

CHAPTER III

MATERIALS AND METHODS

Scope of Investigation

1. Collection of all possible plant materials as well as crude drugs of which both entitled “Reo” (bastard cardamom) distributed in Thailand.
2. Describing the characteristic features of the collecting specimens and correlating with those established specimens of type species deposited in well recognized herbarium collections as well as with recorded data in floras.
3. Pharmacognostic study on external appearance (macroscopic) and histological (microscopic) characters of the crude drugs (“Reo”).
4. Qualitative evaluation of each crude drugs (“Reo”) concerning the specification as required in pharmacopoeia such as foreign matter, moisture content, total ash and acid-insoluble ash will be performed.
5. Determination of volatile oil contents of these drug extracts either by steam distillation or supercritical fluid extraction. The chemical composition of volatile oil from these different sources also determined by using a gas chromatography-coupled with mass spectrometry (GC-MS) method.
6. Identification and conclusion of species of “Reo” (bastard cardamom) that available used in Thailand.

Apparatus

- Sieve no. 60
- Slide and cover slips
- Forceps
- Beaker
- Crucible
- Stirring rod
- Vial

- Ashless filter paper Whatman®
- pH paper
- Hot plate
- Stage micrometer
- Compound microscope Zeiss model Axiostar
- Digital camera Sony Cyber-shot DSC-S85
- Muffle furnace Gallenkamp Size 2
- The apparatus for volatile oil content determination
- The apparatus for determination of water by Azeotropic distillation method
- Supercritical fluid extractor model SFE 400; Supelco
- Gas chromatography-Mass spectroscopy Instrument model Varian Saturn 3

Part I Collecting Specimens and Plant Materials

1. Collecting Specimens

Two species of the *Amomum* plants are called “Reo” or other local name that means the same were collected from different locations in Thailand, collected dates are shown together below.

Amomum sp. A - From Leang nok tha district, Yasothon province in July, 2003.

- From Soi dow district, Chantaburi province in August, 2003.

Amomum sp. B - From Dong luong district, Mukdahan province in July, 2003.

The specimens were inspected about the characteristic feature and identified by comparing with those described in Flora of British India (1954) and Prosea: Plant Resources of South East Asia (1999) and also compared with the authentic herbarium specimens preserved at the Forest herbarium(BKF), national park, wildlife and plant conservation department, Bangkok. The fresh authentic fruits were peel and dried in a hot air oven at 45^oC, then kept in a well-closed container, ground and passed through a sieve with mesh number 60 before examined.

2. Crude drugs

Crude drugs which are called “Reo” were purchased from traditional drugstores throughout Thailand (Table 3).

Table 4 Reo which were purchased from traditional drugstores

No. of sample	Local name	Place	Purchased date
1	Reo	Chiang Mai province (1)	November, 2002
2	Reo	Chiang Mai province (2)	November, 2002
3	Reo	Sukhothai province	May, 2003
4	Reo	Bangkok province (1)	November, 2002
5	Reo	Bangkok province (2)	May, 2003
6	Reo	Bangkok province (3)	May, 2003
7	Reo	Bangkok province (4-a)	August, 2003
8	Reo	Nakhon Pathom province	May, 2003
9	Reo	Chon Buri province	May, 2003
10	Reo	Chanthaburi province	October, 2002
11	Reo	Khon Kaen province	November, 2002
12	Reo	Roi Et province	November, 2002
13	Reo	Ubon Ratchathani province	November, 2002
14	Reo	Surat Thani province	January, 2003
15	Reo	Songkhla province	March, 2003
16	Reo-yai	Bangkok province (4-b)	August, 2003

Part II Pharmacognostic Study

Pharmacognostic study consist of the macroscopic and microscopic characterization. In the macroscopic evaluation, organoleptic sensation is used to determine the size, shape, color, odor and taste of the crude drugs. The microscopic method revealed plant histology.

The macroscopical method

Procedure:

Determined the size, shape, color, odor and taste of the authentic and crude drugs.

The microscopical method

Procedure:

Cross section and powder of authentic were taken photographs by digital camera which is attached to microscope. Most of microscopic powdered were taken photographs from preparations of the powdered which had been first mounted in water. Subsequently sections should be cleared by means of chloral hydrate and some stained as follows:

Chloral hydrate solution BP

A valuable and widely used clearing agent. This dissolves starch, proteins, chlorophyll, resins and volatile oils, and causes shrunken cells to expand. Chloral hydrate does not dissolve calcium oxalate and is therefore a good reagent for detection of these crystals.

Iodine solution

This give a blue color with starch and hemicelluloses.

Phloroglucinol solution and Hydrochloric acid

A solution of phloroglucinol with hydrochloric acid is used to detect lignified tissues. Mounted the section in the solution of alcoholic phloroglucinol and allowed to stand for about 2 minutes; removed any alcohol which has not evaporated with a piece of filter paper; added concentrated hydrochloric acid, covered and examined. All lignified walls stain pink or red.

Hydrochloric acid is a powerful clearing agent and it must be remembered that it will dissolve many cell contents, including calcium oxalate. To prevent damage to the microscope either by liquid contact or by vapors, preparations be removed from the microscope stage as soon as possible.

Part III Volatile Oil Content and Composition

Volatile oil content and composition of extracts from two different methods were determined and compared. One, extract using steam distillation, the method described in the Thai Pharmacopoeia : determination of volatile oil (Ministry of Public Health, 1987) and supercritical fluid extraction, the method using CO₂ for extract.

Steam distillation method

The apparatus for the determination of volatile oil (Figure 1) consists of a round-bottomed flask (A), a condenser assembly that closely fits the flask and consists of the following parts fused into one piece: a vertical tube (BD); a bent tube (DEF); a bulb-condenser (GH); a vented stopper (M) and a tube (N) that coincides with the vent; a tube (HJ) that making a 30° to 40° angle (HJN) with the tube (JN); a graduated tube (LP) to give 1 ml subdivided in 0.01 ml; a bulb-shaped swelling (P); a three way tap (Q) and a connecting tube (CQ) fitted in the middle with a filling funnel (R), a suitable heating device, allowing a fine control and a vertical support with horizontal ring covered with insulating material.

Procedure:

Fifteen grams of each sample were put into a 500 ml round bottom flask. Then added water into the flask to about half full. The content of the flask was distilled until two consecutive reading taken at one hour interval showed no change in oil content. After cooling, the oil volume was measured, calculated and expressed as milliliter of the oil per one hundred grams of sample. The volatile oil obtained was then collected and stored at 4° C until being analyzed for its chemical composition by GC-MS.

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Supercritical fluid extraction method

The supercritical fluid extractor model SFE 400 (Figure 2).

Procedure:

Fifteen grams of each sample were placed in the vessel that covered two side by cotton. The vessel connected to the supercritical CO₂ extraction system and samples were extracted in a static mode. An extraction performance was obtained via operating at 90 bar and 40°C for 30 min. The extracts were collected in vials and stored at 4°C until being analyzed for their chemical compositions by gas chromatography- mass spectrometry (GC-MS).



Figure 2 Supercritical fluid extractor

Gas chromatography – Mass spectroscopy analysis

For the identification of the compositions of volatile oils, a gas chromatography-coupled with mass spectrometry (GC-MS) was used. The volatile oil was diluted to 1 : 100 in hexane before being injected into GC-MS system. The condition of GC-MS was described below. The spectra were recorded and compared with the Nist library program.

GC-MS condition	
Instrument model	Varian Saturn 3
Column	fused silica capillary column (30 m x 0.25 mm i.d.) coated with DB-5 (95% dimethyl 5% diphenyl plysiloxane) (J&W). film thickness 0.25 μm
Column programming	50°C hold 3 min. 50-150°C rate 5°C/min 150-250°C rate 10°C/min hold 5 min
Injector temperature	220°C
Detector temperature	230°C
Helium carrier gas	1 ml/min
Split ratio	100: 1
Accelerating voltage	1700 volts
Sample size	1 μl
Solvent	Hexane (HPLC grade)

Part IV Qualitative Evaluation

Qualitative evaluation of each crude drugs (“Reo”) concerning the specification as required in Thai pharmacopoeia. This investigation was performed according to the monograph of the mentioned pharmacopoeia. (Ministry of Public Health, 1987):

Foreign matter

Vegetable drugs should be free from moulds, insects and other animal contamination. Calculated the percentage of foreign matter.

Foreign matter is material consisting of any or all of the following:

(i) Part of the organ or organs from which the drug is derived, other than the parts named in the definition and description.

(ii) Any organs, other than those named in the definition and description.

Procedure:

Weighed 100 g of the substance being examined and spread it in a thin layer. Detected the foreign matter by inspecting with the unaided eye or with the use of a 6X lens. Separated, weighed and calculated the percentage present.

Moisture content determination

The determination of moisture by azeotropic distillation method is useful to detecting volume of water in products. The apparatus (Figure 3) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1 ml subdivisions so that the error of reading is control or an oil-bath. The upper portion of the flask and the connecting tube may be insulated with asbestos.

Procedure:

Introduced 200 ml of toluene and 2.0 ml of water into a 500 ml round-bottom flask. The solvent was distilled for 2 hours and cooled for 30 minutes before reading the water volume to accuracy of 0.05 ml. 15 g of ground sample were then added to the flask with a few pieces of boiling chips. The flask was heated until the water and toluene were completely separated, The volume of water was recorded and calculated the percentage present in the substance using the formula

$$\frac{100 (n' - n)}{p}$$

where p = the weight in g of the substance to be examined,

n = the volume in ml of water obtained in the first distillation, and

n' = the total volume in ml of water obtained in the second distillations.

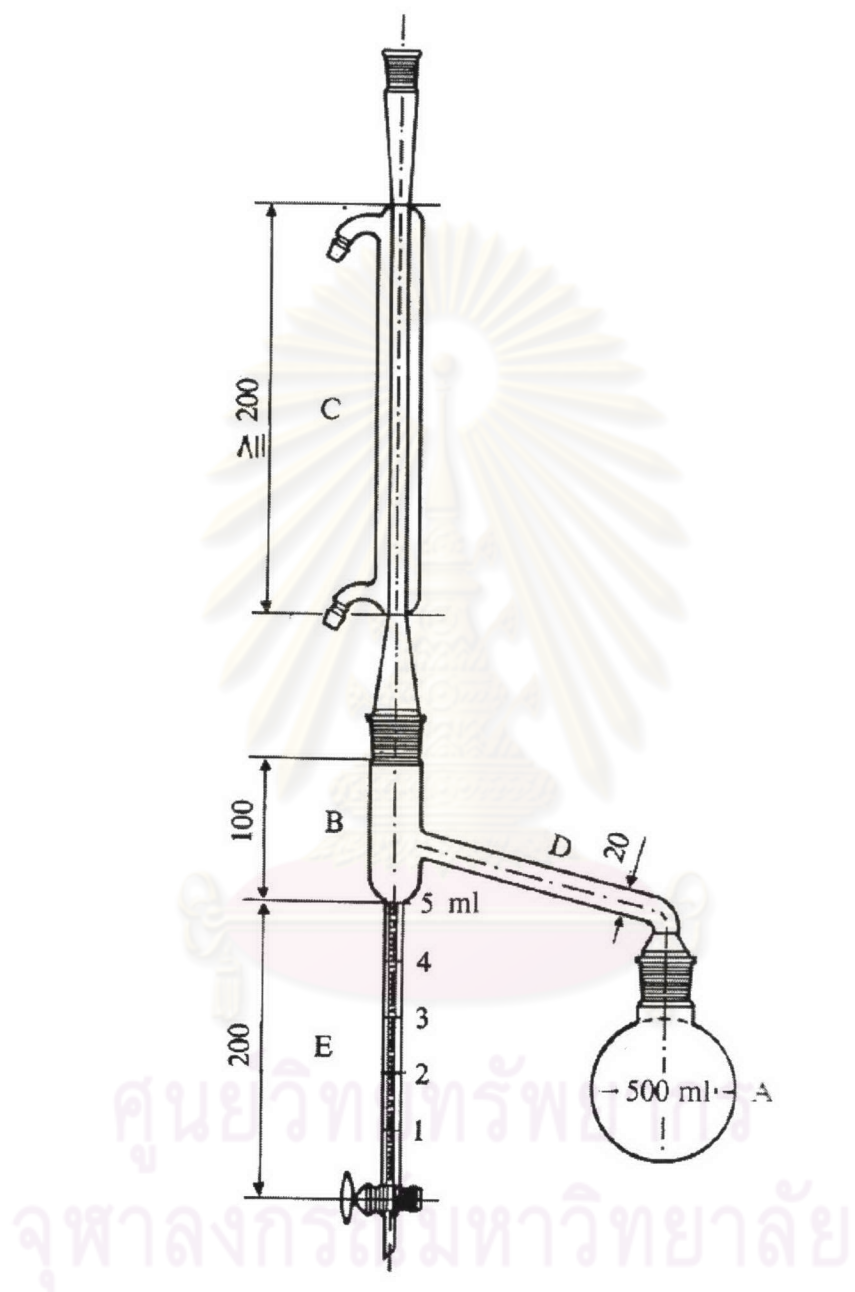


Figure 3 Apparatus for determination of water by Azeotropic distillation method

Total ash and acid-insoluble ash

The determination of ash is useful to detecting low-grade products, exhausted drugs and excess of sandy or earthy matter; it is more especially applicable to powdered drugs. Different types of ash figures are used such as total ash and acid-insoluble ash and water soluble ash. A total ash figure is useful to exclude drugs which have been coated with chalk, lime or calcium sulphate to improve their appearance. The acid-insoluble ash, i.e., the ash insoluble in dilute hydrochloric acid, is often of more value than the total ash. The majority of drugs contain calcium oxalate, sometimes in large amounts and the amount is often very variable. Since, however, the calcium oxide or carbonate, yielded by the incinerated oxalate, is soluble in hydrochloric acid, one can remove all the variable constituent of the ash by means of dilute hydrochloric acid and weigh the residue, which is known as the acid-insoluble ash (Wallis, 1960). The apparatus for incineration is the muffle furnace Gallenkamp size 2.

Procedure:

Total ash

Placed 2 to 4 g of the ground sample, accurately weighed in a suitable tared crucible (usually of platinum or silica), previously ignited, cooled and weighed. Incinerated the sample by gradually increasing the temperature, not exceeding 450°C , until free from carbon; cooled and weighed. If a carbon-free ash cannot be obtained in this way, cooled the crucible and moisten the residue with about 2 ml of water or a saturated solution of ammonium nitrate. Dried on a water-bath and then on a hot plate and incinerate to constant weight. Calculated the percentage of total ash with reference to air-dried substance.

Acid-insoluble ash

Boiled the total ash for 5 minutes with 25 ml of diluted hydrochloric acid, collected the insoluble matter on an ashless filter paper, washed with hot water until the filtrate was neutral, and ignited at about 500°C . Calculated the percentage of acid-insoluble ash with reference to air-dried substance.

Each crude drug sampling was tripled for ash content, but was seconded for moisture content.