CHAPTER III

MATERIALS AND METHODS

1. Study on the geographic distribution of *P. mirifica* plants

1.1. Geographic distribution study

The geographic distribution of *P. mirifica* was studied in some area in the northern, north-eastern and central part of Thailand during 1998-2000, focused on the distributions, habitats and environments in each source.

The mature tuberous roots of a weight more than 2-kg were collected for TLC analysis.

The seeds were collected for characterization under the stereo microscope and also counted for genetic segregation analysis.

1.2. *P. mirifica* tuberous powder preparation

The collected tuberous roots of *P. mirifica* were sliced, dried in a hot air oven at 70 °C and subsequently ground into powder at a size of 100 Mesh.

1.3. Phytochemical technique for the collected tuberous roots

1.3.1. Preparation of *P. mirifica* crude extracts

The 10 g of the powder was stirred in warm (40 °C), 100 ml of 95% aqueous methanol for 12 hours. The extract solutions were filtered through a Whatman filter No.1 folded filter. The filtrate was evaporated in a rotary evaporator until completely dried. The 0.05 g of crude extract was dissolved in the 500 µl of the same solvent.

1.3.2. Qualitative analysis by TLC methods

Thin Layer Chromatography (TLC) was submitted for the chemical characterization of *P. mirifica* crude extracts. The methods is as follow

1.3.2.1. Development of the chromatotank

Before ascending the chromatrograms, the chromatotank was equilibrated with the chloroform: methanol at ratio 90: 10 at 25-30 °C.

1.3.2.2. Preparation and descending of Chromatograms

The 2.5 µl of sample was dropped by the aid of a micropipette on the starting line at approximately 1.5 cm. from the lowerside and 1.0 cm from the edge of the plate. The distance between the adjacent spot was 1 cm.

The samples on the chromatograms were air dried and then placed in the chromatotank filled with chloroform: methanol at the ratio of 90: 10

1.3.2.3. Detection of bands from the chromatograms

After completion of the running, the chromatograms were removed from the chromatotank and dried by the dryer. The detection of the chromatogram was performed under the UV- visible light at 254 and 365 nm.

The TLC conditions were described briefly as follows

Technique	:	one way
Absorbant	:	aluminium sheet Silica gel 60 F254 (precoated, Merck)
Plate size	:	10 x 15 cm
Layer thickness	:	0.2 cm
Solvent system	:	chloroform: methanol (90: 10)
Sample size	:	2.5 µl
Distance	:	10 cm
Developing time	:	30-45 min

Temperature : 25-30 °C

Detection : UV-visible light at 254 and 365 nm

The location of bands and colors were recorded on the output data and then calculated for the R_f value as follows

$$R_f \text{ value} = \frac{\text{Distance of sample}}{\text{Solvent front}}$$

1.3.3. Quantitative analysis by TLC densitometric methods

TLC Densitometry was carried out to quantitate the amount of containing puerarin in *P. mirifica* crude extracts.

1.3.3.1. Standard preparation

The 0.01-mg of puerarin (Sigma, USA) was dissolved in 200 µl of AR grade methanol. The standard solution was serial duluted to establish the standard curves at the concentration range of 0.98-15.63 mg/ml

1.3.3.2. TLC condition

TLC condition to separate puerarin from the crude extracts was carried out with the same process as 1.3.2 excepted for the solvent system which was chloroform: methanol : H_2O at ratio 40 : 40 : 2 .

1.3.3.3. TLC densitometry condition

The conditions of TLC densitometer were described briefly as follows

Model	:	Shimadsu Dual Wavelength Model CS-930
Wave length	:	258 nm
Scan width		
- "X" width	:	6 mm
- "Y" width	:	0.1 mm
Slit width	:	6 mm
Sensitivity	:	medium

By the TLC densitometry, the location of bands were recorded on the output data and then calculated for the R_f value.

2. Study on the genetic differences of *P. mirifica* plants

2.1. Seed source

P. mirifica seeds were collected from two jungles in Chiangmai and Kanchanaburi provinces and thus classified into 2 distinct varieties with 4 clones in each variety.

2.2. The seed germination

The collected seeds from each source were surface sterilizated with 50% Chlorox solution with a few drops of Tween 20, shaken for 30 minutes and washed three times with sterile distilled water. The seeds were germinated on WPM medium (see appendix I) in 10 cm Petri dish. All chemicals for WPM medium were obtained from Sigma Chemical Company, U.S.A. They were incubated for 3 days and transfered to the culture room, with the control atmosphere of 25 °C under 16 hour illumination with fluorescent light, 2800 Lux per day. The seeds were removed from the media, washed with water and soaken in anti-fungal solution for a few minutes and transferred to nurse in the soil pots. Plantlets were kept in the greenhouse for 3 months and then transferred into field plot at Amphur Ban Pong, Ratchaburi province.

2.3. The plantaion in the field plot

The field plot at Amphur Ban Pong, Ratchaburi province was assigned for two plot sites, adjacent to each other, for Chaingmai and Kanchanaburi variety. The 25 plantlets of each clone were planted in the plot. The width between row was 1.5 m and the plantlets were planted 10 cm deep from the surface. The spacing between plantlets was set at 1 m .

The plants were allowed to grown naturally without herbicide, pesticide and fertilizer treatment. Watering was supplied only from rainfall. The growth of the plants were monthly observed.

The flowers were obtained and allowed to seed set. The mature pods were collected for seeds for further characterization study under the stereo-microscope. Then the eighteen-month old plants were measured for the length of peristem, 5 plants of each clone were randomly dug up for measuring the number and weight of the tuberous roots.

The tuberous roots were collected and processed into a powder according to the process 1.2 for the proximate and TLC analysis.

2.4. Proximate analysis

The dried powder tuberous root of four clones from each variety were submitted to the Cassava and Starch Technology Research Unit, Kasetsart Agricultural and Agro-Industrial Product Improvement Institue for the analysis of the percentage of starch, fiber, ash, fat and protein analysis.

2.5. Phytochemical technique for the tuberous roots from the field grown plants

2.5.1. Preparation of *P. mirifica* crude extracts

P. mirifica crude extracts were prepared as same as the process 1.3.1

2.5.2. Quantitative analysis by TLC densitometry

Thin Layer Chromatography (TLC) was the method for separation of the puerarin containing in *P. mirifica* crude extracts. The methods was determined according to the process 1.3.2 except the solvent system was chloroform : methanol : H_2O at ration 40 : 40 : 2 and

The chromatograms were carried to quantitate the amount of puerarin by the TLC densitometer. The methods is as follow:

2.5.2.1. Standard preparation

The 0.01 mg of puerarin (Sigma, USA) was dissolved in 200 µl of AR grade methanol. The standard solution was serial duluted to establish the standard curves at the concentration range of 0.98-15.63 mg/ml

2.5.2.2. TLC densitometry conditions

The conditions of TLC densitometer were described briefly as follows

Model	:	Shimadsu Dual Wavelength Model CS-930
Wave length	:	258 nm
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- "X" width	:	6 mm

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Slit width : 6 mm

Sensitivity : medium

By the TLC densitometry, the location of bands were recorded on the output data and then calculated for the R_f value.

3. Statistical analysis

The experiment was designed using a completely randomized design, the data was analyzed by one-way analysis of variance using a computer SPSS. The significance level was taken at P = 0.05