CHAPTER III

MATERIALS AND METHODS

1. Plant Material

The dried seeds of *Gloriosa superba* were obtained from various parts of Thailand as follow :

Northern : Chiang Mai

Central : Lop Buri

Eastern : Chanthaburi and Prachin Buri

Southern : Chumphon and Songkhla

The details of the plant material used in this study are shown in Table 7.

In all of these provinces except Chiang Mai and Chanthaburi, the *G. superba* plants were cultivated in experimental fields using the tubers of wild-grown plants as starting materials. In Chiang Mai's experimental field, the *G. superba* seeds which were originally obtained from India was used instead of the tuber for cultivation. In Chanthaburi, the *G. superba* seeds from Amphur Khlung were collected from wild-grown plants whereas the seeds from Thai Commodities Co.Ltd. were from the cultivated plants.

Table 7 Various sources of *G. superba* seeds and plant materials used in this study

Province	Experimental field	Method of plantation	Plant part used in this study
Northern :			
Chiang Mai	Department of Biology, Chiang Mai University (c/o Dr. Arayar Jatisatienr)	cultivated from the seeds of Indian *G. superba*	seeds
Central :			
Lop Buri	Field Station at Tambol Khok Toom	cultivated from the tubers of Thai *G. superba*	seeds
Eastern :			
Chanthaburi	1. Field Station of Thai Commodities Co. Ltd. at Amphur Tamai	- cultivated from the tubers of Thai *G. superba*	seeds
	2. Natural *G. superba* field at Amphur Khlung	- wild-grown plants	seeds and all plant parts
Prachin Buri	Field Station at Amphur Wattana Nakhon	cultivated from the tubers of Thai *G. superba*	seeds

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Table 7 (continued)

Province	Experimental field	Method of plantation	Plant part used in this study
Southern :			
Chumphon	Field Station of Thai Commodities Co. Ltd. at Amphur Tasae (3 samples; CPa1, CPa2, CPa3)	cultivated from the tubers of Thai *G. superba*	seeds
Songkhla	Medicinal plant garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University	cultivated from the tubers of Thai *G. superba*	seeds

For various plant parts, the whole *G. superba* plants were collected from the wild-grown ones at Amphur Khlung, Chanthaburi. The plants were separated into seeds, pericarps, flowers, tubers, leaves and stems before drying at 50 °C for 48 hr.

2. Chemicals

Colchicine was purchased from Sigma (St.Louis, MO, U.S.A.). Authentic colchicoside , 3-demethylcolchicine were kindly provided by Professor M.H. Zenk of the Institute of Pharmaceutical Biology, University of Munich, Munich, Germany. Organic solvents used for the mobile phase in HPLC (acetonitrile and methanol) were

chromatographic quality (HPLC grade) obtained from Merck (Damstadt, Germany). Water was triple distilled in glass. All other solvents used in extraction, TLC solvent systems and spectrophotometry were analytical grade. Silica gel 60 F_{254} plates were purchased from Merck (Damstadt, Germany).

3. Preparation of Standard Solutions

For HPLC work , solution of colchicine and colchicoside (100 µg/ml) were prepared in methanol (HPLC grade) protected from light to serve as standard stock solutions. From these stock solutions, various concentrations of the standards were prepared. Standard curves in the range of 5-100 µg/ml and 5-50 µg/ml were then constructed for colchicine and colchicoside, respectively.

For TLC-densitometric analysis of colchicine, the 100 µg/ml stock solution of colchicine in 95% ethanol was diluted to the concentration range of 5-60 µg/ml and then the standard curve in the range of 0.025-0.30 µg/5µl was constructed for colchicine.

For spectrophotometric analysis, the 100 µg/ml stock solution of colchicine was prepared in 95% ethanol. A stepwise half-dilution of the stock solution was

performed and the concentration range of 0.39-50 µg/ml was used for constructing the calibration curve of colchicine.

4. Sample Preparations

The dried materials from various parts of *G. superba* including seeds, pericarps, flowers , tubers, leaves and stems, were powdered in a grinder circulated with cool water (Ika A10, Germany). After passing a sieve no. 20, an appropiate amount of each powdered material (40 mg seeds , 40 mg pericarps , 200 mg flowers, 200 mg tubers, 1 g leaves, 1 g stems) was extracted with 10 ml 95% ethanol under reflux at 60-70 °C for one hour. After cooling, the extracts were filtered through Whatman no.1 filter in a Buchner funnel and adjusted to the volume of 10 ml with 95% ethanol in a volumetric flask.

For HPLC analysis of colchicine in *G. superba* seeds, the ethanolic extract of each sample was passed through a membrane filter (0.45 µm) before subjected to the analysis.

For spectrophotometric analysis of total colchicine derivatives in *G. superba* seeds, the ethanolic extracts were diluted ten times before subjected to the analysis.

5. HPLC Conditions in the Determination of Colchicine in *G. superba* Seeds

The HPLC apparatus consisted of a Varian Model 9010 ternary solvent delivery system attached to a Model 9050 variable-wavelength UV-VIS detector. Samples (see sample preparations) were automatically injected by Varian Model 9095 autosampler and the data were recorded by Varian Model 4400 integrator (Varian , Sugar Land , Texas , U.S.A.). Analytical separation was performed by using a reverse phase C-18 stainless - steel column (Varian SP-C18 , 15 cm x 4 mm I.D., particle size 5 µm). All of the solvents , standard solutions and sample preparations have to be passed membrane filter (0.45 micron pore size) prior to use. The analysis was carried out by using step-gradient elution from a 10 % solution of acetonitrile in water (eluent B) to a mixture of 70 % eluent B plus 30 % eluent A (pure acetonitrile) as follow :

0-4 min : 100 % B

4-10 min : from 100 % B to 95 % B plus 5 % A

10-20 min : run to 80 % B plus 20 % A

20-25 min : run to 70 % B plus 30 % A

25-30 min : maintained 70 % B plus 30% A

Injection volume of each standard solution (see preparation of standard solutions) or sample was 20 µl.

Detection was at 350 nm and the flow-rate of 1 ml/min was constantly throughout the analysis.

6. Spectrophotometric Conditions in the Determination of Total Colchicine and Its Derivatives in *G. superba* Seeds

The spectrophotometer (Perkin - Elmer Model Lambda 3B) was set to the wavelength of 350 nm. Each standard colchicine solution (see prepration of standard solutions) and sample solution (see sample preparations) was read for the absorbance against 95 % ethanol as blank solution. The total colchicine and its derivatives in the seeds of *G. superba* were then calculated based on the calibration curve of pure colchicine.

7. TLC-Densitometric Analysis of Colchicine in Various Parts of *G. superba*

The stationary phase was silica gel 60 F_{254} plate (Merck , Damstadt , Germany). Five microlitres of each standard colchicine (see preparation of standard solutions) and ethanolic extracts of various plant parts (see sample preparations) were spotted on a TLC plate. The mobile phase was chloroform : methanol : 10 % acetic acid ; 85:15:1. Then the plate was developed until the solvent front was approximately 10 cm from the origin. After drying , colchicine in each sample in the TLC plate

was quantitated by densitometric method using Shimadzu Dual - Wavelength TLC scanner Model CS - 930 under the following conditions :

Lamp : deuterium

Scan mode : linear

Determination mode : absorption

Scan width : x = 10.0 mm

y = 0.2 mm

Sensitivity : medium

Slit width : 1.2 x 1.2 mm^2

Wavelength detector : 350 nm