff's , Kraut's and Marme's reagents. The colour of the precipitates were observed and all test tubes showed positive tests. Either the crude extract or compound 7 showed all positive tests.

Steroid test : the unknown compound was tested with the Libermann - Berchard Reaction . The colour of the solution changed from pink to violet , blue or green .

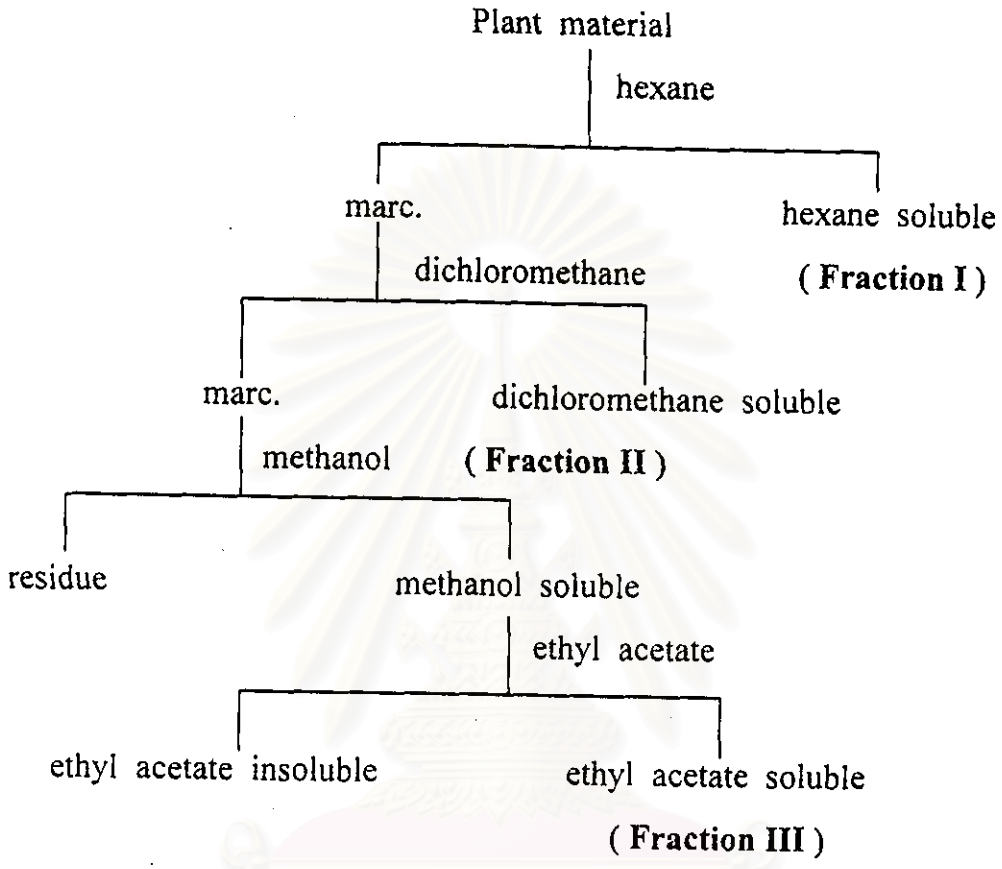
2.4 Extraction

The air - dried roots of *A. racemosus* (13 kg) were minced and extracted with the solvents. The plant was soaked in each solvent for about 4-5 days several times until the solvent became clear. It was then filtered and the solvents were evaporated.

The plant was initially extracted with hexane. The hexane crude extract (Fraction I) was a yellowish - brown material (43.56 g). The hexane insoluble part was extracted with dichloromethane in the same way and yielded the dichloromethane crude extract as a light brown material, 154.67 g. (Fraction II).

The marc was soaked in methanol to obtained the methanol soluble part. This part was partitioned with ethyl acetate and gave the ethyl acetate crude extract, (Fraction III), a sticky pale brown material, 62.74 g.

Scheme 1 Extract procedure of the roots of *A. racemosus* Willd.



2.5 Bioassay Experiments

2.5.1 The inhibitory effect for tumor cell lines.

Preliminary screening of the methanol crude extract of the roots of A. racemosus have been carried out by workers at Beijing Medical University , Beijing , China. They reported the use of the MTT assay to study the inhibitory effect of the extract on tumor cell lines. The 7 cell lines were Human Nasopharyngeal Carcinoma (KB) , Human Carcinoma of the Stomach , Human Leukemia (HL-60) , Human Mammary Cancer , K 562 , Human Carcinoma of the Esophagus and Human Pulmonary Carcinoma.

Cell lines were cultured under conventional conditions : 37°C , 5% CO_2+95%Air , 100% relative humidity , in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum , Penicillin 100 IU. ml^{-1} and Streptomycin 100 $\mu g.ml^{-1}$.

The tetrazolium dye (MTT = 3,4,5-dimethylthiazol-2,5-diphenyltetra zolium bromide) assay is based on a reduction of MTT formazan by living cells. The reduced formazan can be measured with a microplate spectrophotometer.

MTT assay:

Cell lines were seeded in 96 well microtitre plates in wells with 5×10^4 cells. A stock solution of the extract was added to each well, and 8 replicate wells without extract serverd as controls . The plates were incubated for 72 hr. After the incubation , 20 µl of PBS solution with MTT 5 $mg.ml^{-1}$ were added to each well and the plates were reincubated for a further 4 hr. The plates were then inverted on blotting paper to removed the medium. The formazan crystals formed were dissolved in 200 µl of acid-isopropanol . The plates were read on a Model 450 Microplate reader at 570 nm.

2.5.2 The Anti-oxytocin effect

The Anti-oxytocin effect of Compound 7, Asparagamine A , was determined by postponement of parturition in rats . Oxytocin is an important hormone involved in induction of parturition in many mammalian species including rats. Its effect is to induce uterine muscle contraction resulting in parturition . Therefore Asparagamine A. , an anti-oxytocin agent may prevent the contraction of uterus muscle which may cause the delay or postponement of parturition in pregnant rats.

Asparagamine A. preparation

Asparagamine A in olive oil was prepared by dissolving the substance first in a small amount of absolute ethanol. Then , adding a desired amount of olive oil. The solution was warmed gently (45-50° C) to evaporate ethanol.

Animals

Adult female wistar rats (60-90 day-old , 150-200 g body weight) of The Department of Biology , Faculty of Science , Chulalongkorn University , were used . They were housed in an air-conditioned room illuminated between 6.00-20.00 hours. Palleted food (Phokphan Co.) and water were offered without restriction. Pregnant rats were obtained by subjecting proestroue rats to mate with fertile male rats. The next day when a sperm plug had been found in the vagina was designated as day 1 of pregnancy (P_1).

Pregnant rats were subcutaneously administered daily with Aparagamine A. , an oxytocin agent (5 mg , 10 mg / 0.2 ml / rat) for 6 days starting from day 16 of pregnancy (P_{16}) . Parturition was observed from the morning of P_{21}.