CHAPTER 3
MATERIALS AND METHODS

3.1 Apparatus and instruments

1. Analytical balance (Sartorius® Ac 210 S, Germany)
2. Camera lucida (Olympus®, Japan)
3. Carbolite oven (Carbolite®CWF 1100, England)
4. Cellulose membrane (Sigma®, USA)
5. Differential Scanning Calorimeter (Perkin Elmer® SSC7, USA)
6. Franz static diffusion apparatus (PermeGear®, India)
7. Heating mantle (Thermo scientific®, UK)
8. Hot air oven (Beschickung-loading® Model 100-800, USA)
9. Mechanical pipette (Vipro® 10-100/100-1000 µL, Malaysia)
10. Microplate 96 well (Nune®, USA)
11. Microscope (Olympus® CH-2, Japan)
12. Moisture Analyzer (Sartorius® MA50, Germany)
13. Microtiter plate reader (Beckman Coulter® DTX880, Australia)
14. pH meter (Eutech® pH 510, Singapore)
15. Orbit shaker (Junior orbit shaker® Labline Inc., India)
16. Rheometer Plate&Plate Type (Brookfield® , England)
17. Rotary vacuum evaporator (Eyela® Japan)
18. UV-VIS Spectrophotometer (Shimadzu® UV-2450, Japan)
19. TLC silica gel 60 F254 plate (Merck®, Germany)
20. Ultrasonic bath (Powersonic®, Malaysia)
21. UV chromatographic viewer (UVP®, USA)
22. Vortex mixer (Scienctific®, USA)

3.2 Chemicals

1. 1,10-phenanthroline (Merck, Germany)
2. 2,2'-Azino-bis(3-ethylbenzo-acid)diammonium salt (Sigma, Germany)
3. Butylated hydroxyl toluene (O.V. Chemical and supply, Thailand)
4. Cabopol Ultrez 21 (O.V. Chemical and supply, Thailand)
5. Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (Sigma, Germany)
6. Disodium hydrogen orthophosphate anhydrous; Na_2HPO_4 (Ajax Finechem, Australia)
7. Ethylenediaminetetraacetic acid disodium salt dihydrate; $C_{10}H_{14}N_2Na_2O_8.2H_2O$ (Rankem, India)
8. Ethanol 95% (O.V. Chemical and supply, Thailand)
9. Ferrous sulfate anhydrous; $FeSO_4$ (Sigma, Germany)
10. Folin-Ciocalteu reagent (Merck, Germany)
11. Gallic acid (Sigma, Germany)
12. Hydrogen peroxide; H_2O_2 (Merck, Germany)
13. Isopropyl myristate (O.V. Chemical and supply, Thailand)
14. Kaempferol (Fluka, Germany)
15. Trolox (Sigma, Germany)
16. Lutrol® F127 (BASF, Germany)
17. Menthol (O.V. Chemical and supply, Thailand)
18. Potassium persulfate; $K_2S_3O_8$ (Sigma, Germany)
19. Propylene glycol (O.V. Chemical and supply, Thailand)
20. Quercetin (Sigma, Germany)
21. Sodium carbonate; Na_2CO_3 (Ajax Finechem, Australia)
22. Sodium dihydrogen orthophosphate; $NaH_2PO_4.2H_2O$ (Ajax Finechem, Australia)
23. *T. catappa* Linn. red leaves (Thailand)
24. Ursolic acid (Fluka, Germany)

3.3 Methods

3.3.1 Plant materials

T. catappa Linn. red leaves were collected from the Faculty of Pharmacy, Chiang Mai University during July and August, 2009. The leaves were oven-dried at 40°C for 24 hours and grounded to powder. The dried powder was sieved through a sieve No. 40 mesh and retained on sieved No. 60 mesh. The method was carried out based on Specification of Thai Medicinal Plant Vol.1, 1986.

3.3.2 Pharmacognostic study

The pharmacognostic study of *T. catappa* Linn. and the red leaf powders were examined based on Specification of Thai Medicinal Plant Vol.1, 1986 and Thai Pharmacopoeia Vol 1, 1987.

3.3.2.1 Identification of plants

(1) Macroscopic characteristic study

The plant specimen was collected from the Faculty of Pharmacy, Chiang Mai University. Then, the specimen was dried at 50 °C for a week. It was then fixed on the paper and covered with methylene chloride. The specimen was stored at the Herbarium in the Faculty of Pharmacy, Chiang Mai University.

(2) Microscopic characteristic study

The dried red leaves were powdered and passed through a sieve of No. 60 mesh and used to observe microscopic characters and the leaf constants were calculated (56, 57).

A. Microscopic character of the plant powders

The components of the leaf were stained with suitable solution and observed under 40X magnification as following:

Distilled water: To observe starch, crystal, parenchyma and other cells.

Chloral hydrate: To remove pigment, starch and aleurone grain.

Phloroglucinol: To stain cells with lignin and appear red pink color.

B. Leaf constant

The 30 leaves of *T. catappa* Linn. were randomly collected. The 4×4 millimeters piece was cut from the region between the leaf margin and midrib. One piece from one leaf was then cleared with 4 g of chloral hydrate in 1 mL of water. The sample was observed under optical microscope and calculated leaf constant after washed in 20% glycerin.

Stomatal number is an average number of stomatal among 1 mm^2 of epidermis.

$$\text{Stomatal number} = \frac{\text{Number of stomatal}}{\text{Area of epidermis}} \qquad (1)$$

Stomatal index

$$\text{Stomatal index} = \frac{\text{Number of stomatal}}{\text{Number of epidermis cells+Number of stomata}} \qquad (2)$$

Palisade ratio is an average number of palisade cells under 1 epidermis cell.

$$\text{Palisade ratio} = \frac{\text{Number of palisade cells}}{4} \qquad (3)$$

Vein-islet number *is* a ratio of vein-islet to 4 mm^2.

$$\text{Vein} - \text{islet number} = \frac{\text{vein} - \text{islet}}{4} \qquad (4)$$

Veinlet-termination number is a ratio of veinlet-terminal number to 4 mm^2

$$\text{Veinlet termination number} = \frac{\text{veinlet termination}}{4} \qquad (5)$$

C. Phytochemicals test

The plant powder was boiled in distilled water and the extract was then filtered. The filtrated was collected and used in screening test.

a) Tannins

Tannins screening test, 2 mL of the filtrate was tested by tannin testing solution about 2-10 drops. The reaction was then observed.

For testing with Vanillin-HCl reagent, the filtrate was evaporated on evaporating disk. Then it was dropped with the reagent and observed.

For testing with Formalin-HCl reagent, 4 mL of the filtrate was added by 3 drops of 40% formalin and 6 drops of 10% HCl. Then it was boiled in water bath for 1-2 minutes and observed on changing in color.

Positive result is to precipitate with 0.5% gelatin solution, 1% lead acetate, 1% quinine sulfate solution, blue-green precipitate with ferric chloride T.S., crimpson color with vanillin-HCl and red-orange color with formalin-HCl.

Whereas 0.5% gelatin solution, 1% lead acetate, 1% quinine sulfate solution and ferric chloride T.S. tested for hydrolysable tannins. Vanillin-HCl and formalin-HCl tested for condensed tannins.

b) Flavonoids

About 10 mL of the filtrate was mixed with 2-3 drops of HCl and magnesium ribbon. The changing in color was observed.

Positive result is to appear red-pink color.

c) Triterpenoids

The reaction mixture contained 3 drops of acetic anhydride and 3 drops of sulfuric acid. The changing in color was observed.

Positive result is to appear blue-green color for sterols and red pink or red purple for triterpenes.

3.3.2.2 Specification of raw materials

Parameters such as moisture content, ash values (total ash and acid insoluble ash) and extractive values (ethanol extractive value, water extractive value) were determined based on the Thai Pharmacopoeia Vol.1, 1987, the Thai Herbal Pharmacopoeia Vol. 1, 1995 and Specification of Thai Medicinal Plant V.1.

(1) Moisture content by gravimetric method

The using method followed as Specification of Thai Medicinal Plant V.1. About 2-6 g of the plant powder was accurately weighed and transferred to weighing bottle. Then the sample was dried at 105±2 °C for 5 hours and allowed to cool in desiccator at room temperature. The drying process was continued until getting constant weight lower 0.0005 g of difference. The percentage of loss on drying was calculated as following:

$$\%\ \text{Loss on drying} = \frac{\text{Lost weight}}{\text{Initial weight}} \times 100 \qquad \text{6}$$

(2) Total ash

The using method followed as Thai Pharmacopoeia Vol.1, 1987 and Specification of Thai Medicinal Plant V.1. About 2.0000 g of the plant powders was transferred to constant weight porcelain crucible. The sample was then ignited at 500 °C until white ash became and weight was constant. The residue was accurately weighed and the percentage of total ash determined as follow:

$$\text{Total ash}\ (\%) = \frac{\text{Ash weight}}{\text{Plant weight}} \times 100 \qquad \text{7}$$

(3) Acid-insoluble ash

The ash from total ash determination was filled with 25 mL of 2 M HCl and then boiled for 5 minutes. The sample was filtered through ashless filter paper and rinsed by 25 mL of hot water. The filtered paper with residue was transferred to porcelain crucible and ignited at 500 °C until white ash became and weight was

constant. The residue was accurately weighed and the percentage of acid insoluble ash was determined as follow:

$$\text{Acid} - \text{insoluble ash (\%)} = \frac{\text{Ash weight}}{\text{Plant weight}} \times 100 \qquad \text{——————} \quad 8$$

(4) Extractive value

a) Ethanol extractive value

About 5 g of the plant powders was transferred to iodine flask 250 mL and added with 100 mL of 95% ethanol. The sample was shaken for 6 hours and allowed to stand for 18 hours. The extract was then filtered and kept for 20 mL to be evaporated until dry. The 95% ethanol extractive value was calculated as follow:

$$\text{Extractive values (\%)} = \frac{\text{Dried residue as 100 mL of the extract}}{5} \times 100 \quad \text{——} \quad 9$$

b) Water extractive value

The procedure was the same as ethanol extractive value. The solvent was distilled water.

3.3.3 Plant extraction

The powder was extracted by 95% ethanol using a soxhlet apparatus for 40 hours. The extract was filtered and the filtrate was then evaporated until it was dry.

3.3.4 TLC chromatogram determination (58, 59)

The TLC conditions of Saric and colleagues, 2004 were tried as shown in the Table 3.1. The selected condition was chosen for developing TLC.

Table 3.1 TLC conditions used in preliminaly chromatographic determination

System	Hexane	Petroleum ether	Carbon tetrachloride	Toluene	Chloroform	Acetone	Ethyl acetate	Methanol	Formic acid	Acetic acid
1				36			12		5	
2	30						15		5	
3	31						14		5	
4				36			12			5
5				38		10			5	
6	31						14			5
7	40						40	20		
8	80				40			20		

3.3.5 Anti-inflammatory activity study and determination of antioxidant activity

3.3.5.1 Anti-inflammatory study

(1) Anti-inflammatory screening test on cyclooxygenase 1 and cyclooxygenase 2 enzymes inhibition

The anti-inflammatory screening test on COX-1 and COX-2 enzymes inhibition was examined by National Science and Technology Development Agency (NSTDA).

(2) Ethyl phenylpropiolate (EPP)-induced ear edema in rat (60)

EPP-induced ear edema in rats is the widely used model for evaluation the anti-inflammatory effect of test compounds. The edema formation induced by EPP is caused by the release of inflammatory mediators and increase of vascular permeability, which is a primary step of acute inflammation.

The study was carried out following the method described by Brattsand *et*

al., 1982. Male Sprague-Dawley rats weighing 40-60 g were used. They were divided into 6 groups of 3 animals (6 ears per group). Phenylbutazone and the plant extract were dissolved in acetone and ethanol respectively.

Group 1: Ethanol

Group 2: Acetone

Group 3: Phenylbutazone 1 mg/ear

Group 4-6: *T. catappa* Linn. red leaf extract at the dose of 0.5, 1 and 3 mg/ear, respectively.

Rat ears were applied topically on the inner and outer surfaces with either vehicle (ethanol or acetone), a standard drug (phenylbutazone), or the plant extract at the volume of 20µL/ear. Immediately after application of test drugs, ear edema was induced by topical application with EPP 1 mg/ear. Ear thickness was measured by vernier calipers before and at 15, 30, 60 and 120 minutes after EPP application. The ear edema volume and the percent edema inhibition of the test drugs were calculated and recorded.

3.3.5.2 Total phenolic contents (61)

The Total phenolic contents were determined using a modified Folin-Ciocalteu method. A sample was diluted in ethanol with an accurate concentration, then 0.5 mL of sample solution was mixed with 5 mL Folin-Ciocalteu reagent solution (1:10 mL of distilled water) and 4 mL of 7.5% Na_2CO_3 was added. The mixture was stored in the dark for 30 minutes before the absorbance was measured at 765 nm using a UV-VIS spectrophotometer (Shimadzu UV-2450). Gallic acid was used as standard and prepared at the concentration of 25, 50, 75, 100, 125 and 150 mg/L. Total phenolic contents are expressed as gallic acid equivalent (GAE) in milligram per gram of dry sample.

3.3.5.3 Determination of antioxidant activity

(1) Hydroxyl radical (OH^-) scavenging activity (62)

Hydroxyl radical scavenging activity of the extract was measured by the method of Bai *et al*., 2011 with slightly modification. The reaction was mixed with 400 µL of sample solution, 250 µL of 10 mM $FeSO_4$, 250 µL of 10 mM 1, 10-

phenanthroline, 400 µL of 0.15% H_2O_2 and 3000 µL of phosphate buffer pH 7.2. The mixture was then incubated at 37°C for 1 hour. After that the absorbance was measured at 536 nm using UV-VIS spectrophotometer before calculation by the following equation.

$$\% \text{ Hydroxyl radical scavenging activity} = \frac{(A_S - A_1)}{(A_0 - A_1)} \times 100 \quad \text{——— 10}$$

Where A_0, A_s and A_1 are absorbance of blank solution, sample and control, respectively.

(2) 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (28)

The DPPH radical scavenging activity method of Povichit *et al*., 2010 was slightly modified for this study. The sample was mixed with 180 µL of DPPH in 95% ethanol and incubated for 30 minutes. The absorbance was measured at 520 nm using a multimode detector (Beckman Coulter DTX880). The percent inhibition of the DPPH radical was calculated according to equation 11. Trolox was used as a standard.

$$\% \text{ Inhibition} = \frac{(P-N)-(S-B)}{(P-N)} \times 100 \quad \text{——————————— 11}$$

Where P is absorbance of positive control; N for negative control; S for sample or standard; and B for blank of sample or standard.

(3) 2,2'-Azino-*bis* (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radical scavenging assay (63)

ABTS assay was carried out according to the method of Re *et al*., 1999 with slightly modification. The working solution was prepared as following: 7 mM ABTS solution was prepared in DI water and mixed to 2.45 mM $K_2S_2O_8$. The ratio of ABTS solution and $K_2S_2O_8$ is 1:1.4 mL. The mixture was allowed to stand in the dark at room temperature for 24 hours to generate the radical cation ($ABTS^{\bullet+}$). The

absorbance measurement, $ABTS^{\bullet+}$ solution was diluted with DI water to obtain an initial absorbance of 0.7-0.8 at 734 nm using the UV-VIS spectrophotometer. Samples and standard were reacted with the $ABTS^{\bullet+}$ solution for 1 hour and measured by UV-VIS spectrophotometer at 734 nm. Trolox was used as a standard. The percentage of inhibition was calculated (Equation 1).

The results were expressed as trolox equivalent antioxidant capacity (TEAC) in mg trolox per gram sample in dried form.

$$\% \text{ Inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad 12$$

Where A_0 and A_1 are absorbance of control and sample respectively.

3.3.6 Preformulation study

3.3.6.1 Physicochemical properties

(1) Characteristic of the crude extract

Oder, color and physical character of the crude extract were observed by sensory organ.

(2) pH

The crude extract about 0.01 g was dissolved in 10 mL of deionized water before pH measurment.

(3) Solubility

The crude extract about 0.01 g was transferred to test tube and added with solvent including distilled water, 95% ethanol, glycerin, propylene glycol, PEG 400, phosphate buffer pH 7.4, 1% tween 20, mineral oil, isopropyl myristate and caster oil. The volume of solvent was shown in the Table 3.2. Then the sample was mixed by vortex mixer.

Table 3.2 The volume of solvent by step of solubility evaluation

Series	Volume of solvent (μL)	Series	Volume of solvent (μL)	Series	Volume of solvent (μL)
1	10	6	1000	11	1000
2	90	7	1000	12	1000
3	200	8	1000	13	1000
4	700	9	1000	14	1000
5	1000	10	1000	15	1000

Table 3.3 Values for estimating drug solubility based upon USP-NF definition

Solubility	**Solvent volumn (μL)**
Very soluble	<1
Freely soluble	1-10
Soluble	10-30
Sparingly soluble	30-100
Slightly soluble	100-1000
Very slightly soluble	1000-10000
Insoluble	>10000

(4) Chemical reaction

a) Acid reaction

0.1M of HCl solution was used to react with the extract and physical character was observed.

b) Base reaction

0.1M of NaOH solution was used to react with the extract and physical character was observed.

c) Redox reaction

2.5 μM of $KMnO_4$ solution was used as oxidizing agent and 0.1 M of $FeSO_4$ solution was used as reducing agent. The reaction was observed after mixed with the extract.

3.3.6.2 Compatibility study

Differential scaning calorimetry (DSC) was used for studying compatibility between the crude extract and gelling agents, carbopol ultrez 21 and poloxamer 407. The mixture of the extract and gelling agent was 1:1 by weight.

3.3.7 Formulation of gel containing *T. catappa* Linn. red leaf extract

Gel base preparations are shown in the Table 3.4. The crude extract was dissolved in 95% ethanol to make concentration 0.26 g/mL and incorporated in gel base as 3 %w/w. The use of enhancer was varied as following:

Table 3.4 All 7 Formulas of gel base

Ingredients	**Formula number**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Carbopol ultrez 21 (%w/w)	1	1	1	1	1	1	1
95% Ethyl acohol (%w/w)	-	20	20	20	-	20	20
Propylene glycol (%w/w)	-	-	5	5	5	5	10
Isopropyl myristate (%w/w)	-	-	-	5	-	10	5
Menthol (%w/w)	5	5	5	5	5	5	5
BHT (%w/w)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
EDTA (%w/w)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Conc. Paraben (%w/w)	1	1	1	1	1	1	1
Triethanolamine	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Distilled water q.s. to (g)	100	100	100	100	100	100	100
Last concentration of ethanol after incorporate the extract (%w/w)	8.54	26.54	26.54	26.54	8.54	26.54	26.54

Note: Conc. Paraben is the mixture of 1% of 10 % methyl paraben and 2% propyl paraben in propylene glycol.

3.3.8 Evaluation of gel containing *T. catappa* Linn. red leaf extract

Each formula was evaluated as following parameters: physical character

(odor, color, viscosity, pH and spreadability), stability, antioxidant activity and *in vitro* releasing test.

3.3.8.1 Physical properties

Odor, color, removable and spreadability of gel were observed by sensory organ. pH was measured by pH meter and universal pH paper. The viscosity was also measured by Brookfield® rheometer (plate/plate type).

3.3.8.2 Stability study

The gel bases and sample gels were kept in 45±2 °C and 4±2 °C for each of 48 hours consequently 6 cycles. Each formula was examined before and after finished the cycles. The stability was evaluated using physical character (odor, color, viscosity and pH) and total phenolic contents (for gel containing the extract).

3.3.8.3 Antioxidant of gel containing *T. catappa* Linn. red leaf extract

Hydroxyl radical scavenging activity of gel containing the extract was determined.

3.3.8.4 *In vitro* releasing test using Franz static diffusion cells (64)

The study was carried out based on the method of Nuno *et al*., 2012 with modification. Cellophane 1200 Dalton pore was used as membrane, soaking in receiver fluid (DI water pH 7.0±0.2: 95% ethanol, 60:40) for 24 hours before using. Sample gel was transferred on the donor about 0.1 g and Franz static diffusion cell was filled with the same receiver fluid and continuously agitated with magnetic stirrer. Laboratory was set as the Figure 3.1. After the experiment was started, the sample solution from receiver fluid was collected about 1 mL at 1, 2, 4, 6, 8, 12 and 24 hours and replaced by the new receiver fluid. The sample solution was determined total phenolic contents using Folin-Ciocalteu reagent.

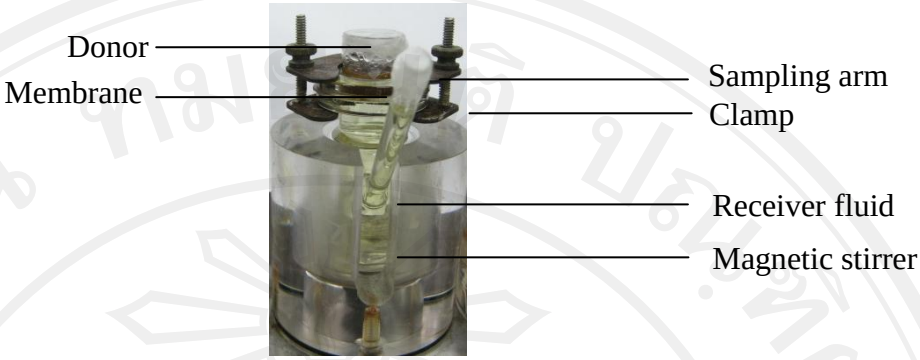

Figure 3.1 Franz static diffusion cell

3.3.8.5 Long term stability test of gel containing *T. catappa* Linn. red leaf extract at 4°C

The sample gels were storaged at 4±2 °C for 3 months. Each formulation was examined at 0, 1, 2 and 3 months. The stability was evaluated using physical character (viscosity and pH), total phenolic contents and hydroxyl radical scavenging activity. The results were compared to 0 month of each formula using paired sample T-test (Appendix B).

The results then statistic analyzed using T-test.