CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and equipments

3.1.1 Plant materials

The recipes were prepared from a variety of plants which were indigenous in 7 upper northern provinces of Thailand (Chiang Mai, Chiang Rai, Lamphun, Lampang, Phayao, Phrae and Nan). All medicinal plants are commercially available. The fresh plants were collected from a herbal garden at Mae Tang district, Chiang Mai. These plants were authenticated and the herbarium voucher specimens were prepared and deposited in the herbarium of Faculty of Pharmacy, Chiang Mai University. The recipes and the characteristics of the plants used in this study were listed in Table 3.1.

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant parts used	Lanna uses
1	25	*Cassia alata* L. Roxb.	Leguminosae-Caesalpinioideae	010086	Fruit	Anti-inflammation for insect

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant parts used	Lanna uses
1		*Datura metel* L.var.*fastuosa* (Bernh.) Danert	Solanaceae	010087	Stem, leaf	
		Jatropha gossypifolia L.	Euphorbiaceae	010089	Stem, leaf	
2	105	*Aegle marmelos* (L.) Correa ex Roxb.	Rutaceae	010096	Fruit	Anti-inflammation for insect sting and bite
		Azadirachta indica A. Juss.var.siamensis Valeton .	Meliaceae	010095-1	Flower	
		Cyperus rotundus L.	Cyperaceae	010092	Rhizome	
		Oryza sativa L.	Poaceae	-	Water	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant Parts used	Lanna uses
2					from washing uncooked rice	
	105	*Phyllanthus emblica* L.	Euphorbiaceae	010090	Fruit	
		Piper chaba Hunt.	Piperaceae	010093	Seed	
		Terminalia chebula Retz.	Combretaceae	010091	Fruit	
		Zingiber officinale Roscoe.	Zingiberaceae	010094-1	Rhizome	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant parts used	Lanna uses
3	192	*Anethum graveolens* L.	Apiaceae	-	Seed	Anti-inflammationfor insect sting or bite
		Baliospermum solanifolium (Burm) Suresh.	Euphorbiaceae	010098	Leaf	
		Croton Oblongifolius Roxb.	Euphorbiaceae	010097-2	Leaf	
		Croton tiglium L.	Euphorbiaceae	010095-2	Leaf	
		Dioecrescis erythroclada (Kurz.) Tirveng	Rubiacieae	010096	Root/ Stem	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant parts used	Lanna uses
3	192	*Foeniculum vulgare* Mill. var. vulgare (Miller) Thell.	Apiaceae		Seed	
		Lepidium sativum L.	Brassicaceae	-	Seed	
		Nigella sativa L.	Ranunculaceae	-	Seed	
		Oryza sativa L.	Poaceae	-	Fruit	
		Phyllanthus emblica L.	Euphorbiaceae	010090	Fruit	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant Parts used	Lanna uses
3	192	*Piper chaba* Hunt.	Piperaceae	010093	Seed	
		Piper nigrum L.	Piperaceae	-	Seed	
		Plumbago indica L.	Plumbaginaceae	010097-1	Whole	
		Plumbago zeylanica L.	Plumbaginaceae	010099	Whole	
		Terminalia chebula Retz.	Combretaceae	010091	Fruit	
		Zingiber officinale Roscoe.	Zingiberaceae	010094-1	Rhizome	
4	346	*Caesalpinia digyna* Rottle.	Leguminosae - Caesalpinioideae	-	Root	Acne abscess

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant Parts used	Lanna uses
4	346	*Lagenaria siceraria* (Molina) Standl.	Cucurbitaceae	-	Calyx	
		Passiflora foetida L.	Passifloraceae	010100	Stem, leaf	
5	717	*Caryota bacsonensis* Magalon.	Arecaceae	010102	Root	Skin abscess, Insect sting or bite
		Cassia occidentalis L.	Leguminosae - Caesalpinioideae	010105	Flower	
		Dregea volubilis (L.F) Hook.f.	Asclepiadaceae	010101	Whole	
		Fagraea fragrans Roxb.	Loganiaceae	010104	Stem bark	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipesne (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant Parts used	Lanna used
5	717	*Psophocarpus tetragonolobus*	Leguminosae Papilionoideae	-	Pod	
		Quisqualis indica L.	Combretaceae	010103	Leaf, Seed	
6	895	*Cassia alata* L.	Leguminosae-Caesalpinioideae	010086	Fruit	Gum abscess
		Gardenia turgida Roxb. Terveng.	Rubiaceae	-	Leaf, Root	
		Sauropus androgynus L. Merr.	Euphorbiaceae	010106	Leaf	
		Siphonodon celastrineus Griff.	Celastraceae	010109	Root	
		Tiliacora triandra Diels.	Menispermaceae	010108	Leaf	

Table 3.1 List of plant, characteristics, botanical names, family, herbarium voucher specimen numbers, plant part used and uses in Lanna traditional medicine of the selected recipes (continued)

No.	Recipe No.	Botanical names	Family	Voucher specimen numbers	Plant Parts used	Lanna used
7	896	*Coccinia grandis* L. voigt.	Cucurbitaceae	010110	Leaf	abscess and tooth pain
		Sesamum indicum L.	Pedaliaceae	-	Oil	
		Vitex trifolia L.	Verbenaceae	010111	Leaf	
		Zingiber officinale Roscoe.	Zingiberaceae	010094-1	Rhizome	

3.1.2 Chemicals

- Acetone AR grade (Labscan, Doublin, Ireland)
- Acetic anhydride (Sigma Chemical Co., St. Louis, MO, USA)
- Alpha naphthol (Fluka, Madrid, Spain),
- Ammonium hydroxide (Fluka, St. Louis, MO, USA)
- Antimony III chloride (Fluka, Buchs, Switzerland)
- Bismuth nitrate (Merck, Darmstadt, Germany)
- Cadmium iodine (Merck, Darmstadt, Germany)
- Calcium hydroxide (Merck, Darmstadt, Germany)

- Carrageenan (Lambda Type IV, Sigma Chemical Co., St. Louis, MO, USA)
- Chloroform (Labscan Asia, Bangkok, Thailand)
- Copper sulfate (Fluka, St. Louis, MO, USA)
- Cupric acid–Aldrich (Sigma Co, St. Louis, MO, USA)
- Cyanidin (Sigma Co, St. Louis, MO, USA)
- Ethanol (Merck, Darmstadt, Germany)
- Ethyl phenylpropiolate, 5 ml, (Fluka, St. Louis, MO, USA)
- Ferric chloride (Sigma Co, St. Louis, MO, USA)
- Formalin (Merck, Darmstadt, Germany)
- Gelatin salt reagent (Fluka, St. Louis, MO, USA)
- Gelatin (Fluka, St. Louis, MO, USA)
- Glacial acetic acid (Merck, Darmstadt, Germany)
- Hydrochloric acid AR grade (Labscan, Dublin, Ireland)
- Hydrogen peroxide (Sigma Chemical Co., St. Louis, MO, USA)
- Iodine (Sigma Chemical Co., St. Louis, MO, USA)
- Lead acetate (Sigma Chemical Co., St. Louis, MO, USA)
- Magnesium ribbon (Merck, Darmstadt ,Germany)
- Mercuric chloride (Fluka, St. Louis, MO, USA)
- Methanol (Merck, Darmstadt, Germany)
- Morin ((Merck, Darmstadt, Germany)
- Nitric acid (Fluka, St. Louis, MO, USA)
- Phenylbutazone (Fluka, St. Louis, MO USA)

- Picric acid (Sigma Chemical Co., St. Louis, MO, USA)
- Potassium iodide (Sigma Chemical Co., St. Louis, MO, USA)
- Potassium bismuth iodide (Sigma Chemical Co., St. Louis, MO, USA)
- Potassium hydroxide (Sigma Chemical Co., St. Louis, MO, USA)
- Modena Prednisolone acetate (Fluka, Buchs,Switzerland)
- Quinine sulfate (Fluka, Buchs,Switzerland)
- Red mercuric iodide (Sigma Chemical Co., St. Louis, MO, USA)
- Resorcinol crystal (Sigma Chemical Co., St. Louis, MO, USA)
- Rutin (Merck, Darmstadt, Germany)
- Sodium carbonate (Sigma Chemical Co., St. Louis, MO, USA)
- Sodium citrate (Sigma Chemical Co., St. Louis, MO, USA)
- Sudan IV (Sigma Chemical Co., St. Louis, MO, USA)
- Sulfuric acid (Sigma Chemical Co., St. Louis, MO, USA)
- Tannic acid (Fluka, St. Louis, M, USA)
- Volatile lactone glycoside (Sigma Chemical Co., St. Louis, MO, USA)
- Zinc dust (Sigma Chemical Co., St. Louis, MO, USA)
- Tween 80 (Uniquema, Bromborough, UK)

3.1.3 Equipments

- Rotary evaporator (Buchi, R-200, Burladingen, Germany)
- Freeze-dryer (Christ, Billerbeck, Germany)
- Vernier caliper 6 inch, 0-150 mm. (Mitutoyo, Tokyo, Japan)
- Plethysmometer 7150 (Ugo Basile, Modena, Italy)

- Centrifuge tube 50 ml **(**TPP, Trasadingen, Switzerland**)**
- Ultracentrifuge (Univeral 32 R, Hettich Zentrifugen, Germany)

3.1.4 Animals

Sprague Dawley rats were purchased from National Laboratory Animal Centre, Mahidol University, Salaya Campus, Nakhon Pathom, Thailand**.** The hind paw edema test used male rats 180-250 g with the age of 6 weeks. The rat ear edema test used the male rats with weight ranging from 50-70 g and the age of 3 weeks.

3.2 Methods

The scope of this study was divided into four parts as shown in Figure 3.1. The detailed methods are as follows:

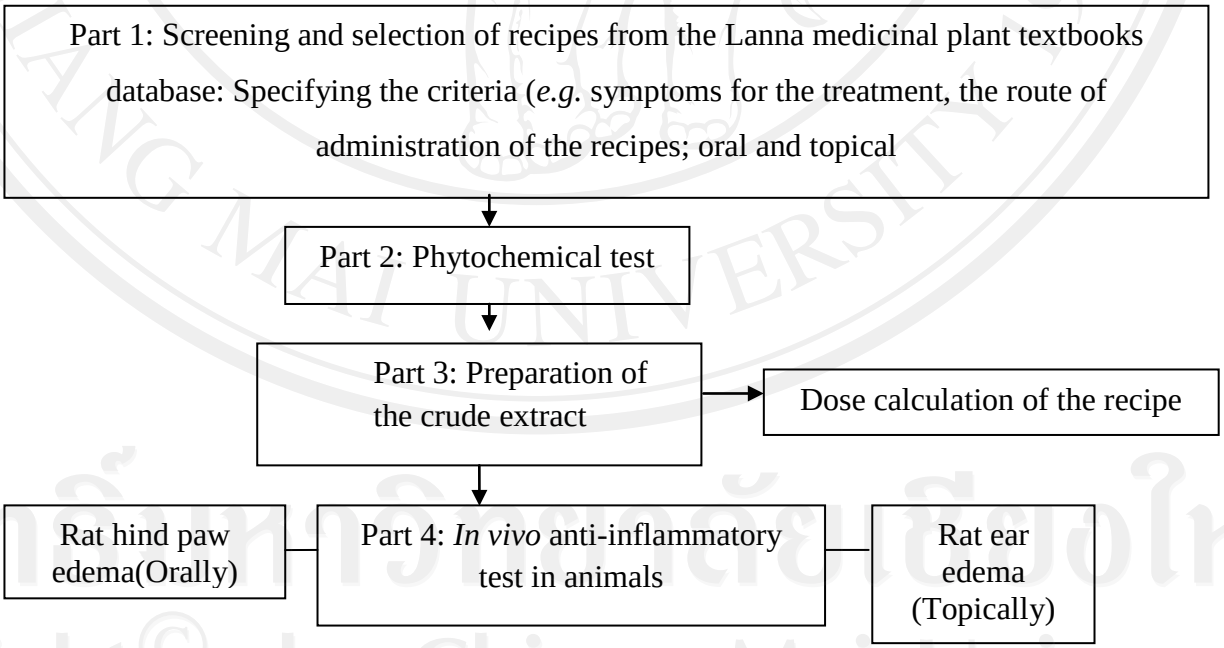

Figure 3.1 Scope of the study

3.2.1 Screening and selection of recipes from the Lanna medicinal plant textbooks database MANOSROI II

Step 1 Selection the recipes from the Lanna medicinal plant textbooks from " MANOSROI II" database. The selection criteria were as follows:

1) Specify the criteria (*e.g.* symptoms for the treatment) to select the anti-inflammatory recipes such as swelling, redness, edema, pain on the external skin, insect/animal bite, acne, boil, gum edema (abscess), body and tendon disturbance (Kumar, 2005).

2) Select the recipes which had both oral and topical routes of administration.

3) Select the recipes from the 11,130 translated recipes in the database collected from seven provinces, Chiang Mai, Chiang Rai, Lamphun, Lampang, Phayao, Phrae and Nan in the upper north of Thailand.

Step 2 The selected recipes were screened and ranked for the recipes selection. The criteria and conditions of scoring and ranking were set as describes:

1) Determine the plant which appeared with high frequency in most recipes. This was ranked by the score from high to low with 4 levels of score point. The priority for frequency in most recipes were 7, 4, 3, 2 and 1, the full score was set at 20 points for the frequency of 7. For example, the frequency of the same plant used in the recipe was 4, thus the score was 11.4 points (4 X 20/7).

2) Specify the selection criteria for the ease availability of plants in the local scrub forests or markets. The score and priority for recipe selection was set at 10 points

as full score when the recipe contained the plants as specify in the recipe of Lanna medicinal plant textbooks and easy availability in the local scrub forests or markets. For example, the recipe contained of the 8 plants, but the 2 plants were unable to collect from the local scrub forests or markets, thus the score of this recipe was 7.5 points (10 X 2/8).

3) Specify the selection criteria for completeness of the recipes from the Lanna medicinal plant textbooks. The 4 selection criteria were the composition, dosage, preparation and indication uses of the recipes. The scores for these criteria were set at 5 points for each condition of the completeness of the recipes. The recipes were ranked and then the top seven recipes were selected for the study.

3.2.2 Phytochemical Test

The phytochemicals in the recipes were identified by a standard phytochemical test (Farnsworth, 1966). Chemical screening tests are the basis for discovering the chemical compounds as shown in Table 3.2.

Table 3.2 The reagents and methods for the phytochemical test

Chemical Compound group	Reagent	Method	References
Alkaloid	Dragendorff's	Bismuth nitrate solution (8gm of bismuth nitrate was dissolved in 30% w/v Nitric acid 12ml) was	Rosin, 1967.

Table 3.2 The reagents and methods for the phytochemical test **(continued)**

Chemical Compound group	**Reagent**	**Method**	**References**
Alkaloid		mixed together with potassium iodide solution (27.2 gm of potassium iodide in 50 ml of distilled water). The mixture was adjusted to 100 ml by using distilled water. A positive test is orange in color.	
	Hager's	Dissolve 1 g of picric acid in 100 ml of distilled water then drop 1-2 ml into a preparation tube recipe solution. A positive test is yellow in color.	Lide D.R., 2004
	Meyer's reagent	Mercuric chloride solution (1.36 gm of mercuric chloride in 60 ml of	Rosin, 1967.

Table 3.2 The reagents and methods for the phytochemical test **(continued)**

Chemical Compound group	**Reagent**	**Method**	**References**
Alkaloid		distilled water) was mixed with potassium iodide solution (5 gm of potassium iodide in 10 ml of distilled water). Then, the solution was adjusted to 100 ml by using distilled water. A positive test is white in color.	
	Wagner's reagent	Potassium iodide solution (2 gm of potassium iodide in 10 ml of distilled water) was mixed to 1.27 gm of Iodine. Then, the solution was adjusted to 100 ml by using distilled water. A positive test is orange brown jn color.	Onwukaeme, *et al*., 2007

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Anthraquinone glycosides	Modified Borntrager's	0.5N KOH 10 ml and 3%H_2O_2 1 ml were added to 500 mg of each recipe. This mixture was heated on a water bath for 10 minutes. Then, the mixture was filtrated and glacial acetic acid was added until it resulted in an acidic solution. After that the solution was transferred to a separator funnel and extracted with 10 ml of benzene. The benzene layer was collected and divided it to 2-5 ml of each of two	Onwukaeme *et al*., 2007.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Anthraquinone glycosides		tubes. Tube no. 1 was control tube while Tube no. 2 had NH_4OH T.S. added and mixed with the solution. A positive test is pink-red color in basic layer.	
Carbohydrate	Benedict's solution	Benedict's solution: Dissolve 173gm of sodium citrate, 17.3 gm of copper sulfate and 100 gm of sodium carbonate with distilled water and adjust volume to 1000 ml. Then heated the crude drug with Benedict's solution. A positive test is a brick-redcolor precipitate indicates presence of the	Dickson, 1998

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Carbohydrate		aldehyde group.	
	Molisch's test	α-naphthol is dissolved in ethanol. This reagent (2 drops) and 2 ml of distilled water are added to each recipe (0.1 gm) and mixed together in test tube. Then, this test tube is laid at an angle of 45 degrees and 1 ml of sulfuric acid is added to the tube; the acidic layer is a placed under the water layer. The tube is left to stand for a moment. A positive test is brownish-purple ring between two layers.	Dickson, 1998.
		1. $C_5H_{10}O_5$ (pentose) +	

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Carbohydrate		conc. $H_2SO_4 \rightarrow C_5H_4O_2 + 3\ H_2O$ 2. $C_5H_4O_2$ (furfural) + 2 $C_{10}H_8$-OH (α-naphthol) → colored product	
	Barfoed's reagent Copper acetate Acetic acid	198 ml of distilled water and 2 ml of glacial acetic acid are added to 13.3 gm of cupric acid. 1ml of this	Dickson, 1998.
		mixture is added to each recipe and mixed. Then, the solution is heated on a water bath. A positive test is red precipitate (monosaccharide).	
	Seliwanoff' s test Resorcinol HCl	A small portion of resorcinol crystal is added to 1ml of each recipe solution in the test tube	Dickson, 1998.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Carbohydrate		and mixed. Then, 1 ml of conc. HCl is added and the solution heated on a water bath for 5 minutes. A positive test is orange-red color (ketone hexose).	
Cardiac glycosides	Liebermann-Burchard	0.5 gm of each recipe was dissolved in 10ml of methanol. Then, the solution was heated on the water bath for 5 minutes. After that the solution was filtered and the solvent evaporated by evaporator to give the residue. This residue was dissolved with 2 ml of acetic anhydride and mixed with 1ml of conc. sulfuric acid. A	Harborne, 1998.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Cardiac glycosides		positive test is the brownish-red ring between two layers of acetic anhydride and sulfuric acid.	
	Keller-Kelliani's	Each recipe was extracted with 3ml of chloroform. Then, 3ml of $FeCl_3$ reagent (0.3 ml of 10% $FeCl_3$ in 50 ml glacial acetic acid) was added and mixed in a test tube. The test tube was placed upright for a moment. Next, the test tube was laid at an angle of 45 degrees and 5-6 drops of sulfuric acid were added to the tube. A positive	Onwukaeme *et al.*, 2007.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Cardiac glycosides		test is brown, red or green color between two layers of reagents which diffused to glacial acetic acid layer.	
Carotenoid	-Concentrated sulfuric acid. -Antimony III chloride	A: Carotenoid extraction Each recipe was extracted with ethanol. The ethanolic extraction was collected. Then, the ethanolic extraction of each recipe was extracted with ether in separator funnels, and the ether layer was collected. Ether was evaporated at room temperature and residue was found. B: Chemical test 1. The extraction in A	Harborne, 1998.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Carotenoid		was dissolved with chloroform. Then, conc. H_2SO_4 was added to the solution. A positive test is blue or greenish-blue in color. 2. The extraction in A was dissolved with chloroform. Then, absolute antimony III chloride was added to the solution. A positive test is dark blue –red in color.	
Lactone glycosides (Coumarins)	20% NaOH	Distilled water is added to 2 gm of each recipe in a round-bottom flask until humid, and then is covered with filter paper soaked with 20%	Trease and Evans, 1989.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Lactone glycosides (Coumarins)		NaOH. Then, the solutions are heated on a water bath for 5 minutes. After that, the filter paper is placed under UV long wave. The positive result shows fluorescence on paper (volatile lactone glycoside). Then, the residue in the round-bottom flask is mixed with 5-10 ml of 95% ethanol and warmed on a water bath for 15 minutes. The solution is filtrated and evaporated it until give the concentrated solution. This solution is spotted to filter paper soaked with	

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Lactone glycosides (Coumarins)		20% NaOH.After that, the filter paper is placed under UV long wave. A positive test is fluorescence on paper (non volatile lactone glycoside).	
Flavonoid glycosides	-Magnesium ribbon -Concentrated HCl -Zinc dust -2 N HCl	-2 ml methanolic extraction of each recipe was divided to 1 ml/test tube for 2 tubes.Tube no. 1: 0.1 gm of Mg ribbon + conc. HCl 1 ml(Result = positive color: Orange) Tube no. 2: 0.5 gm of Zn dust + 2 N HCl (2 drops) (Result = positive color: Red) -Benzo-γ-pyrone nucleus was determined as	Harborne, 1998; Onwukaeme *et al*., 2007.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Flavonoid glycosides		following: Mg ribbon was added to a methanolic extraction from each recipe. Then, these were heated and added with conc. HCl. (Result = positive color as belows:-Reddish-orange (flavone) - Red-crimson (flavonol) -Crimson-purplish-red (flavanone) - Red (Flavanonol) Negative color: Yellowish-green	
Lipid	Sudan IV test	Add 2 ml of each sample, 2 ml of water and 1 drop of Sudan IV. Agitate each tube. A positive test is	Trease and Evans, 1989.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Lipid		floating red droplets or a floating red layer colored by Sudan IV. 500 mg of each recipe was added with 10 ml of boiling water and this mixture was placed aside to cool.	
Saponin	Froth test	Then, it was shaken for 10 seconds and 1-2 drops of 2N HCl were added. A positive test is observed if there is foam on the mixture.	Onwukaeme *et al*., 2007.
Tannin	-0.5% Gelatin solution -Ferric chloride T.S.	1.Preparation of extraction 100 ml of distilled water was added to 5 gm of each recipe and heated until boiling. The solution was filtrated and collected for	Trease and Evans, 1989; Harborne, J.B., 1998.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	Reagent	Method	References
Tannin	-1% lead acetate solution -10% Sodium chloride solution -1% Quinine	further tests. 2. Tannin determination Divide the extraction from 1 to each tube for 1 ml and adjusted to 6 ml of each tube by using distilled water. Add with 2-10 drops of the test reagent as follows: 0.5% Gelatin solution, 1% lead	Onwukaeme, D.N. *et al*., 2007;
	sulfate solution -Formalin-HCl reagent -Vanillin reagent	acetate solution, 1% Quinine sulfate solution, Ferric chloride T.S. A positive test is the dark blue color and/or precipitate. 3.Tannin determination (tannin assembly type) Vanillin reagent: Divide	

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	**Reagent**	**Method**	**References**
Tannin	-Concentrated-Hydrochloric acid -Tannic acid	the extraction from 1 to evaporating dish for 10 ml and evaporate on water bath until dry. Add 1 ml of vanillin reagent and 1 drop of conc. • HCl. A positive test is crimson color. Formalin–HCl reagent: Divided the • extraction from 1 for 2 ml and added with 3 drops of 40% formalin and 6 drops of 10% HCl. Then, heated them on water bath for 1-2 minutes. A positive test is red	

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	Reagent	Method	References
Tannin		color of precipitate.	
Xanthone	5% Potassium hydroxide - Cyanidin - Mg ribbon - H_2SO_4	A: Xanthone extraction Each recipe was extracted with ethanol and heated on boiling water for 5 min.The extraction was then filtrated. B: Chemical test 5% KOH was dropped to the extraction in A The result is positive color: yellow 1. .Ethanol was evaporated from the extraction in A by using boiling water until it gave dried residue. This residue was dissolved with	Harborne, 1998.

Table 3.2 The reagents and methods for the phytochemical test (continued)

Chemical Compound group	Reagent	Method	References
Xanthone		methanol. Then, Mg ribbon was added to this solution and heated. Then, conc. H_2SO_4 was added to the heated solution. A positive test is pink color.	

3.2.3 Preparation of the crude extract

1) Extract preparation

The extracts of each recipe was performed according to the instruction in the recipe. The ground plant materials (approximately 20 gm of each plant) were mixed and soaked in 700 ml of distilled water for 1 hour and then boiled for 1 hour. The extractions were filtered and evaporated under reduced pressure in a rotary evaporator (Buchi, Italy, R-200) at 50 ºC and lyophilized by a freeze-dryer (Christ, Germany). The percentage yield of the recipes was calculated. The freeze-dried extracts were collected and stored in small glass bottles for further evaluation. The diagram of the extraction was shown in Figure 3.2.

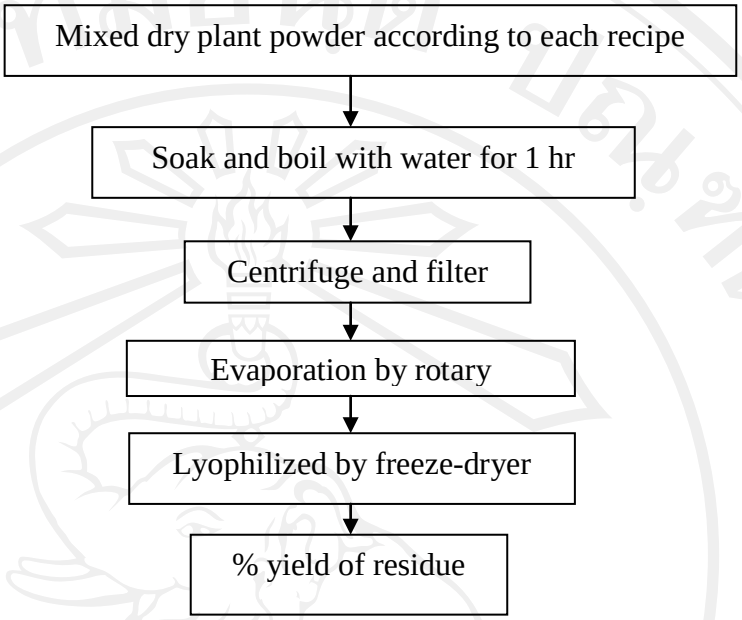

Figure 3.2 Diagram of the preparation of the crude extract of the recipes

The percentage yield of the crude extract was described as follows:

$$\text{Percentage Yield} = \frac{\text{Extract weigh/g}}{\text{Plant recipe weigh/g}} \times 100\%$$

2) Dose calculation of the recipes

Two anti-inflammatory, which were the rat hind paw edema and rat ear edema method were performed. The doses of all recipes used in both experiments were calculated as follows:

2.1 The doses of the recipes for the experiments were on rat hind paw edema (Table 3.3) were calculated based on the recommended dose given in the Lanna medicinal recipes. Taken into consideration, the route of administration, which is orally, recommended dose in humans confer the maximum volume of drug for oral feed in rats (1 ml). The calculation of a dose for oral feed in rats is as follows: (Appendix A)

$$\text{Volume for oral feed (ml)} = \frac{\text{weight of a rat (g) x recommended dose (mg/g)}}{\text{Concentration of drug preparation (mg/ml)}}$$

The positive control was prednisolone acetate with the dose in the range of 1-4 mg/kg/day (Lacy *et al*., 1999) when the weight of rats was approximately 250 mg each.

Five different doses of double concentration of each recipe were investigated. The first group of rats used as the primary dose calculation and the doses increased 2 times in the following groups. The doses each recipes were shown in Table 3.3. For the negative control group, 1 ml of distilled water was used while the positive control group, various pre-calculated concentrations of prednisolone acetate at 1.5, 2 and 4 mg/kg were used (Agarwal and Rangari, 2003).

Table 3.3 The doses of the recipe extracts for the rat hind paw edema assay

Recipe no.	**Doses (mg/kg BW)**				
	G1 mg/kg b.w.	G2 mg/kg b.w.	G3 mg/kg b.w.	G4 mg/kg b.w.	G5 mg/kg b.w.
25	1.97	3.94	7.88	15.76	31.52
105	2.59	5.18	10.38	20.76	41.52
192	1.33	2.66	5.32	10.64	21.28
346	1.62	3.24	6.48	12.96	25.92
717	2.03	4.06	8.12	16.24	32.48
895	1.67	3.34	6.68	13.36	26.72
896	1.87	3.74	7.48	14.96	29.92

Note: Please see the dose calculation in the Appendix A

2.2 The doses in all recipes for the rat ear edema method were recommended from the Thai Lanna medicinal recipes. The first group of rats used as the primary dose and the doses increased 2 times in the following group. Ethyl phenylpropiolate (EPP) was used to induce rat ear edema. For the control groups, the negative control group was water and the positive control was phenylbutazone (1 mg/20 µL/ear) (Barik *et al*., 1976). The doses of each recipe were lower, equal and higher than the standard drug as shown in Table 3.4.

Table 3.4 The doses of the recipes extract for the rat ear edema assay

Recipe No.	**Doses (mg/20 µL/ear)**		
	G3 mg/20 µL/ear	G4 mg/20 µL/ear	G5 mg/20 µL/ear
25	0.5	1.0	2.0
346	0.5	1.0	2.0
717	0.5	1.0	2.0

3.2.4 *In vivo* anti-inflammatory tests

The male Spraque Dawley rats were purchased from National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand, weight between 180 - 250 gm for the hind paw edema assay and 50 - 70 gm for the ear edema assay. The female rats were excluded from the test with regard to the inflammatory action of serotonin mediator (Carlsson *et al.*, 1985). The animals had free access to food and water

and were kept on a 12/12 hour light/dark cycle. Groups of four animals were used in each test group of hind paw edema test. For the ear edema tests, groups of four animals were used in each test group. The negative control animals of both the hind paw edema test and ear edema test received vehicle only.

For the anti-inflammatory test in animals, the recipe extracts which were indicated for topical used were tested both in rat hind paw edema and rat ear edema. The test was compared with the positive control using the standard anti-inflammatory drugs, prednisolone acetate for oral administration and phenylbutazone for topical administration. The percentage inhibition of inflammation of the extract and the reference drug was calculated using the following equation (Palanichamy and Nagarajan, 1990):

Percentage inhibition of inflammation = (A-B)/A) x100

Where A was the average degree of inflammation of the control and B was the average degree of inflammation of the extract.

A. Hind paw edema assay

Carrageenan – induced rat paw edema assay was used. A carrageenan solution (conc.1% w/v) 0.1 ml was injected into the right hind paw of each rat at the sub plantar region (Winter et al., 1962). The male rats with the weight ranging from 180–250 gm and the age of 6 weeks were used in the experiment. The animals were divided into 9 groups. Group 1 (control) was given distilled water. Group 2 was given orally a standard drug (prednisolone acetate). Groups 3 – 9 (test groups) were given orally various doses of the extracts. Paw volume was determined by means of a volume displacement

technique using a plethysmometer (Model 7150, Ugo Basile, Modena, Italy). The right hind paw was immersed into measuring chamber containing 0.05 % NaCl in distilled water, exactly to an ink mark at the anatomical hair line. Each paw volume was obtained from the average of five readings. The rat hind paw volume was measured five times at each interval from 0 to 4 hours after the carrageenan injection. The test drug was given orally 30 minutes before the injection of carrageenan. The volume of the right hind paw was measured before injection and at 1, 2, and 3 hrs after induction of inflammation. The edema volume of the paw (paw volume (ml) at time x- paw volume (ml) at time 0) and the percentages of edema inhibition for each test recipe extracts versus the control group were calculated. The schematic diagram of the hind paw edema procedure was shown in Figure 3.3.

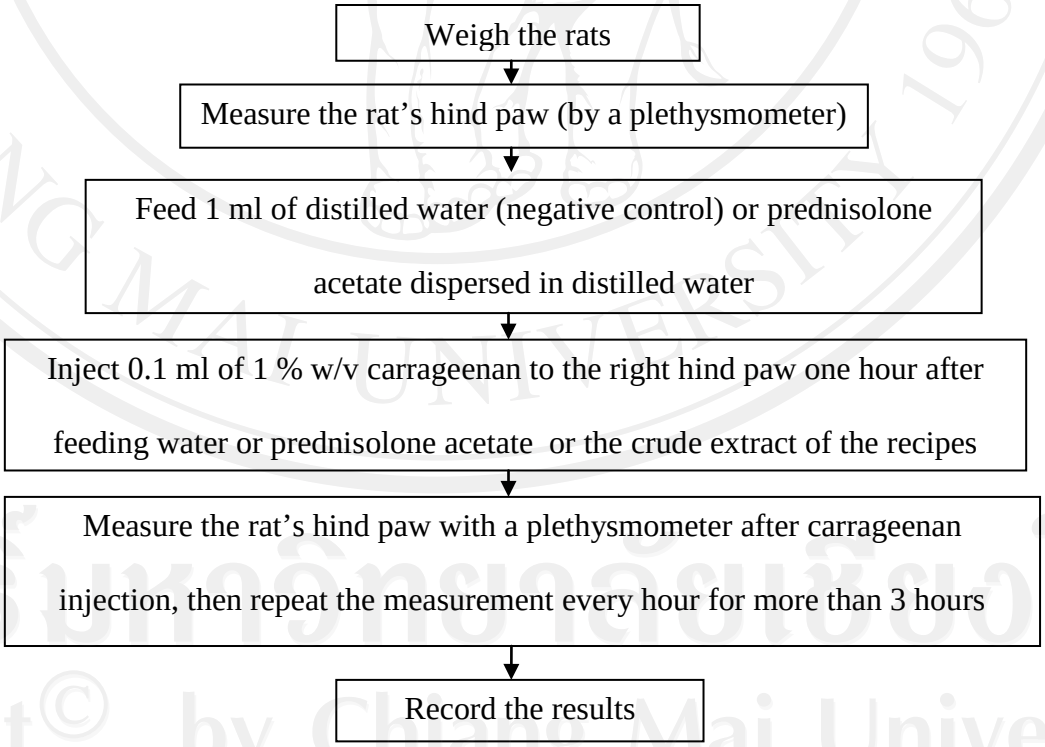

Figure 3.3 Schematic diagram of the rat hind paw edema assay

B. Ear edema assay

The three selected recipes (recipe nos. 25, 346 and 717) which indicated the topically use and gave the anti-inflammatory activity from the rat hind paw experiment were tested for anti-inflammatory by the rat ear edema method. The edema was induced by the application ethyl phenylpropiolate (EPP), (Brattsand *et al*., 1982). The EPP solution was applied to each inner and outer surfaces of both ears. A vernier caliper was used to measure the thickness of the rat's ear. The male rats with the weight of 50 gm and the age of 4 weeks were used in the experiment. The animals were divided into 5 groups. Group 1 (negative control) received distilled water and acetone. Group 2 (positive control) received the standard solution of phenylbutazone. Groups 3-5 (tested groups) received the extracts from the recipes. For ear thickness determination, a vernier caliper (Mitutoyo, Toky, Japan) was applied on the tip at inner and outer surface of the ear. Measurements were taken from 0-24 hours after EPP-induced ear edema. The schematic diagram of the ear edema assay procedure was shown in Figure 3.4.

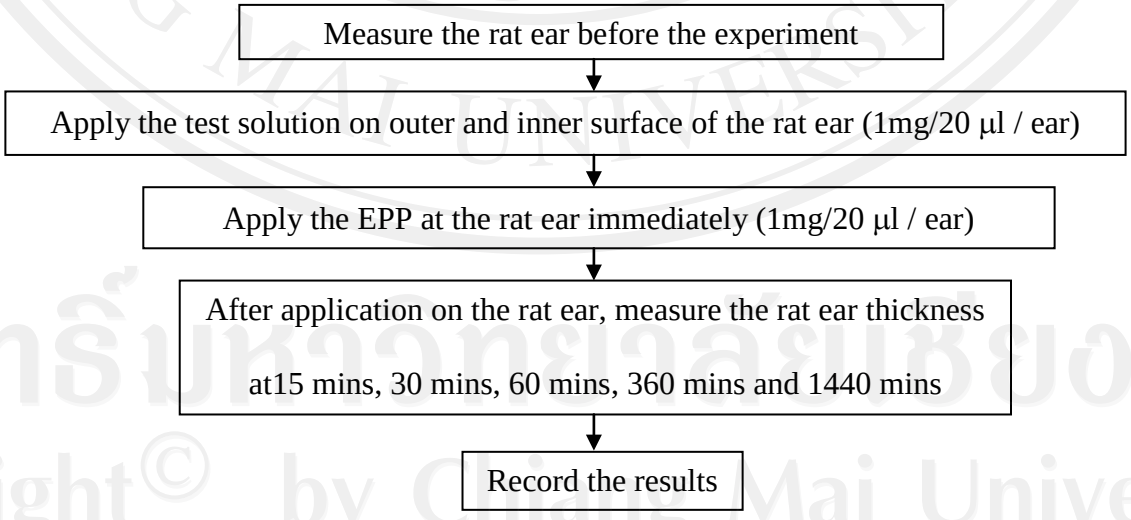

Figure 3.4 Schematic diagram of the rat ear edema assay