CHAPTER III

Materials and Methods

1. Setting up the total isoflavone analysis

1.1 Instrumentation and column

Ultrasonic bath, Mettler, USA. Rotatory evaporator, Buchi model R-114, Swizerland were used to assist methanol extraction of the crude extract. All HPLC analyses were performed using a Shimadzu instrument (Model CLASS-LC10 analytical workstation, Model LC10AD liquid chromatograph, Model SIL-10A autosampler, Model SPD-10A ultraviolet-visible detector). The reversed phase C_{18} column was filled with Spheri 5 μm in 220 x 4.6 mm (Brownlee, USA). The filter set was Millipore. The filter membrane of Millipore was 0.45 μm pore size, 13 mm diameter for the sample and 47 mm diameter for mobile phase, HA type for aqueous solution and HV type for organic solvent.

1.2 Sample preparation

The collected sample were cleaned, sliced, dried in a hot air oven at 70 °C and subsequently pulverized to powder at 100 mesh. Five grams of plant powder was dissolved in 50 ml methanol and sonicated in ultrasonic bath for 15 min. The supernatant was filtered through Whatman # 1 filter paper. The pellet was extracted with 25 ml methanol and sonicated in ultrasonic bath for 15 min. The second supernatant was filtrated and mixed with the first one. The final filtrate was evaporated under vacuum with a rotary evaporator and adjusted volume with 15 ml methanol. The crude extract was filtered with 0.45 μm pore size, 13 mm diameter filter membrane.

1.3 Assessment of analytical condition

Methods for HPLC fingerprint has been modified from Oshima *et al.*, 1988 by setting the gradient system from 100 : 0 to 0 : 100 of 1.5 % (v/v) acetic acid : acetonitrile, 100 min with reference standard isoflavones including puerarin, daidzin, genistin, daidzein and genistein. The analytical time was set from retention time of the last eluted reference standard. The wavelength for the analytical was performed at the wavelength of 254 nm and 280 nm for comparison absorbance of isoflavone based on retaintion time, spike peak between sample and standard.

1.4 Chromatographic condition

The mobile phase was optimized using the linear gradient system. It consisted of 1.5 % (v/v) acetic acid and acetonitrile. The solvent gradients created by pumping system with the varying amount of solvent as 1.5% (v/v) acetic acid : acetonitrile (100:0 to 55:45) for 45 min, with flow rate of 1.0 ml/min. The plant extract dissolved in methanol was injected at the volume of 15 µl. Isoflavone content was detected with a UV detector at 254 nm.

1.5 Standards and solvents

Puerarin, Daidzin, Genistin, Daidzein and Genistein were purchased from Sigma, USA. Methanol, Acetonitrile and Acetic acid were analytical grade from Merck, USA.

1.6 Standard preparation and calibration curve

Commercialized standards of puerarin, daidzin, genistin, daidzein and genistein (5 mg each) was dissolved in 2 ml methanol and used as standard stock solutions for generating calibration curves. The stock solution (0.5 mg/ml) was diluted 1:1, 1:2, 1:4, 1:8 and 1:16 times with methanol by serial

dilution method to afford the concentration of 0.25, 0.125, 0.0625, 0.03125 and 0.015625 mg/ml solutions, respectively. The five concentrations of standard solution were injected to generate a five points calibration curve for the standard compounds separately. Calibration curves were obtained for most isoflavone with high linearity ($r^2 > 0.995$) by plotting the standard concentration as a function of peak area from HPLC analysis of 15 µl injection. The concentrations of standard were chosen to cover isoflavones in the samples.

1.7 Calculation for the amount of isoflavones

The samples were run in triplicate. The calibration curves were used to calculate for the concentration of isoflavone. The results were expressed as mg isoflavone per 100 g powder of the sample. Statistical analysis was done by the aid of SPSS program.

2. Qualitative analysis of the varied sample preparation

The powder from *P. mirifica* clone Chiang Dao (as described in 1.2) was extracted with methanol, ethanol and water and treated as mentioned. The sample from spray dry preparation of the same material was also submitted to the mentioned HPLC fingerprint analysis.

3. Isoflavone HPLC fingerprint analysis of the wild *P. mirifica* from 29 provinces

The tuberous root of wild *P. mirifica* was collected from the northern, north - eastern, central and southern parts of Thailand during March – April 2000-2002. The survey was done by driving through the mountainous-based route as well as a pedal survey through the mountainous-base areas. The tuberous roots of a weight not less than one kilogram was collected from 3 plants. They were prepared as discribed in 1.2. The collected provinces are shown in Figure 6. (Wichai Cherdshewasart personal communication)

4. The study of the influence of different collected site on total isoflavone content of *P. mirifica* collected from the same province

P. mirifica was collected from 5 districts in Chiang Mai province, 3 districts in Lampang province, 3 districts in Kanchanaburi province and 3 sites from 2 districts in Saraburi province. Three plants of each clone were prepared according to the mentioned methods and submitted to HPLC analysis. The collected sites are shown in Figure 8, 9, 10 and 11, respectively.

5. The study of the influence of field location on total isoflavone content of the field grown *P. mirifica*

The one year old field grown tuberous roots of 3 plants from clone Doi Tao were collected from the field trials in Chiang Rai, Bangkok and Ratchaburi province and clone Chaiprakarn were collected from the field trials in Chiang Rai and Ratchaburi province in summer 2001. The tuberous materials of each clone were prepared and analyzed according to the mention above.

6. **The study of the influence of season on total isoflavone content of the field grown *P. mirifica***

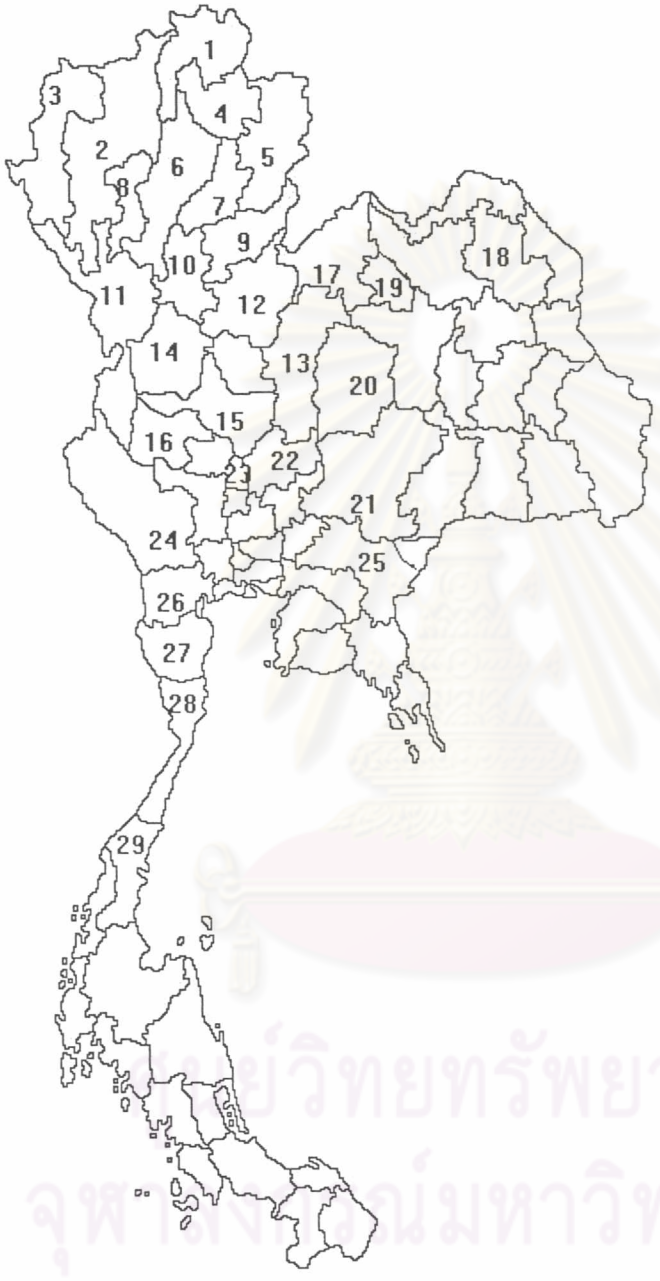
The 2 years old field grown tuberous roots of 3 plants weight not less than one kilogram from clone, Doi Tao and Chaiprakarn, were collected in July 2000, November 2000 and March 2001 from the field trial in Ratchaburi province. The tuberous materials of each clone were prepared and analyzed according to the mentioned above.

7. The study of the influence of genetics on total isoflavone content of the field grown F_1 *P. mirifica*

The one year old field grown tuberous roots from 3 plants of clone Doi Tao and Sai Yoke were collected from the field trial at Ratchaburi province at the same period as 5 and submitted to the mentioned preparation.

8. The study of tuber differentiation on total isoflavone content of the field grown sub-clone *P. mirifica*

Three sample of clone Doi Tao in 6 was individually analyzed and compared for total isoflavone content.



No.	**Provinces**
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
4	Phayoa
5	Nan
6	Lampang
7	Phrae
8	Lamphun
9	Uttharadith
10	Sukhothai
11	Tak
12	Phitsanulok
13	Phetchabun
14	Kamphaeng Phet
15	Nakhon Sawan
16	Uthai Thani
17	Loei
18	Sakon Nakhon
19	Nong Bua Lam Phu
20	Chaiyaphum
21	Nakhon Ratchasima
22	Saraburi
23	Lop Buri
24	Kanchanaburi
25	Phrachin Buri
26	Ratchaburi
27	Phetchaburi
28	Prachuap Khiri Khan
29	Chumphon

Figure 6 Distribution of *P. mirifica* in Thailand
(Cherdshewasart unpublished data)

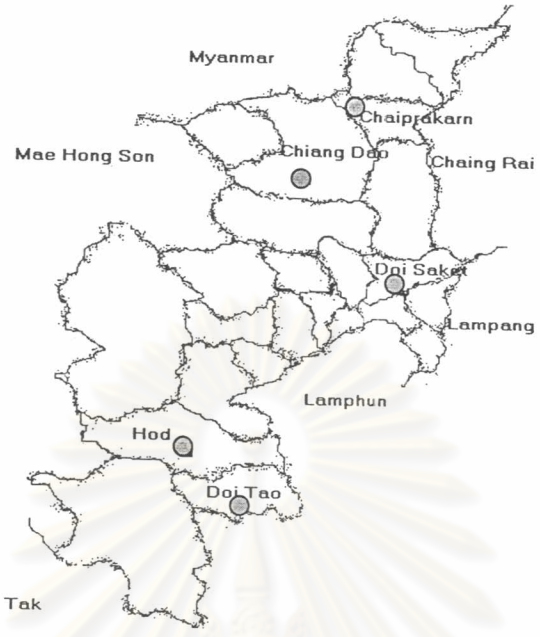


Figure 7 Collected sites of *P. mirifica* in Chiang Mai province

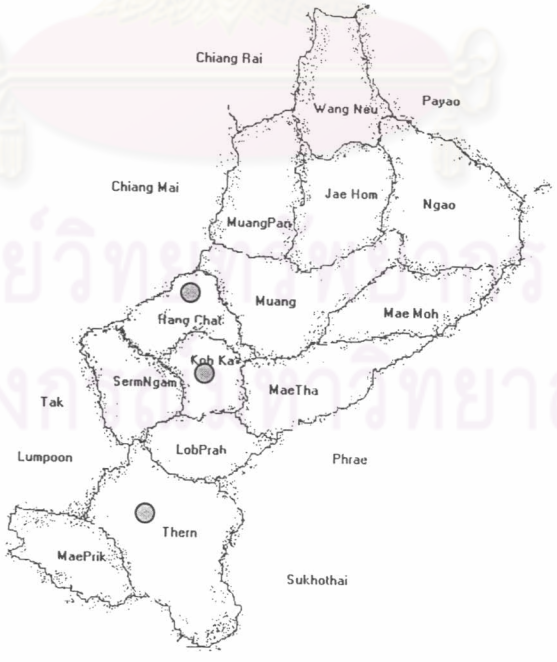


Figure 8 Collected sites of *P. mirifica* in Lampang province

Figure 9 Collected sites of *P. mirifica* in Kanchanaburi province

Figure 10 Collected sites of *P. mirifica* in Saraburi province

9. Setting up *B. superba* HPLC fingerprint analysis

9.1 Sample preparation

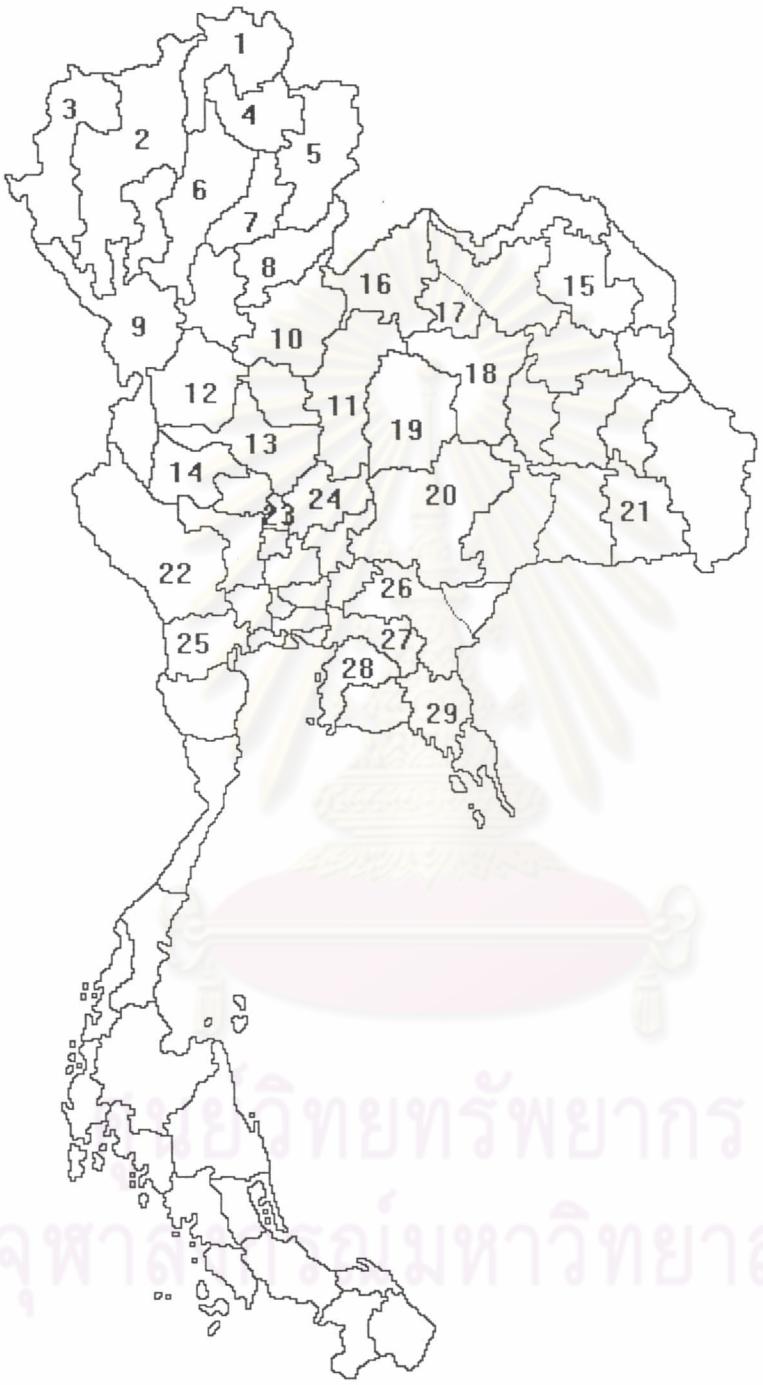
The tuberous root of *B. superba* were prepared and analyzed according to 1.2 .

9.2 Assessment of analytical condition

The condition was set according to 1.4. The wavelength for the analytical was performed at the wavelength of 254 nm and 270 nm for comparison absorbance of *B.superba* extract.

9. 3 HPLC fingerprint analysis of the wild *B. superba*

The tuberous root of wild *B. superba* was collected from the northern, north-eastern and central parts of Thailand during March – April 2000-2002. The collected provinces are shown in Figure 11 (Wichai Cherdshewasart personal communication). The sample from Lampang, Ratchaburi, Khon Kaen and Chanthaburi was selected for analyzed. Three plants of each clone were prepared and analyzed according to the mention above.



No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Mae Hong Son
1	Phayoa
5	Nan
6	Lampang
7	Phrae
8	Uttharadith
9	Tak
10	Phitsanulok
11	Phetchabun
12	Kamphaeng
13	Nakhon Sawan
14	Uthai Thani
15	Sakon Nakhon
16	Loei
17	Nong Bua Lam
18	Khon Kaen
19	Chaiyaphum
20	Nakhon
21	Srisaket
22	Kanchanaburi
23	Lop Buri
24	Saraburi
25	Ratchaburi
26	Prachinburi
27	Chachoengsoa
28	Chonburi
29	Chantaburi

Figure 11 Distribution of *B. superba* in Thailand

(Cherdshewasart unpublished data)

10. Setting up *M. collettii* HPLC fingerprint analysis

10. 1 Sample preparation

The stem of *M. collettii* were prepared and analyzed according to 1.2.

10. 2 Assessment of analytical condition

The condition was set according to 1.4. The wavelength for the analytical was performed at the wavelength of 254 and 270 nm for comparison absorbance of *M. collettii* extract.

10. 3 HPLC fingerprint analysis of the wild *M. collettii*

The stem of *M. collettii* was collected from the northern and central part of Thailand during March – April 2000-2002. The collected provinces are shown in Figure 12. The samples from Chiang Rai, Lampang and Kanchanaburi were submitted for HPLC analysis. (Wichai Cherdshewasart personal communication). Three plants of each clone were prepared and analyzed according to the mentioned methods.

No.	Provinces
1	Chiang Rai
2	Chiang Mai
3	Lampang
4	Kanchanaburi
5	Nakhon Ratchasima*
*	In the Naironal Park

Figure 12 Distribution of *M. collettii* in Thailand
(Cherdshewasart unpublished data)