CHAPTER III

EXPERIMENTAL

Materials and Techniques Used in the Investigation

1. Crude Drug Samples from Market

In order to investigate Khaao-Yen-Nuea Khaao-Yen-Tai in commercial market, crude drugs were bought from traditional drug stores located in Bangkok (12 stores, 19 samples) and in 10 provinces (15 stores, 21 samples) (see Table 6, page 78-79). Since rhizome is the main part used of Khaao-Yen-Nuea Khaao-Yen-Tai, it is thus necessary to compare the rhizome samples of crude drugs sold in the market with the rhizome obtained from a known plant.

2. Collection and Identification of Authentic Plants

The four plant samples as the authentic drugs are mostly collected wild from the following sources :

1. Ya-Hua (local name) collected from the forest in Loei.

2. Khaao-Yen-Nuea (local name) collected from Doi Suthep in Chiang Mai.

3. Khon Kra-Tae (local name) collected from Amphur Am-nat Charoen, Ubon Ratchathani.

4. Khaao-Yen-Tai (local name) collected from Chantha-buri Medicinal Plant Garden, Chanthaburi.

Their morphological characters are noted and the flowering and fruiting branches preserved as voucher specimen. The specimens of all investigated plants have been deposited at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences Chulalongkorn University.

Identification of plant species is effected by comparative study on morphological characters with the following specimens at the Forest Herbarium, Division of Silviculture, Royal Forest Department, Ministry of Agriculture and Cooperatives, Thailand.

1. *Smilax glabra* Roxb.
2. *Smilax corbularia* Kunth
3. *Pygmaeopremna herbacea* (Roxb.) Mold.

And the last one is compared with specimen deposited at Botany section, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand.

4. *Dioscorea birmanica* Prain et Burkill.

3. Preparation of Drug Samples

The crude drugs selected for this study are carefully separated from soil and other plant parts and then cleaned. Healthy pieces are set aside for the macroscopical study. The rest of the sample is reduced to small pieces and dried in an oven at 45°-60° C. The first portion for microscopic study is grinded and passed through a No. 40-60 sieve mesh. Whilst the second portion passed through a No. 20-40 sieve mesh is used for chemical study.

4. Macroscopical Study

The prepared sample drugs were selected to study the shape, size, external and internal color, marking, fracture, odor and taste.

5. Microscopical Study

5.1 Histology

Investigation of histology is carried out by the following methods :

5.1.1 Slide preparation

The crude drug is cut into thin sections by means of sliding microtome. The tissue sections are mounted onto a slide in glycerine water and then examined under the microscope. The dried rhizome are very hard and tough so they should be softened by immersing in the mixture of glycerol and ethanol TS prior to sectioning.

5.1.2 Staining

The section were stained with the following reagents :

- phloroglucinol TS and hydrochloric acid to stain lignified elements.
- aniline blue TS to stain phloem tissues.
- iodine TS to stain starch granules.
- chlorzinciodine TS to distinguish between cellulose and lignified walls. It causes starch grains to swell and colors them blue.

5.1.3 Photographing and drawing

The stained sections are photographed under the camera contacted with the microscope and are drawn with the aid of camera lucida.

5.1.4 Polarized light examination

Crossed nicols, the source of polarized light is used in the examination of starches and the detection of small amounts of calcium oxalate in drug sections. Crystals of calcium oxalate appear bright on a dark background. The different kinds of starches exhibit distinct peculiar polarization crosses in polarized light.

5.2 Powdered drug

Powdered drug used in this study should be passed through a fine sieve (mesh no. 40-60). The procedure involves the following steps :

5.2.1 Organoleptic (sensory character)

The odor, colour, taste and other characteristic features of each drug are record.

5.2.2 Clearing and staining

A small amount of the powder is clearified in a solution of chloral hydrate TS with the aid of heat. The cleared material is then stained in the solution as in 5.1.2

5.2.3 Drawing

The slide is examined under the microscope, characteristic cells and tissues are drawn using a camera lucida. The relative abundance of each type of cells and tissues is also noted.

5.2.4 Without clearing examination

The drug is again examined as in 5.1.2 but without clearing to determine the presence of other cells or tissues with may be destroyed by the clearing agent. These include starch granules, oil droplets and pigments.

5.2.5 Polarized light examination

(see 5.1.4)

6. Chemical Identification test

6.1 Preliminary tests :

6.1.1 The pulverized drug (0.5 g) is shaken vigorously with 10 ml of water in a test tube. The formation of long lasting foam is observed.

6.1.2 The pulverized sample (0.5 g) is warmed with 2 ml of acetic anhydride on a water bath for 2 mins while shaking. After filtration, 1 ml of sulfuric acid is carefully added to the filtrate to make two layers. The occurrence of the color at the zone of contact is observed.

6.1.3 The pulverized sample (0.5 g) is boiled with 10 ml of water for 2 mins. After filtration, 1 ml of Fehling's TS is added to 3 ml of the filtrate and the mixture was warm in a water bath. The appearance of red precipitate is observed.

6.1.4 The pulverized sample (0.5 g) is boiled with 10 ml of water and filtered. One drop of ferric chloride TS is added to 2 ml of the filtrate. The formation of any precipitate is noted.

6.2 Confirmatory test (Thin - layer Chromatographic analysis)

Test solution : Reflux powdered drug (5 g) with ethanol (50 ml) on a water-bath for 30 mins, cool, filter, evaporate to dryness, add dilute hydrochloric acid (20 ml) reflux on a water bath for 3 hrs, cool. extract with chloroform (20 ml) twice, combine the chloroform extract, wash with purified water, then add sodium sulfate anhydrous to absorb water. Evaporate the chloroform extract to dryness, dissolve the residue with 3 ml of chloroform.

Reference standard solution (1 mg / chloroform 1 ml)

Diosgenin, tigogenin, smilagenin, β-sitosterol stigmasterol

Technic : One Way, ascending, tank saturated

Absorbent : TLC plate silica gel GF 254, precoated 20 x 20 cm, 0.25 mm thickness.

Developing solvent : Hexane : ethyl acetate = 2.5 : 1.5

Developing distance: 12 cm

Spotting amount : 5 µl each

Spraying reagent : 1. Anisaldehyde-sulfuric acid in ethanol

2. Phosphoric acid in ethanol

Detection : 1. Visible developing color spots without spraying

2. Under UV light 254

3. Under UV light 365

4. Spray with anisaldehyde-sulfuric acid and heat the plate to 105°-110° C for about 10 mins, record the developing color spots.

5. Spray with phosphopic acid and heat the plate to 120° C for about 10 mins, record the developing color spots.

6. Do as No. 5, detect the alternating developing color spots undder UV light 365

7. Purity Evaluation

7.1 Total ash determination

Place 2-4 g of the ground material in a suitable tared crucible, usually of platinum or silica, previously ignited and weighed. Spread the material into an even layer and weigh accurately. Incinerate the material at a low temperature at first then the temperature gradually raised to 450°-500° C until free from carbon; cool in a desiccator and weigh. If a carbon-free ash cannot be obtained in this way, cool the crucible and moisten the residue with about 2 ml of water or a saturated solution of ammonium nitrate. Dry on a water-bath and then on a hot plate and incinerate to constant weight. Calculate the content in g of ash per 100 g of air-dried material (59).

7.2 Acid-insoluble ash determination

Procedure : to the crucible containing the residue obtained from the determination of ash or sulfated ash, add 25 ml of hydrochloric acid (70 g/l) TS, cover with a watch-glass and boil gently for 5 minutes. Rinse the watch-glass with 5 ml of hot water and add the rinsings to the crucible. Collect the insoluble matter on an ashless filter paper and wash with hot water until the filtrate is neutral. Transfer the filter paper containing the insoluble matter to the original crucible, dry on a hot plate and ignite to constant weight. Calculate the content in g of acid-insoluble ash per 100 g of air-dried material (59).

7.3 Moisture determination

The moisture content loss on drying. About 2-6 g of the test sample are transferred to a tared weighing bottle and accurately weighed. The sample is then dried at 100° - 105° C for 5 hrs allowed to cool in a desiccator containing silica gel and again weighed accurately. The drying process is continued and the sample weighed at 1 hr intervals until the weight of the sample becomes constant. The percentage of loss on drying is then calculated (60).

7.4 Extractive values determination

7.4.1 Water-soluble extractive

Weigh accurately 4 g of the air-dried drug, coarsely powdered, into a suitable glass-stoppered flask. Macerate the drug with 100 ml of the purified water for 24 hrs, shaking frequently during 6 hrs and allowing to stand for 18 hrs. Filter rapidly, taking precautions against loss of

solvent, evaporate 25 ml of the filtrate to dryness in a tared flat-bottomed dish on a water-bath and dry at 105° C for 6 hrs. Weigh and calculate the content of extractives in g per 100 g of the air-dried drug (59).

7.4.2 Ethanol-soluble extractive

The procedure is as described in water-soluble extractive but ethanol of specified strength is used instead of water.