CHAPTER 2
EXPERIMENTS

2.1 Plant material

S. curtisii (Figure 5) were collected from natural habitats in Chumphon Province, Thailand in November 2005. Voucher specimens (specimen no. SKP 182 19 03 01) have been identified by Asst. Prof. Choathip Purintavaragul, department of Biology, faculty of Science, Prince of Songkla University and deposited at the herbarium of the faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Figure 5. Roots and rhizome of *S. curtisii*

Figure 6. Dried root powder of *S. curtisii*

The roots and rhizomes of this plant were washed with tap water to remove the remaining soil and other unwanted materials, cut into small pieces (0.5 cm cubes) and dried in hot air oven at 50 °C for 24 hours. The dried plant was powdered and stored in plastic bags and kept in dark conditions at room temperature (30±2 °C) (Figure 6) until use.

2.2 Chemicals

HPLC grade acetonitrile, analytical grade methanol, 37% hydrochloric acid, and sodium hydroxide were purchased from Labscan (Bangkok, Thailand). Analytical grade triethylamine was obtained from Prolabo (Paris, France). Commercial grade dichloromethane, ethyl acetate, and 95% ethanol were received from High Science distributor (Bangkok, Thailand). Water was purified by passing through a Milli-Q purification system. Commercial grade sodium bisulfite was obtained from Vidhyasom (Bangkok, Thailand). Hydrogen peroxide solution was purchased from Siribuncha (Nonthaburi, Thailand). Pharmaceutical grade lactose monohydrate was received from DMV International Distributor (Thailand). Commercial grade polyvinyl pyrrolidone K-30 (PVP K-30), sodium alginate, sorbitan monooleate (Span 80), and polyethylene sorbitan monooleate (Tween 80) were purchased from Srichand United Dispensary (Bangkok, Thailand). Food grade soy bean oil was purchased from Thai vegetable oil public company limited (Bangkok, Thailand). Analytical grade butylated hydroxytoluene (BHT) was obtained from Sigma (St.Louis, USA).

2.3 Extraction, isolation and identification

The dried root powder (100 g) was macerated with 200 ml dichloromethane at room temperature for 3 days (three times), filtered and evaporated in a rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 40°C. The crude extract (2.286 g) was separated by silica gel column chromatography (Merck® silica gel 60, 0.040 - 0.063 mm) using mixtures of dichloromethane, ethyl acetate, and methanol (70, 25, and 5%, respectively) as solvent. The eluted fractions which afforded pure pyridostemin were combined to give colorless prismatic crystal (54.2 mg) as a major compound. The compound was then identified as pyridostemin by ^{1}H NMR (FT-NMR spectrometer, 500

MHz, Model UNITY INOVA, Varian, USA), MS (MAT 95 XL Mass Spectrometer, Thermofinnigan, Germany), and IR (Fourier Transform Infrared Spectrometer, Perkin Elmer®, USA). Pyridostemin was used as the marker and external standard for quantitative analysis of pyridostemin in the extract and formulations.

2.4 HPLC analysis

The analysis of pyridostemin in the *S. curtisii* extract was performed using Agilent 1100 HPLC system including a quaternary pump, degasser, autosampler, and photodiode array detector (Agilent Technologies, Palo Alto, CA). Chromatographic separations were achieved using Restek® C_{18} column (250×4.6 mm, 5 μm) (Restek Corporation, USA). Data acquisition and analysis were performed by Agilent ChemStation software.

2.4.1 Chromatographic conditions

The mobile phase was a mixture of acetonitrile-water (containing 0.12% triethylamine) (30:70, v/v). The flow rate of the mobile phase was 1.25 ml/min and the injection volume was 20 μl. The column temperature was maintained at 25°C. The quantitation was performed using peak area. The UV detector was used to detect the peak of pyridostemin with the fixed wavelength at 300 nm.

2.4.2 Standard and sample solutions

Standard solutions of pyridostemin (5-25 μg/ml) were prepared in methanol. Each solution was subjected to inject into HPLC system in triplicate. The mean peak areas for each concentration were calculated and standard calibration curve was constructed by plotting concentrations against peak areas.

Approximately 1 mg of the extract was accurately weighed and transferred to a 10 ml volumetric flask and was made up to volume with methanol and sonicated until complete solubilization. The injection volume was 20 μl. The analysis was performed in triplicate.

2.5 Method development and validation

The validation parameters were specificity, precision, accuracy and linearity.

2.5.1 Specificity (modified from Pathare *et al.*, 2007)

Specificity is the ability of the method to measure the response in the present of its potential impurities (ICH, 2000). The specificity of the developed HPLC method for pyridostemin was carried out in the presence of the other components in the crude extract. Stress studies were performed for pyridostemin to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress condition of acid, base, oxidation, and reduction with heat at 50°C for 24 hours to evaluate the ability of the proposed method to separate pyridostemin from its degradation products (ICH, 1995). Peak purity test was carried out for the peak of pyridostemin by using PDA detector in stress samples.

2.5.1.1 Acid hydrolysis

A 4 ml of 1 N hydrochloric acid was added to 1 ml of crude extract stock solution (100 µg/ml) and heated at 50°C for 24 hours. The mixture was then neutralized by 4 ml of 1 N sodium hydroxide and adjusted to 10 ml with methanol. Sample was allowed to cool to room temperature. The resulting solution was then filtered and used for HPLC analysis.

2.5.1.2 Base hydrolysis

A 4 ml of 1 N sodium hydroxide was added to 1 ml of crude extract stock solution (100 µg/ml) and heated at 50°C for 24 hours. The mixture was then neutralized by 4 ml of 1 N hydrochloric acid and adjusted to 10 ml with methanol. Sample was allowed to cool to room temperature. The resulting solution was then filtered and used for HPLC analysis.

2.5.1.3 Oxidation

A 4 ml of 3 %w/v hydrogen peroxide was added to 1 ml of crude extract stock solution (100 µg/ml) and heated at 50°C for 24 hours. The mixture was then adjusted to 10 ml with methanol. Sample was allowed to cool to room temperature. The resulting solution was then filtered and used for HPLC analysis.

2.5.1.4 Reduction

A 4 ml of 10 %w/v sodium bisulfite was added to 1 ml of crude extract stock solution (100 µg/ml) and heated at 50°C for 24 hours. The mixture was then adjusted to 10 ml with methanol. Sample was allowed to cool to room temperature. The resulting solution was then filtered and used for HPLC analysis.

2.5.2 Linearity and range

Linearity of the method was studied by analysis of 5 concentrations of standard solutions prepared in methanol in the range of 5-25 µg/ml (5, 10, 15, 20, and 25 µg/ml) using the HPLC method. The experiment was performed in triplicate. The peak areas versus concentration data were treated by least-squares linear regression analysis. The linearity test was carried out for 3 consecutive days. The minimum acceptable coefficient to establish linearity was set at 0.999 a prior.

2.5.3 Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of three different concentrations of the standard solutions in triplicate on the same day. Intermediate precision of the method was checked by repeating the studies on three different days.

2.5.4 Accuracy

Accuracy of the developed method was tested in triplicate by fortifying a mixture of crude extract solutions with six concentrations of the standard solutions (0, 5, 10, 15, 20, and 25 μg/ml) and determining the recovery of added analyte.

2.6 Extraction of pyridostemin by maceration using organic solvents

The dried root and rhizome powder (10 g) was placed in a conical flask with stopper and macerated with 30 ml of dichloromethane at ambient temperature (30±2 °C) for 3 days with occasional stirring. The solvent was then filtered and evaporated in a rotary evaporator under vacuum at 40°C. The residue was placed in a vacuum desiccator at room temperature until dry. The crude extract was stored in a well-closed container and protected from light and kept in a desiccator at 20°C. The extraction was carried out on three separate occasions (n = 3). The above procedure was repeated using methanol as solvent instead of dichloromethane, again replicated on three occasions (n = 3). Pyridostemin in the crude extracts were analyzed by HPLC.

2.7 Preparation of partially purified *S. curtisii* extract

Pyridostemin in crude extract (30 g) was concentrated by partially purification using silica gel vacuum chromatography (Merck® silica gel 60, (0.040 - 0.063 mm)) and a mixture of dichloromethane, ethyl acetate, and methanol (70, 25, and 5%, respectively) was used as the solvent. The eluted fractions which afforded pyridostemin were combined to give partially purified extract (3.352 g).

2.8 Chemical stability of partially purified *S. curtisii* extract

2.8.1 Accelerated stability study (modified from Lomlim *et al.*, 2003)

The storage stability test of partially purified *S. curtisii* extract was carried out under accelerated conditions. Samples of extract were stored in glass containers at 45, 60, and 70°C with 75 %RH and protect from light. The amount of pyridostemin in each stored sample (n = 3) were analysed at 0, 1, 3, 7, 14, 21, 28, 56, and 84 days after

storage. The amount of pyridostemin was plotted against the storage time in order to calculate the half life ($t_{1/2}$).

2.8.2 Photostability study (modified from Jang *et al.*, 2006)

The photostability of extract was also studied. The extract was exposed to light (36-watt fluorescent lamp at a distance of 40 cm) at room temperature and aliquot was taken at 0, 1, 3, 7, 14, 21, 28 days for analysis of pyridostemin contents. Another set of samples were prepared in the same manner but they were stored in the dark at room temperature.

2.9 Preparation of water dispersible granules containing *S. curtisii* extract

Partially purified *S. curtisii* extract	7 g
PVP K-30	5 g
Tween 80	2 g
Sodium alginate	5 g
Lactose	q.s. to 100 g

The water dispersible granules containing partially purified *S. curtisii* extract prepared by wet granulation technique consisted of partially purified *S. curtisii* extract, PVP K-30, sodium alginate, Tween 80, and lactose as given in the formulation above. The partially purified *S. curtisii* extract was dissolved in a little amount of dichloromethane and PVP K-30 was dissolved in a little amount of ethanol. The obtained solutions were then mixed together and evaporated by rotary evaporator to give a solid mixture. The solid mixture was levigated with Tween 80 and then mixed with other excipients by geometric dilution technique to give a homogeneous powder. Distilled water was added dropwise with continuous mixing to obtain the damp mass. The damp mass was passed through the sieve no. 14 and dried in hot air oven at 40 °C for 1 hour. After that the dried granules were screened through the sieve again. The resulting water dispersible granules were stored in glass containers at ambient temperature and protected from light.

2.10 Evaluation of physicochemical properties of water dispersible granules containing *S. curtisii* extract

The granule samples were evaluated for pyridostemin content, disintegration time, friability and flowability. The viscosity and pH of suspension were determined after disintegration of granules in water.

2.10.1 Pyridostemin content

Approximately 25 mg of granule sample was accurately weighed and transferred to a 25 ml volumetric flask and was made up to volume with methanol and sonicated for 5 min. The solution was filtered and determined for the pyridostemin content by the validated HPLC. The injection volume was 20 µl. The analyses were performed in triplicate.

2.10.2 Disintegration time (modified from Gauthier and Tatin, 2003)

Granule sample (0.5 g) was dispersed in 50 ml of distilled water and stirred at 500 rpm at ambient temperature. The time required for complete disintegration of granules was recorded. Disintegration time of 1.0 g and 1.5 g of samples in 50 ml distilled water was also done under the same condition. Three replicates were performed for each test.

2.10.3 Friability (modified from Wiwattanapatapee *et al.*, 2007)

Measurements of friability were carried out on a Roche Friabilator. Sample (10 g) initially between 14 and 18 mesh in size, was loaded into a Roche drum. Steel-balls of 0.6 cm diameter (25 balls) were also loaded into the drum, which was then attached to the friabilator. The sample was subjected to 400 rotations, where each rotation causes the sample to fall a distance of 15 cm. Afterwards, the sample was pass through a sieve with 45 mesh, and the weight of sample remaining above 45 mesh was measured. A friability index was calculated from the following equation. Three replicates were performed for each test.

$$\text{friability index} = \frac{\text{weight of granule remaining above 45 mesh}}{\text{total weight of loaded sample}} \times 100$$

2.10.4 Flowability (The United States Pharmacopeial Convention, 2007)

The flowability of the granules was analyzed by measuring the angle of repose (α) using a fixed base method. A funnel was set at 10 cm height from the table and the granules were passed through the funnel to the paper that has a circle of 5 cm radius until the granules reached the circle. The height of the granules were measured and calculated for angle of repose from the following equation. The evaluation was performed in triplicate.

$$\tan \alpha = \frac{\text{height of granules (cm)}}{5\,(\text{cm})}$$

2.10.5 Viscosity

The viscosity of suspensions was measured by a Brookfield Model DV III Rheometer (spindle no. 1 at 30 rpm) after complete disintegration of 1, 2, and 3 %w/v granules in water. Three replicates were performed for each test at 25°C.

2.10.6 pH

The pH values of suspensions were measured by a pH meter (Mettler-Toledo Co., Ltd., Switzerland) after complete disintegration of 1, 2, and 3 %w/v granules in water. The evaluation was performed in triplicate.

2.11 Stability of water dispersible granules containing *S. curtisii* extract

The prepared water dispersible granules were investigated for both chemical stability and physical stabilities.

2.11.1 Chemical stability of water dispersible granules containing *S. curtisii* extract

The stability during storage of granules containing *S. curtisii* extract was tested under accelerated conditions. Samples were stored in glass containers at 45, 60, and 70°C with 75 %RH and protect from light. The amount of pyridostemin in each stored sample (n = 3) were analysed at 0, 1, 3, 7, 14, 21, 28, 56, and 84 days after storage. The amount of pyridostemin was plotted against the storage time in order to calculate the shelf life ($t_{90\%}$).

2.11.2 Physical stability of water dispersible granules containing *S. curtisii* extract

Granule samples were stored in well-closed container at ambient temperature and protected from light. Physical stabilities such as disintegration time, friability, flowability, viscosity, and pH during storage were determined in similar manners to that described in section 2.10.1 - 2.10.6. Each stored samples were evaluated for their physical stabilities at 0, 1, 2, and 3 months after storage.

2.12 Preparation of emulsifiable concentrate containing *S. curtisii* extract

The pesticide emulsifiable concentrate prepared by simple mixing the components as follow:

S. curtisii extract	10 g
Tween 80	35 g
Span 80	25 g
BHT	0.1 g
Soy bean oil	29.9 g

S. curtisii extract was levigated with Tween 80 and Span 80 in a mortar and then mixed with other excipients to give a homogeneous emulsifiable concentrate. The

resulting emulsifiable concentrate was stored in glass containers at ambient temperature and protected from light.

2.13 Evaluation of physicochemical properties of emulsifiable concentrate containing *S. curtisii* extract

The emulsifiable concentrate samples were evaluated for pyridostemin content. The particle size of internal phase, viscosity, and pH of 1, 2, and 3 %w/v emulsifiable concentrates in water were also measured.

2.13.1 Pyridostemin content

Approximately 25 mg of emulsifiable concentrate sample was accurately weighed and transferred to a 25 ml volumetric flask and was made up to volume with methanol and sonicated until complete solubilization. The pyridostemin content was determined by validated HPLC. The injection volume was 20 µl. The analysis was performed in triplicate.

2.13.2 Particle size

Emulsifiable concentrate (0.5 g) was dispersed in 50 ml of distilled water by stirring with a magnetic stirrer at 500 rpm for 1 minute to complete emulsion forming at ambient temperature. The obtained emulsion was evaluated for oil droplet size by Mastesizer E (Malvern Instruments Ltd, UK). Samples containing 1.0 g in 50 ml water and 1.5 g in 50 ml water were also evaluated using the same condition. The evaluations were performed in triplicate.

2.13.3 Viscosity

The viscosity of emulsion obtained from 1, 2, and 3 %w/v emulsifiable concentrate in water were measured by a Brookfield Model DV III Rheometer (spindle no. 1 at 30 rpm). Three replicates were performed for each test.

2.13.4 pH

The pH values of emulsion obtained from 1, 2, and 3 %w/v emulsifiable concentrate in water were measured by a pH meter (Mettler-Toledo Co., Ltd., Switzerland). The evaluations were performed in triplicate.

2.14 Stability of emulsifiable concentrate containing *S. curtisii* extract

2.14.1 Chemical stability of emulsifiable concentrate containing *S. curtisii* extract

The stability during storage of emulsifiable concentrate containing *S. curtisii* extract was carried out under accelerated conditions. Emulsifiable concentrate samples were stored in glass containers at 45, 60, and 70°C with 75 %RH and protected from light. The amount of pyridostemin in each stored sample (n = 3) were analysed at 0, 1, 3, 7, 14, 21, 28, 56, and 84 days after storage. The amount of pyridostemin was plotted against the storage time in order to calculate the $t_{90\%}$.

2.14.2 Physical stability of emulsifiable concentrate containing *S. curtisii* extract

Emulsifiable concentrate samples were stored in well-closed container at ambient temperature and protected from light. The emulsion (1 %w/v) obtained after dilution of emulsifiable concentrate in water was evaluated for particle size, viscosity, and pH in the similar procedures as described in section 2.13.2 - 2.13.4. Each stored samples were evaluated for their physical stabilities at 0, 1, 2, and 3 months after storage.