CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

- Fruit of *Phyllanthus emblica* Linn., *Solanum trilobatum* Linn., *Terminalia chebula* Retz., *Piper nigrum* Linn. and *Morinda citrifolia* L. (The dried plant materials, Samunprai Lanna, Thailand)
- Underground part of *Zingiber officinale* Rosc., *Curcuma longa* Linn., *Acorus calamus* Linn., *Eurycoma longifolia* Jack. and *Glycyrrhiza glabra* Linn. (The dried plant materials, Samunprai Lanna, Thailand)
- Leaf of *Cassia angustifolia* Vahl., *Andrographis paniculata* Wall. Ex Nees., *Centella asiatica* (L.) Urban., *Pluchea indica* (L.) Less. and *Citrus hystrix* DC. (The dried plant materials, Samunprai Lanna, Thailand)
- Stem of *Tinospora crispa* Miers., *Cinnamomum verum* J.S., *Caesalpinia sappan* Linn. and *Derris scandens* Benth. (The dried plant materials, Samunprai Lanna, Thailand)
- Ethanol (AR Grade) (Lab-Scan, Thailand)
- NaOH (AR Grade) (Lab-Scan, Thailand)
- Distilled water
- Corn starch (C.M. Chemical & Lab supplies, Thailand)
- Microcrystalline cellulose (Avicel® grade pH 101 , JRS Pharma LP., Germany)
- Croscarmellose sodium (Ac-Di-Sol®, Rama Production, Thailand
- Purified talcum (Vechavit, Thailand)
- Magnesium stearate (Riedel-de Haen, Germany)
- Polyvinylpyrrolidone (PVP K90, Serva Feinbiochemica GmbH & Co., Germany)
- Gelatin A (Unionscience Co., Ltd., Thailand)

3.2 Instruments

- Hot air oven (BINDER ED 240/E2, Germany)
- Vacuum pump (MEDI-PUMP, THOMAS Industries, USA)
- Sieve (U.S.A. standard testing sieve no.60, U.S.A.)
- Moisture balance (Sartorius type MA 50, Germany)
- Jolting volumeter (J. Engelsmann AG, Germany)
- Single stroke tableting machine (HANSEATEN Wilhelm Fette, Germany)
- Hardness tester (ERWEKA® type TBH 100, Germany)
- Roche friabilator (Pharma test® type PTF 20 E, Germany)
- Disintegration tester (Pharma test® type PTZ-AUTO 3, Germany)
- Digital vernier caliper (MS Scientific instrument Co., Ltd, Thailand)
- Analytical balance (Scaltec type SBC 31, Germany)
- Electronic balance (Sartorius type LA 230 S, Germany)
- Light microscope (Olympus C 001, Japan)
- Hot plate stirrer (IKA® C-MAG HS 7, Germany)

3.3 Methods

3.3.1 Selecting of herbal plants

Selection of the herbal plants used for the study was primarily based on categorization of parts used for medication, namely fruit, leaf, stem, and underground part (root or rhizome). Four or five medicinal plants from the list of herbs for primary health care were chosen for each part used as follows:

Fruit: *P. emblica*, *S. trilobatum*, *T. chebula*, *P. nigrum* and *M. citrifolia*

Underground Part: *Z. officinale*, *C. longa*, *A. calamus*, *E. longifolia* and *G. glabra*

Leaf: *C. angustifolia*, *A. paniculata*, *C. asiatica*, *P. indica* and *C. hystrix*

Stem: *T. crispa*, *C. verum*, *C. sappan* and *D. scandens*

3.3.2 Preparation of herbal powders

The plant samples were dried at the appropriate temperature before pulverization and screening through sieve No.60.

3.3.3 Verification of herbal plants and powders

The verifications of herbal plants and powders were carried out following the official pharmacopoeia and/or standard of crude drug (Vichiara et al., 1995 & 2000; ASEAN countries, 1993; Faculty of pharmacy, Mahidol university,1986; Norman et al.1992)

3.3.3.1 Identity verification

(1) Macroscopic characteristics of herbal plants

The visual observation as well as the photographs of the herbal plants were taken to compare the physical characteristics with the official reference books. (Vichiara et al., 1995 & 2000; ASEAN countries, 1993; Faculty of pharmacy, Mahidol university, 1986; Norman et al.1992)

(2) Microscopic characteristics of herbal powders

The optical microscopic photographs of herbal powders were taken. Suitable staining dyes were used to identify the specific components as follows: (Vichiara et al., 1995 & 2000; ASEAN countries, 1993; Faculty of pharmacy, Mahidol university, 1986; Norman et al.1992)

- **Distilled water:** To observe general compositions of plant powder, i.e. parenchyma cells, starch grain, crystals and other cells for verify the basis of a cell
- **2% Iodine solution:** To stain starch grains into blue or purple color
- **Saturated solution of phloroglucinol in alcohol and 20% HCl:** To stain cells containing lignin like fibers and sclereids into red or pink color and to stain collenchyma and epidermis that contain suberin or cutin into orange red color
- **Alcoholic Sudan III solution:** To stain oil cells into yellow or red color
- **Alcoholic picric acid solution:** To stain aleurone grains into yellow color
- **75% Chloral hydrate solution:** To remove cell contents such as protein, starch, resin, chlorophyll, volatile oil etc., which conceal other more important characteristics of cell structures. Removal of cell contents makes remaining part more transparent and reveals details of other characteristics

3.3.4 Analysis of ash content and herbal powder compositions

3.3.4.1 Ash content

The total ash and acid-insoluble ash of the herbal powder were determined as the following procedures.

(1) Total ash

The total ash method is used to measure the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plants tissue itself, and “non-physiological” ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plants surface. The procedure started with placing about 2-4 g of the air-dried herbal powder, accurately weighed, in a previously ignited and tared crucible. Spread the material in an even layer and ignite it by gradually increasing the heat to 450°C until it is white, indicating the absence of carbon. Cool in a desiccator and weigh. Calculate the content of total ash in mg per g of air-dried material (Vichiara et al., 1995).

(2) Acid-insoluble ash

The acid-insoluble ash is designed to measure the amount of ash insoluble to diluted hydrochloric acid. The procedure started with adding carefully 25 ml of 2 M hydrochloric acid to the ash (obtained as total ash method), boil gently for 5 minutes, collect the insoluble matter on ashless filter-paper for quantitative analysis, wash with hot water until the filtrate is neutral, and dry the residue together with the filter paper. Spread the material in an even layer and ignite it by gradually increasing the heat to 500°C. Cool it in a desiccator and weigh accurately. Calculate the content of acid-insoluble ash in mg per g of air-dried material (Vichiara et al., 1995).

3.3.4.2 Starch Content

Glucoamylase method was used for analyzing the starch content. Briefly, the sample was washed with absolute ethanol until the clear and colorless filtrate was obtained to minimize the inteference from inherited color to the analysis. Then, the test sample was autoclaved to gelatinize starch before the enzymatic hydrolysis by glucoamylase was applied to convert starch to glucose. The glucose content was measured by using colorimetric method and later converted to starch content. This method is fully described in Appendix 1 (AOAC International, 1977).

3.3.4.3 Volatile Oil Content

Volatile oil content was measured by using steam distillation. The distillate oil was measured for its content and calculated into percentage in comparison with the weight of herbal powder (Vichiara et al., 1995).

3.3.4.4 Fiber Content

Crude fiber analysis method was used for analyzing the fiber content. In principle, the method involves dissolving the sample in acid solution followed by alkaline solution. The insoluble matters were filtered with ashless filtered paper, and burned to obtain the ash content, which are the compositions of cellulose and lignin. The details of method are provided in Appendix 2 (Ministry of industry, 1973).

3.3.5 Examination of fundamental pharmaceutical properties of herbal powder

3.3.5.1 Flow properties

(1) Angle of repose

Herbal powder is poured through a glass funnel. Calculate the angle of repose from herbal powder heap. Angle of repose corresponds to flow properties. Lower angle of repose values represents better flow. (Banker et al, 1980)

Table 3.1 Flow properties and corresponding angle of repose (USP 34/NF 29)

Flow property	Angle of repose (degrees)
Excellent	25-30
Good	31-35
Fair	36-40
Passable	41-45
Poor	46-55
Very poor	56-65
Very, very poor	> 66

(2) Compressibility ratio

Approximately 100 ml of herbal powder is poured into a tared graduated cylinder and the initial volume and weight of the material was recorded, then held up 1 inch from a hard wood surface and dropped down on gravity force 3 times at 2 second intervals. The bulk density was calculated from the difference between the

initial volume and the final volume of powder. The graduated cylinder is placed on a tap density tester Jolting volumeter (J. Engelsmann AG, Germany) and the final volume is recorded after 500 taps.

The bulk density and tapped density were calculated and used for the calculation of compressibility ratio by using the following equation

$$\text{Compressibility ratio} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \quad (1)$$

Compressibility ratio values are associated indirectly with relative flow rate, agglomeration and particle size of powder. Lower percent compressibility values represent better flow (Banker et al, 1980).

Table 3.2 Scale of flowability (USP 34/NF 29)

Compressibility index (%)	**Flow character**
≤ 10	Excellent
11-15	Good
16-20	Fair
21-25	Passable
26-31	Poor
32-37	Very poor
> 38	Very, very poor

(3) Flow rate

The flow rate of the herbal powders was determined by measuring the time required for the herbal powder to pass through the funnel orifice with a diameter of 10 mm (n=3). The flow rate was expressed as a ratio of mass (g) to time (s) (Singh et al, 2009).

(4) Kawakita equation

Ten grams of herbal powder was filled into a 50 ml cylinder and the volume of the powder was read before (V_O) and after (V_N) the cylinder was tapped for N times (N= 10, 30, 100 and 300). C value was calculated as the following formula:

$$C = \frac{(V_O - V_N)}{V_O} \quad (2)$$

Then, a graph representing the relationship between N/C value and N value was plotted as shown in the following equation.

$$\frac{N}{C} = \frac{N}{a} + \frac{1}{ab} \quad (3)$$

1/a value is the slope value where a refers to compactibility. It is indirectly related to the flowability, that is, the higher the value is the worse the flowability. In addition, this equation also indicates cohesiveness of the powder through 1/b value which can be calculated from intercept value of the graph (Patra et al., 2008).

3.3.5.2 Compressibility

The examination of compressibility of the herbal powder was conducted by the following method. Firstly, herbal powder was compacted using instrumented single-punch machine (Hanseatea EI, Germany) at a compression force of 15,000 N (punch diameter = 12.7 mm, n=6). Then, the hardness of the tablets was measured. If the hardness value is over 40 N, it can be primarily assumed that the herbal power is good enough for tablet production via direct compressing method or dry granulation.

3.3.6 Statistical analysis of the relationship between herbal powder composition and its fundamental pharmaceutical properties

The statistical analysis of the relationship was done according to Quadratic Model. The second order (quadratic) model as shown below was applied to evaluate the relationship between the independent variables and dependent variables.

$$Y = \beta 0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + e$$

Where: Y = the study responses i.e flow parameters, tablet hardness or Kawakita constant (a); X_1 = Fiber Content (%, w/w); X_2 = Starch Content (%, w/w); X_3 = Volatile Oil Content (%, w/w); β = regression coefficient; e = random error

3.3.7 Development of herbal tablet formulation

3.3.7.1 Tablet production process selection

The selection of tablet production method was chosen according to the results of the flow and compression properties as the assessment criteria in Table 3.3.

Table 3.3 Production process selection by evaluating the flow and compression properties

Assessment criteria	Production process
Flow and compression properties were in accordance with the criteria specified in 3.3.5.1 (Flow property is fair – excellent) and 3.3.5.2 (Hardness ≥ 40 N)	**Direct compression process**
Flow property was in accordance with the criteria specified in 3.3.5.1 (Flow property is fair – excellent) but compression property was not in accordance with the criteria specified in 3.3.5.2 (Hardness < 40 N)	**Dry granulation process**
Flow and compression properties are not in accordance with the criteria specified in 3.3.5.1 (Flow property is very, very poor – passable) and 3.3.5.2 (Hardness < 40 N)	**Wet granulation process**

(1) Direct compression method

Powdered drugs were dry mixing with excipients, including a glidant (purified talcum) and a lubricant (magnesium stearate). Mixed powder was compacted one tablet at a time using instrumented single-punch machine at 15,000 N. Then, the hardness of the tablets was measured. If the hardness value is less than 40 N, it can be primarily assumed that the herbal powder does not have enough features to be produced via direct compression method. This may need to be switched to the preparation by dry granulation or wet granulation methods.

(2) Dry granulation method

Powdered drugs and excipients including glidant and lubricant were mixed and compressed into slug. The granules were produced by using the oscillating granulator. Their angle of repose was evaluated according to the procedures in 3.3.5.1. The granules were mixed with the glidant and lubricant and then were compacted into tablets using instrumented single-punch machine at 15,000 N. After

that, the hardness of the tablets was measured. If the hardness value is less than 40 N, this formulation was switched to the method of wet granulation.

(3) Wet granulation method

The herbal powder was blended with an individual binding solution, namely 10% starch paste, 5% starch paste + 5% gelatin solution, 10% polyvinylpyrrolidone in water and 10% gelatin solution to obtain the wet mass. The amount of each binding solution used was noted. The granules were prepared by pressing the wet mass through sieve No.20. After drying in a tray dryer overnight, the dried granules were compacted using instrumented single-punch machine at 15,000 N. Then, the hardness of the tablets was measured to evaluate the appropriate binder for each herbal powder.

3.3.7.2 The pressure transmission ratio (R)

The efficiency of a lubricant may be quantitatively expressed as the ratio R of the maximum lower punch force to the maximum upper punch force. In addition to the comparison of various lubricants, the R value is helpful in the determination of the concentration of a lubricant that provides an optimum lubricant effect, which for most pharmaceutical lubricants does not exceed 1.

3.3.7.3 Disintegration test

The herbal compacts were tested for disintegration time using the disintegration tester (Pharma test® type PTZ-AUTO 3, Germany). The test condition was in accordance with that specified by USP 34/NF 29. The acceptance criteria for the disintegration time of the tablets was defined within 30 minutes. If the tablet disintegrated slower than the specified criterion time, a disintegrant e.g. croscarmellose sodium was added to improve the disintegrating property.

3.3.8 Scale-up phase

After the acceptable formulations were established, the scale-up batch (100 g) was produced. The tabletting machine was set to run continuously.

3.3.9 Quality control of finished products

The acquired tablets were investigated for the finished product quality control as follows:

3.3.9.1 Tablet appearance

The tablets were evaluated for their appearance such as shape and homogeneity in color.

3.3.9.2 Tablet dimensions

The thickness and diameter of 20 herbal tablets were measured using a Digital vernier caliper (MS Scientific instrument Co., Ltd, Thailand). The range should not exceed ± 5% of the mean value.

3.3.9.3 Weight variation

Twenty tablets were randomly selected from each batch individually, weighed to calculate average weight and compare the individual tablet weight to the average. If no more 2 tablets are outside the percentage limit and if no tablet differs by more than 2 times the percentage limit then it is acceptable. The acceptance percentage deviation used in this study followed USP 34/NF 29.

3.3.9.4 Strength of tablets

(1) Friability

Twenty tablets were weighed and placed in the Roche friabilator (Pharma Test® type PTF 20E, Germany) and apparatus was rotated at 25 rounds per a minutes for 4 minutes, after that difference between pre-weight and post-weight were investigated. Friability of tablets was calculated in percentage unit (%). Typical tablets should have the friability value of less than 1.0%.

(2) Hardness

Ten tablets were measured fot the tablet hardness with Erweka tester (Erweka Type TBH 100, Germany). and the results were reports in Newton unit (N). Hardness of the tablet in general should be sufficient to obtain the friability and disintegration property in the standard range.

3.3.9.5 Disintegration test

Six tablets were tested for disintegration time using the disintegration tester (Pharma test® type PTZ-AUTO 3, Germany). Disintegration time of traditional tablets which produced by modern production process should lie within 30 minutes (Ministry of public health, 2001). The apparatus and conditions of the disintegration test was conformed to USP 34/NF 29.