CHAPTER 2

Materials and Methods

2.1 Drugs, Chemicals and Instruments

The names of drugs, chemicals and instruments used in present study are shown in Appendix A. Reagent preparation methods are shown in Appendix B.

2.2 Plant material

The dry powder of *M. loriformis* was obtained from Abhaibhubejhr's hospital, Prachinburi, Thailand. The plant material was authentic and the voucher specimen (QBG.No.25135) was kept at the herbarium section of Queen Sirikit Botanical Garden.

2.3 Preparation of the ethanol extract from *M. loriformis*

The ethanol extract of *M. loriformis* was prepared as follows: *M. loriformis* (3 kg) was overnight macerated in 80% ethanol at room temperature. The extraction process was repeated 3 times. The ethanol extract was filtered through Whatman's filter paper No.4. After filtration, the filtrate obtained was pooled and concentrated under reduced pressure condition (using a rotating evaporator, 55 °C) and lyophilized. The yield of the ethanol extract was 14.1% of dry powder. The ethanol extract from *M. loriformis* was designated as ML extract.

2.4 HPLC analysis

The characterization of ML extract was carried out using an Agilent 1200 Series high performance liquid chromatography with the Zorbax SB-C18 StableBond Analytical column (4.6 x 150 mm, 5 micron) and UV-Vis Diode array as a detector (at 215 nm). ML extract was dissolved in 100% methanol at the concentration of 50 mg/mL. ML extract (10 μL) was injected into the column and used methanol: deionized (DI) water (0.05% acetic acid) as a mobile phase. The percentage of methanol was increased from

3, 3, 50, 45 and 99% at the time of 0, 1, 21, 26 and 40 min, respectively. The flow rate was 0.5 mL/min. HPLC fingerprint of ML extract is shown in Figure 2.1.

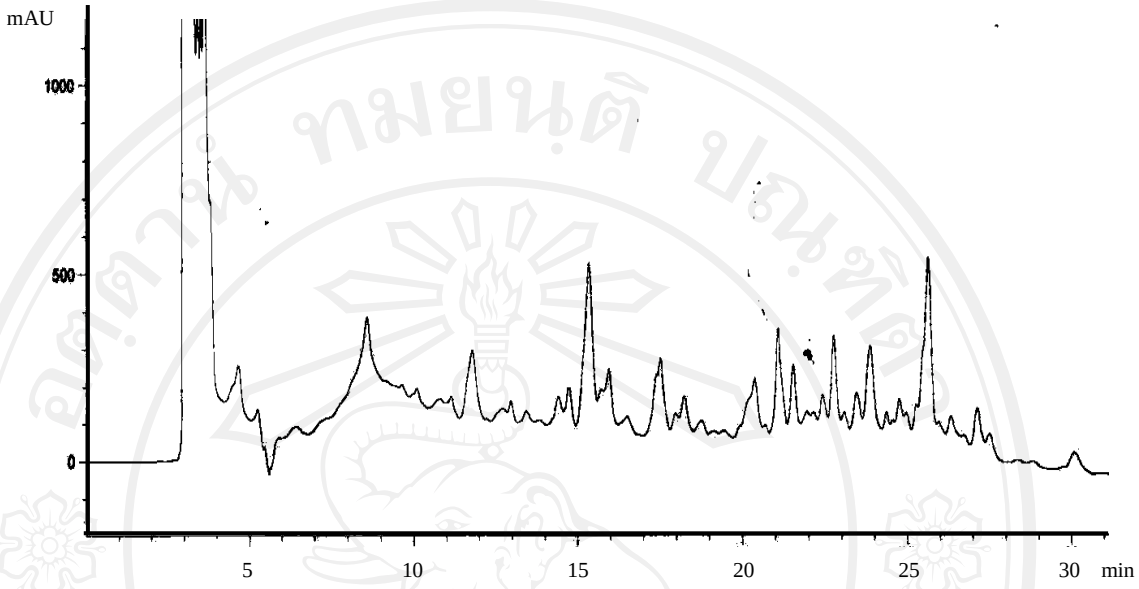


Figure 2.1 HPLC fingerprint of ML extract

2.5 Phytochemical study

Chemical constituents of ML extract were screened by standard phytochemical tests for alkaloids, tannins, and glycosides (i.e. antraquinone, cumarin, flavonol,s flavones, saponins, cardiac glycosides, steroids, terpenes, and anthocyanins) (Ayoola *et al*., 2008; Yadav and Agarwala., 2011). The diagrams of phytochemical methods are presented in Figures 2.2-2.11.

2.5.1 Test for alkaloids (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

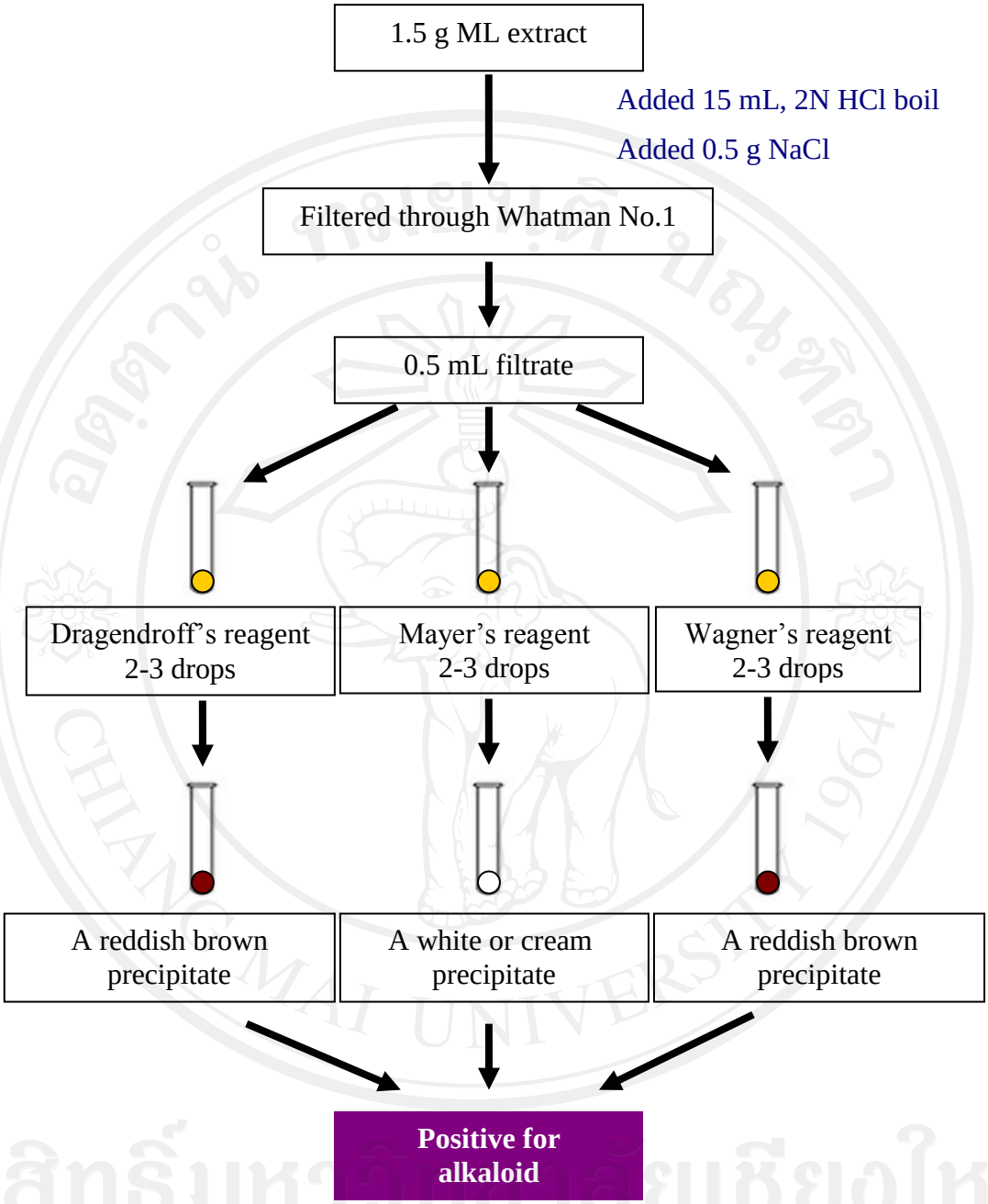


Figure 2.2 Method of alkaloids determination

2.5.2 A confirmatory test for alkaloids (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

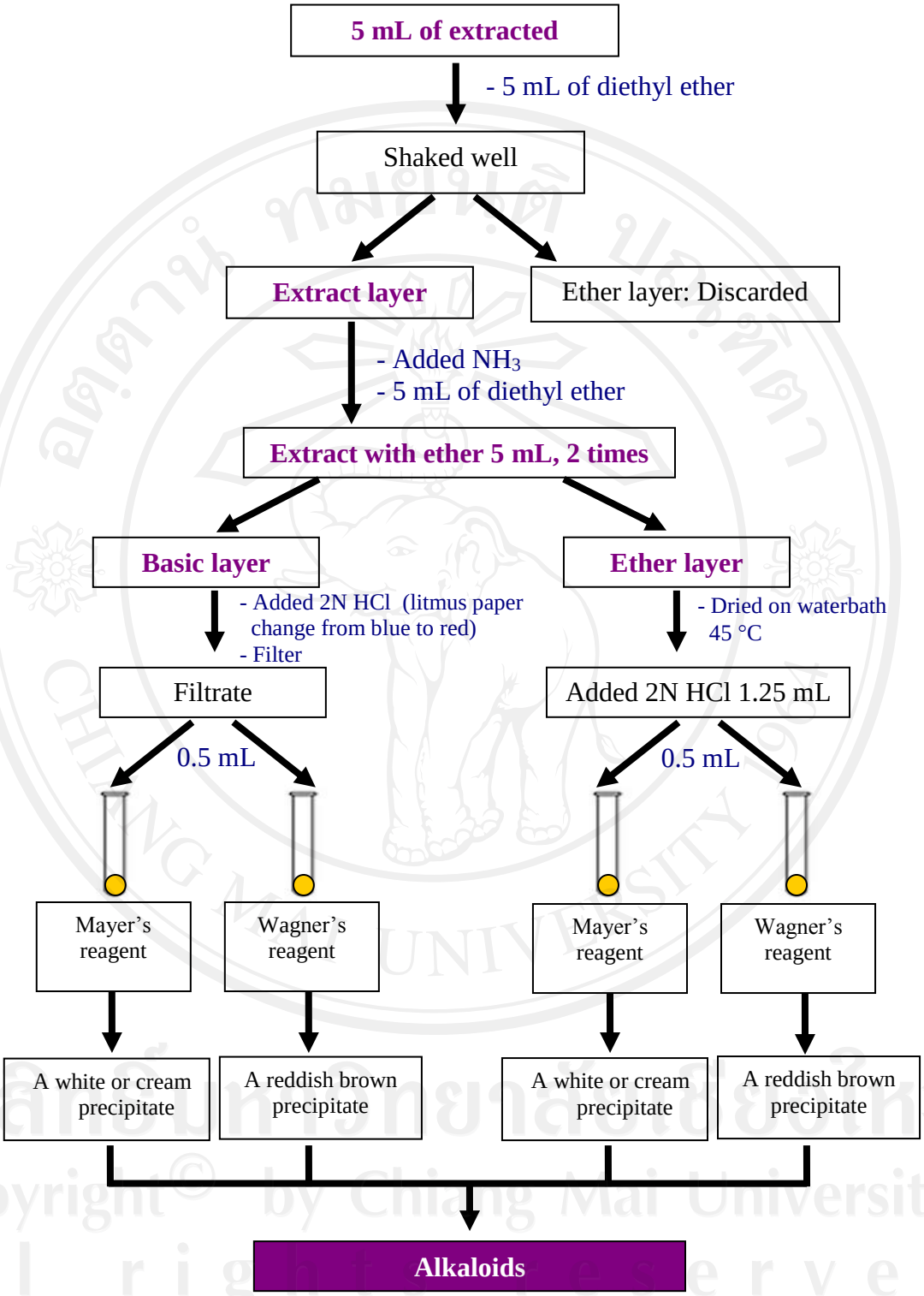


Figure 2.3 Method of alkaloids confirmation

2.5.3 Test for phenolic compounds and tannins (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

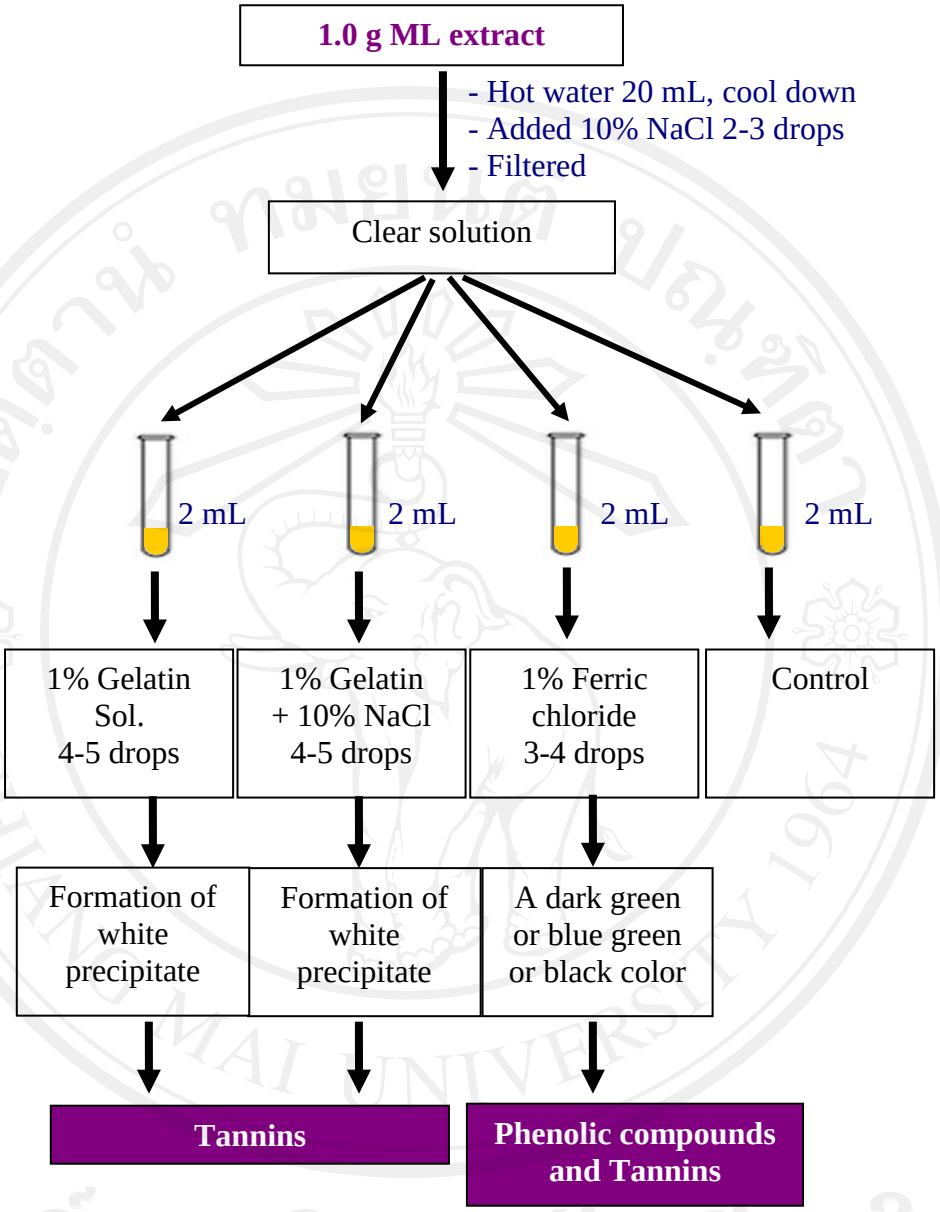


Figure 2.4 Method of phenolic compounds and tannins testing

2.5.4 Test for glycosides (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

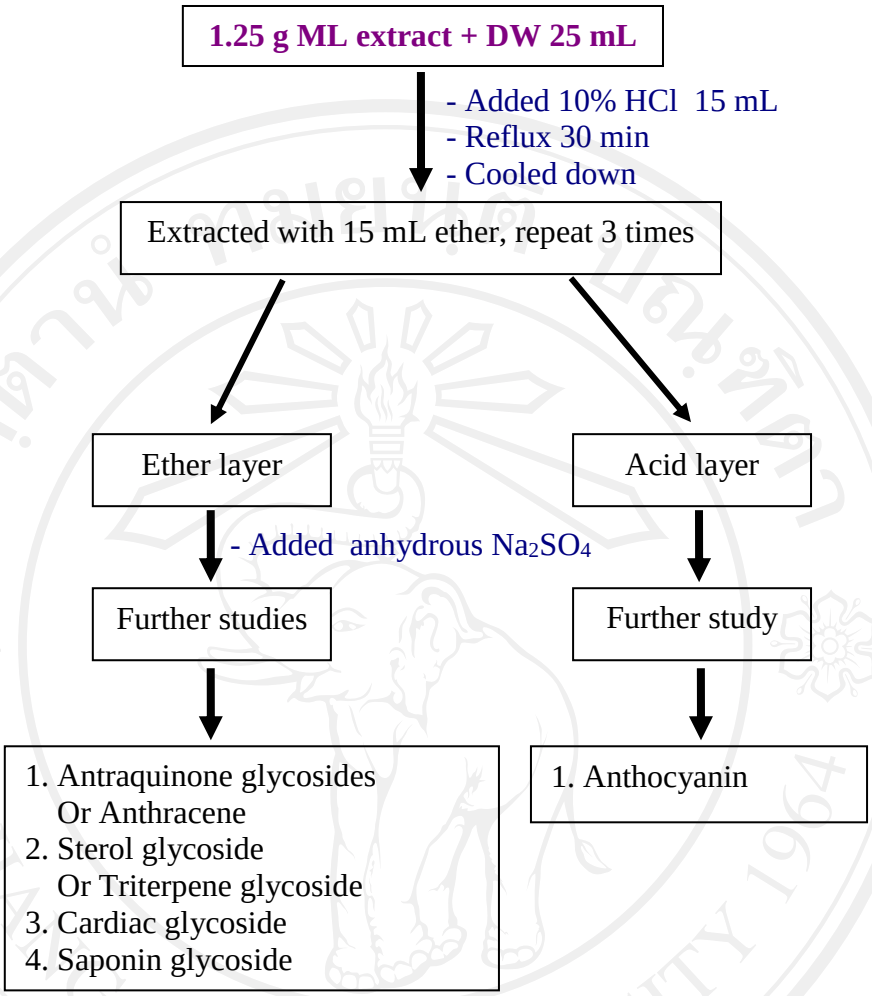


Figure 2.5 Method of glycosides determination

2.5.5 Test for anthraquinone glycoside (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

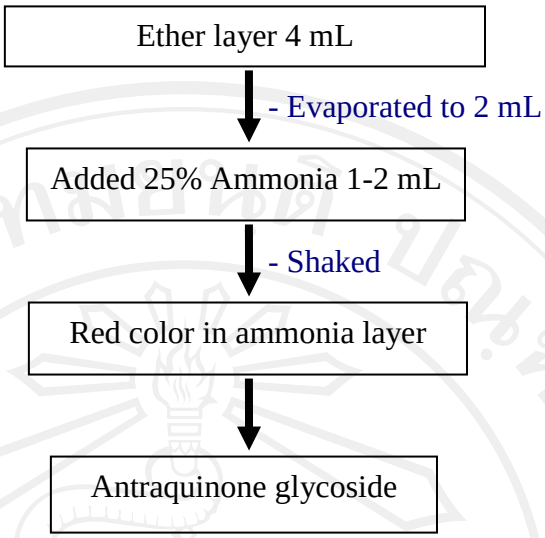


Figure 2.6 Method of antraquinone glycoside determination

2.5.6 Test for sterol glycoside or triterpene glycoside (Liebermann Burchard's reaction) (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

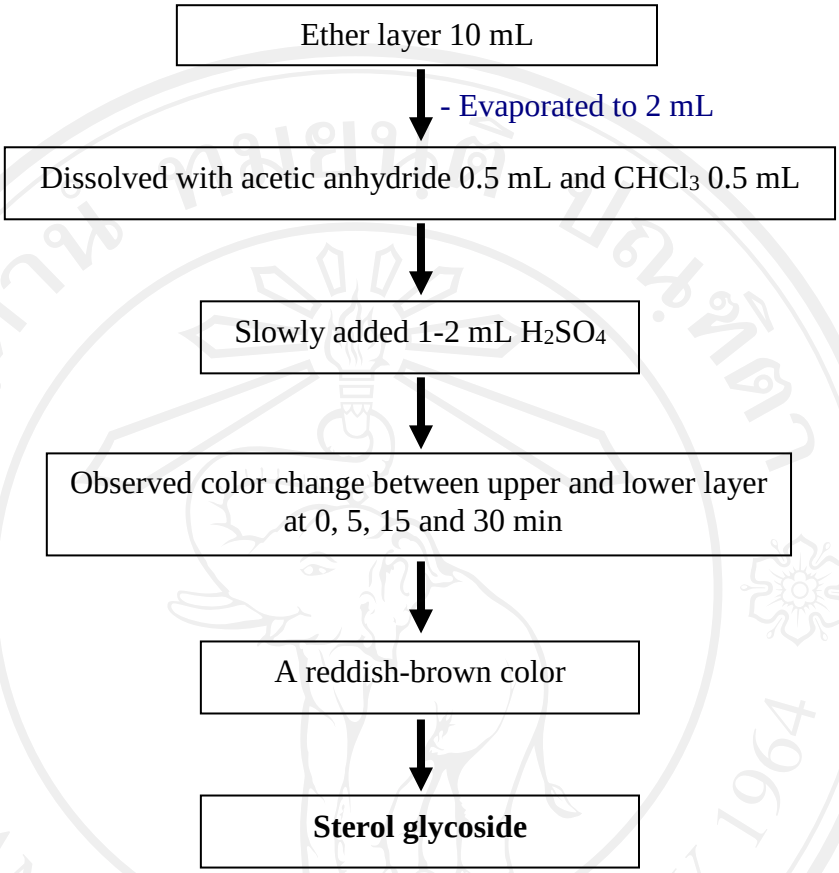


Figure 2.7 Method of sterol glycosides or triterpene glycosides determination

2.5.7 Test for cardiac glycosides (Kedde's test) (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

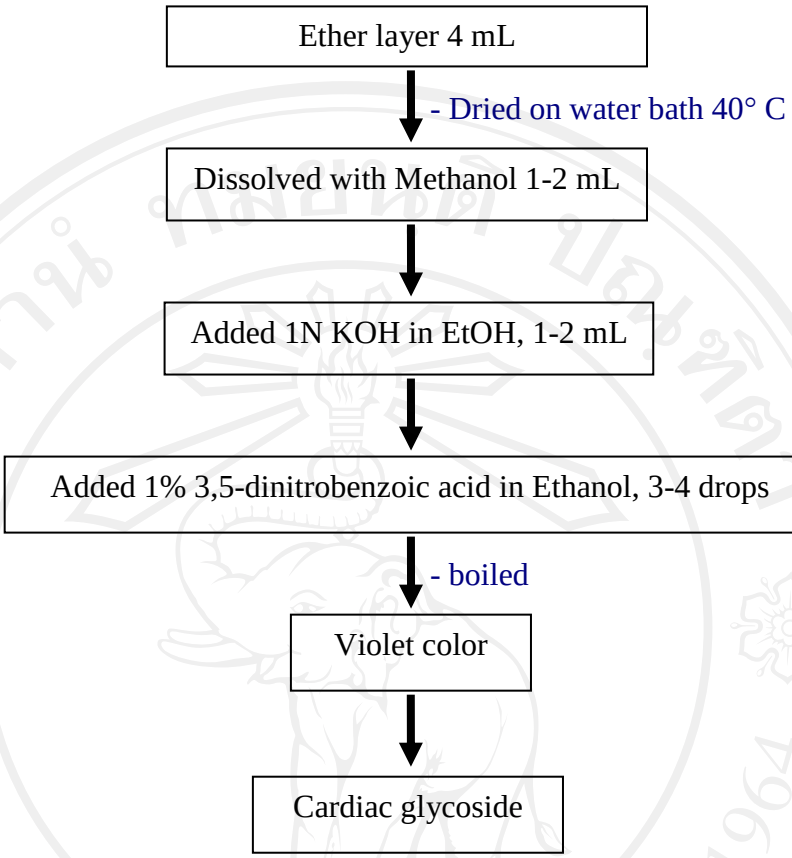


Figure 2.8 Method of cardiac glycosides determination

2.5.8 Test for saponin glycosides (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

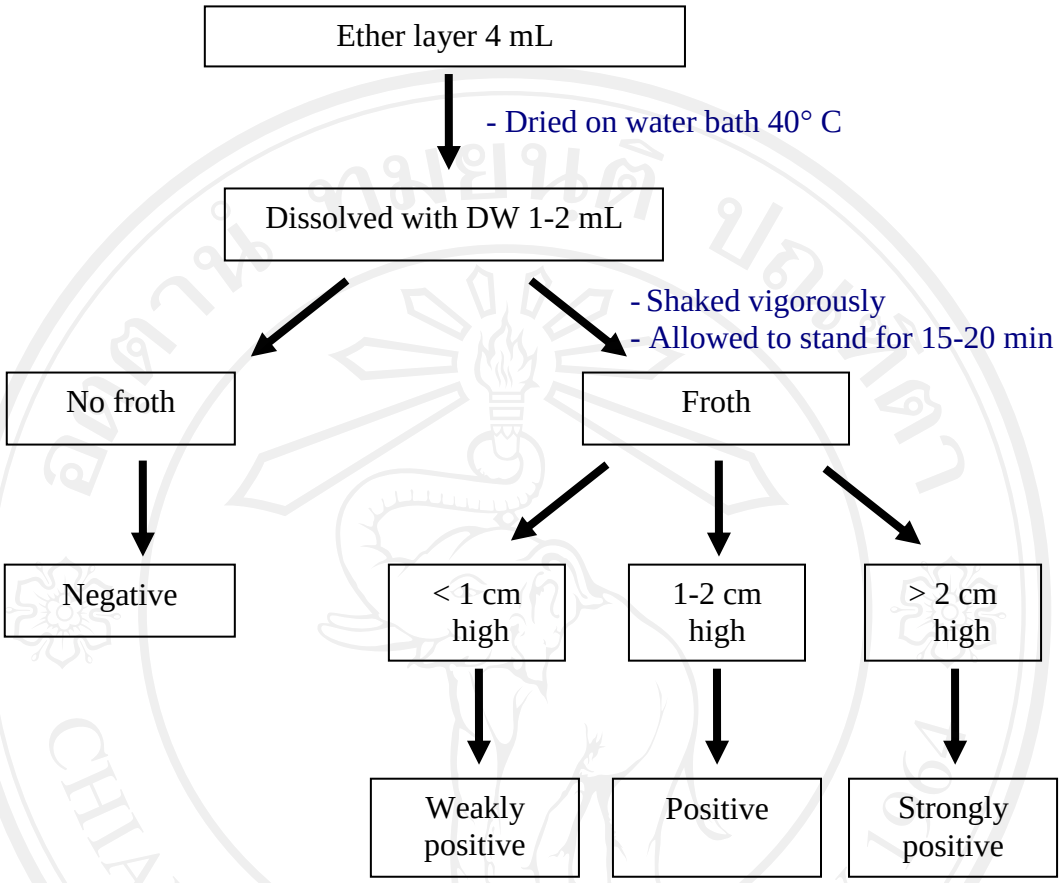


Figure 2.9 Method of saponin glycoside determination

2.5.9 Test for flavonoid glycoside (Shibata's reaction) (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

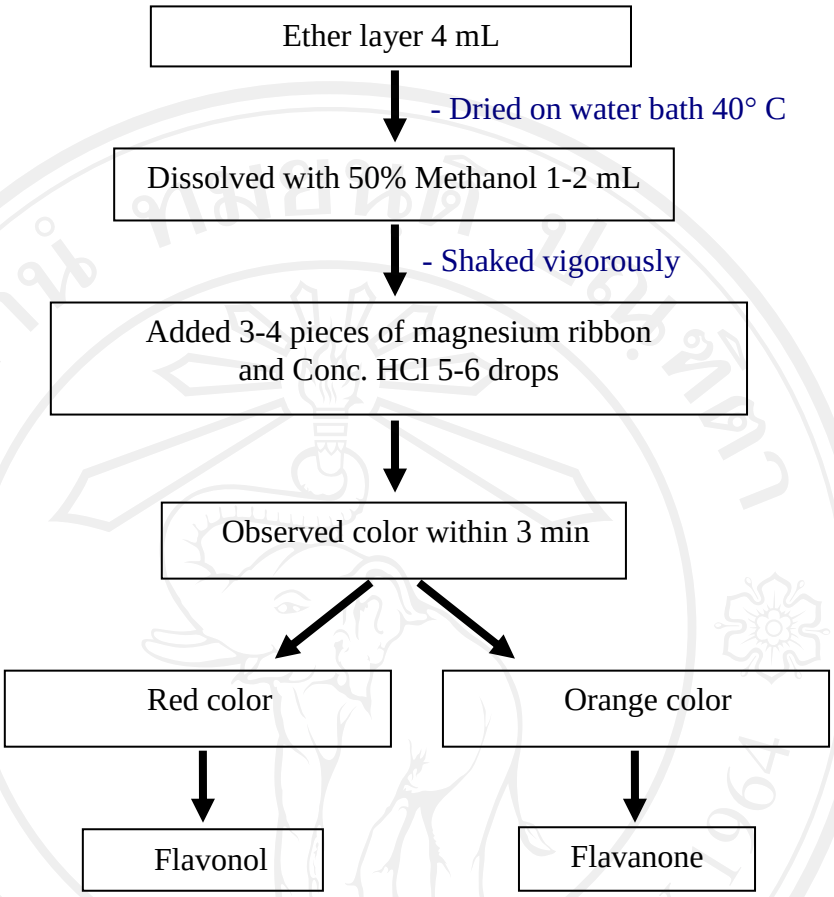


Figure 2.10 Method of flavonoid glycosides determination

2.5.10 Test for anthocyanin (Ayoola *et al*., 2008; Yadav and Agarwala., 2011)

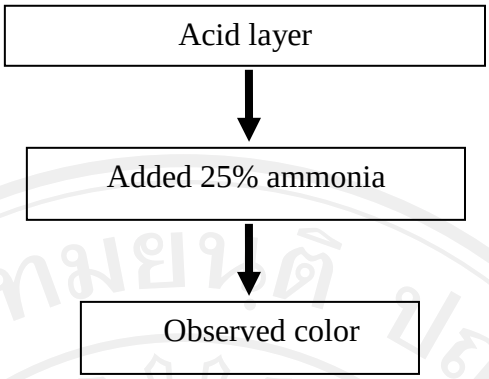


Figure 2.11 Method of anthocyanin determination

2.6 Determination of antioxidant activity

2.6.1 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay (Bozin *et al*., 2006)

The determination of DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging activity of ML extract was performed using the method described briefly as follows: The various concentrations of ML extract (1 mL) were mixed with 1 mL of 90 μM DPPH solution. The mixture was further diluted with 95% methanol, to a final volume of 4 mL. The mixture was kept at room temperature for 1 h under light protection. The absorbance was read at 515 nm by a spectrophotometer. Deionized water was used as the blank; and gallic acid (GA) was used as a positive control. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [(Ab - As) / Ab] \times 100$$

Whereas

Ab = absorbance at 515 nm of the blank

As = absorbance at 515 nm of the sample

All determinations were carried out in triplicate. The antioxidant activity of ML extract was expressed as the half maximal inhibitory concentration (IC_{50}).

2.6.2 Determination of total phenolic content

The estimation of total phenolic contents of ML extract was done using Folin-Ciocalteu reagent (Hammerschmidt and Pratt, 1978). Briefly, 0.2 mL of ML extract was mixed with 1.0 mL of 10% Folin-Ciocalteu solution and 0.8 mL of 7.5% sodium carbonate solution. The mixture was allowed to stand for 1 h at room temperature. The absorbance was measured at 765 nm by a spectrophotometer. Gallic acid (GA) was used as the standard for the calibration curve, and the total phenolic content was expressed as GA equivalent (GAE) per g of ML extract.

2.7 *In vitro* anti-inflammatory models

2.7.1 Cell lines and cell cultures

In this experiment, RAW264.7 macrophage-like cell was used for studying the possible mechanisms of ML extract. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cell line was grown at 37°C, 5% CO_2 in a humidified atmosphere.

2.7.2 Cell viability assay

Cell viability assay was performed using sulforhodamine B colorimetric assay or SRB assay. This method was developed in 1990 by Skehan (Skehan *et al*., 1990).

The treatment protocol for cell viability assay is shown in Figure 2.12. Briefly, RAW 264.7 cells were seeded in 96 well plates in concentration $2x10^4$ cells/well and cultured for 24 h. ML extract (dissolved in DMSO) in various concentration ranging from 3.125-800 µg/mL were added into the cells. The cells were further incubated for 24 or 48 h. After 24 h and 48 h incubation, 10% trichloroacetic acid (TCA) was added to fix cells, and then stained with 0.057% SRB for 30 min. After staining method, 1 % (v/v) acetic acid was added to remove the excess dye. The protein-bound dye was dissolved in 10 mM Tris base solution for OD determination at 510 nm using microplate reader (Vichai and Kirtikara, 2006) (Figure 2.13).

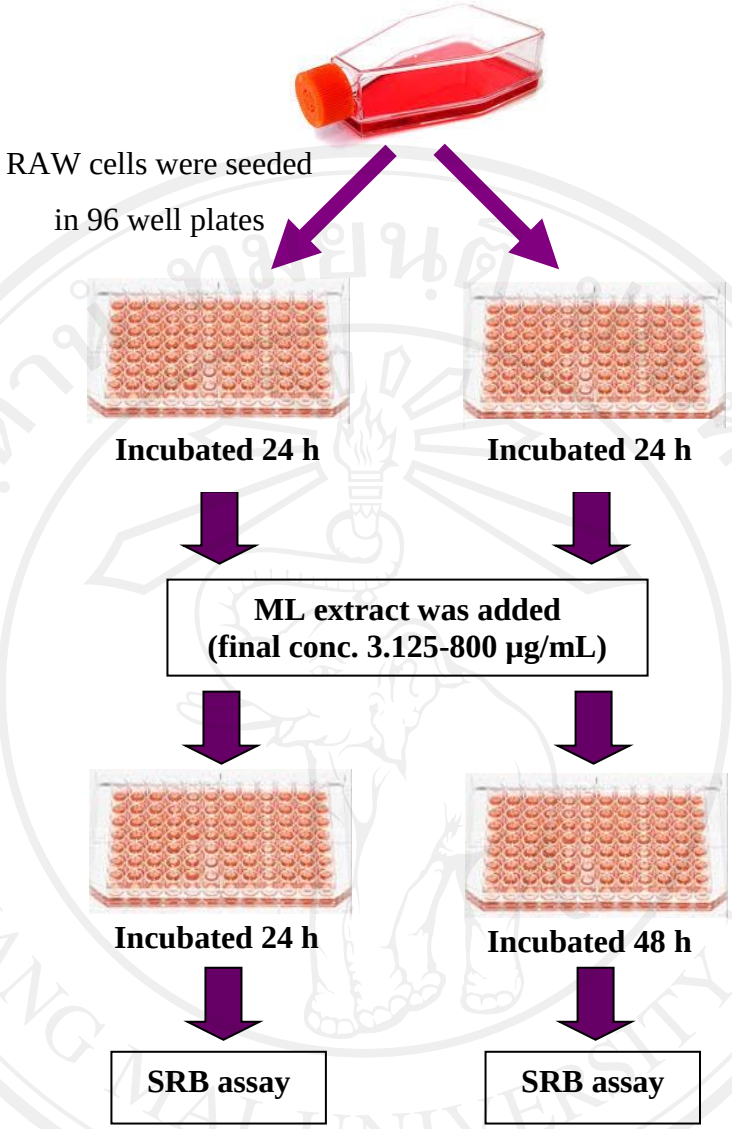


Figure 2.12 Treatment protocol for cell viability assay

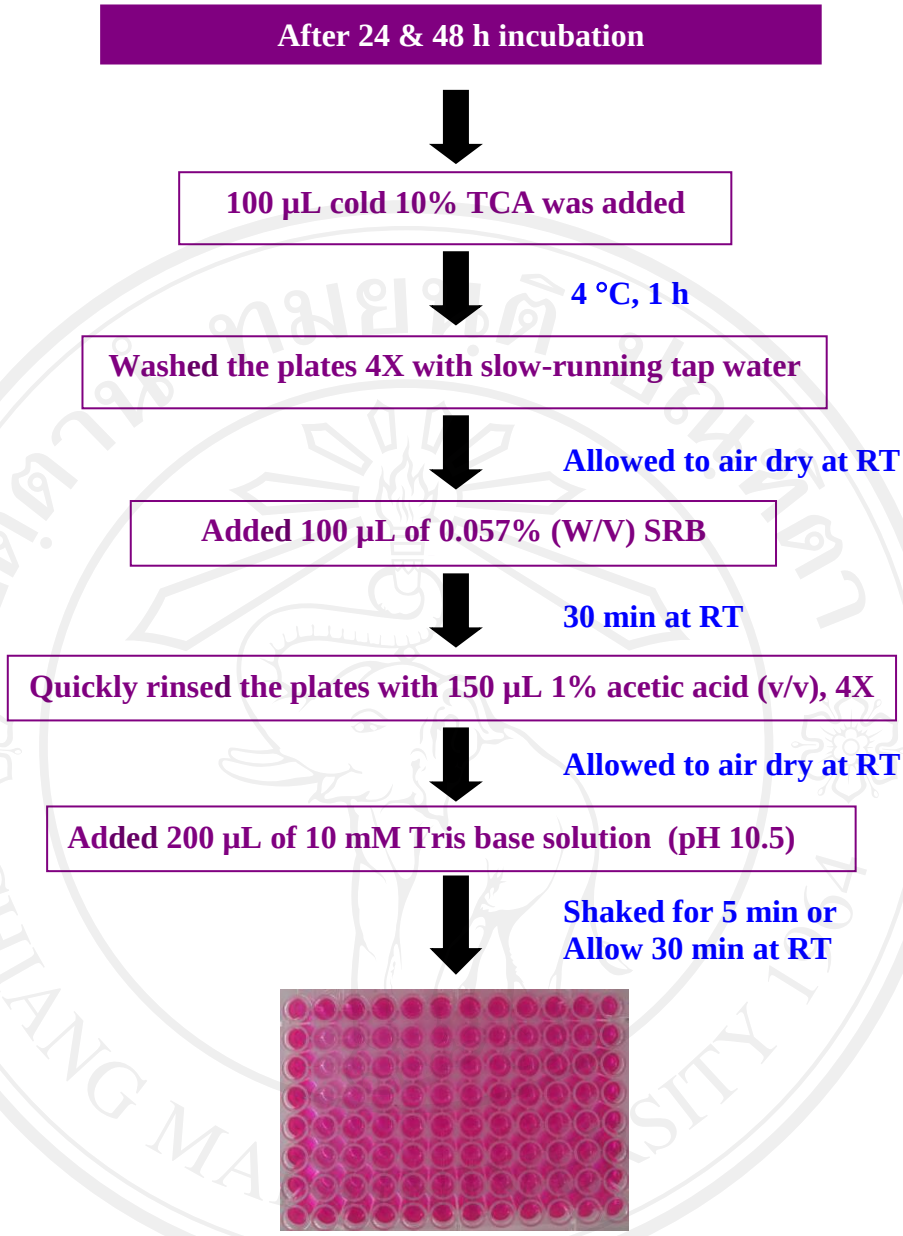


Figure 2.13 SRB assay procedure

2.7.3 Nitrite assay

To evaluate the inhibitory activity of ML extract on LPS-induced NO production, RAW 264.7 cells were seeded in 96-well plates ($2x10^4$ cells/well), and incubated for 24 h. Two experiments of pre-treatment and post-treatment were conducted as follow:

2.7.3.1 Pre-treatment: RAW264.7 cells were treated with various concentrations of ML extract for 1 h and then 1 µg/mL LPS was added. The mixture was further incubated 24 and 48 h. At the end of incubation time, the cultured supernatant was collected and analyzed for nitrite accumulation (a stable of form of NO) (Figure 2.14).

2.7.3.2 Post-treatment: 24 h after cell plating, 1 µg/mL LPS was added to cultured cells line and further incubated for 24 h. After additional 24 h incubation, ML extract in various concentrations were added in culture plate. The media were collected at 2, 4, 6 and 12h thereafter and analyzed for nitrite accumulation by Griess reaction (Ding *et al*., 1988) (Figure 2.15).

Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (Sigma-Aldrich, USA). This mixture was incubated at room temperature for 15 min. Absorbance was measured at 540 nm with a microplate reader (Figure 2.16). Nitrite levels in the samples were determined by comparisons against the standard sodium nitrite curve.

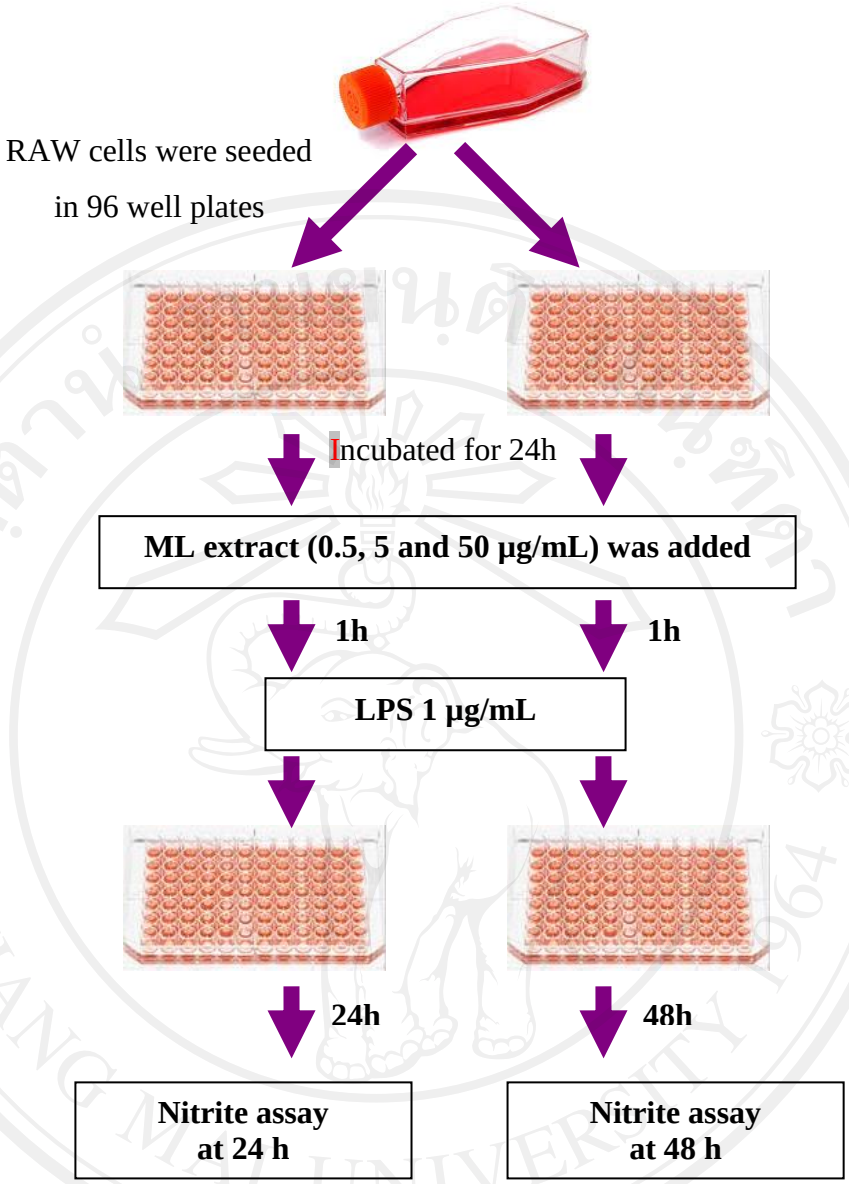


Figure 2.14 NO pre-treatment assay

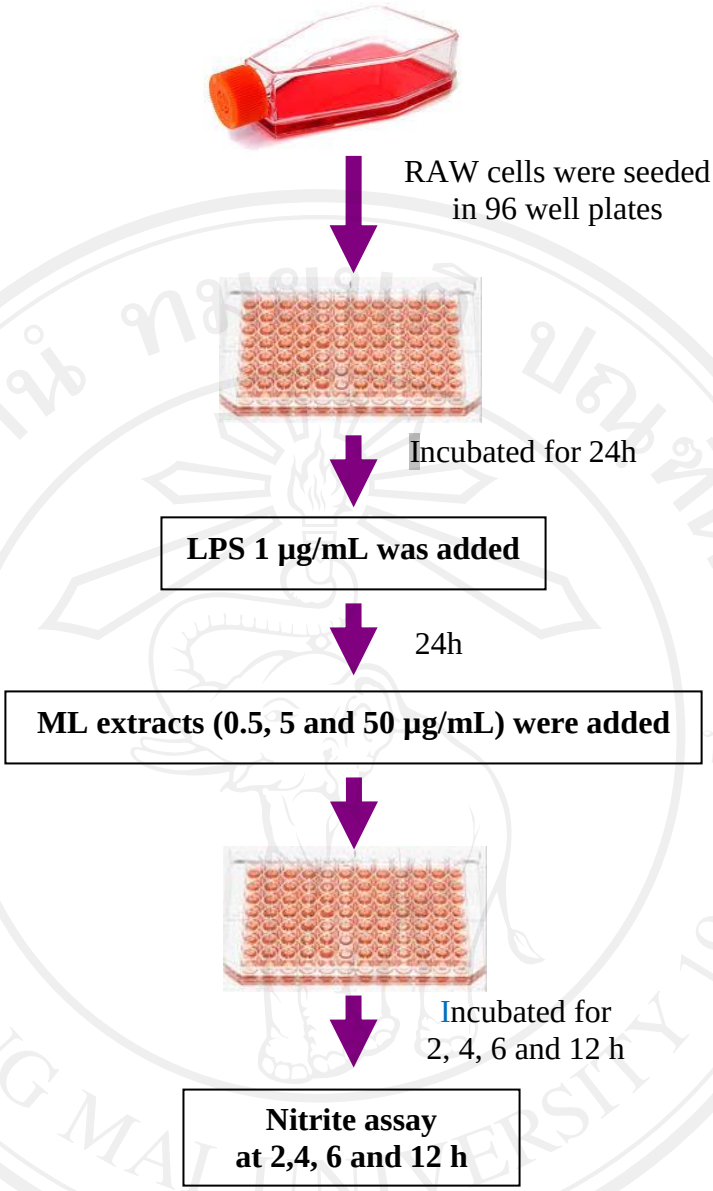
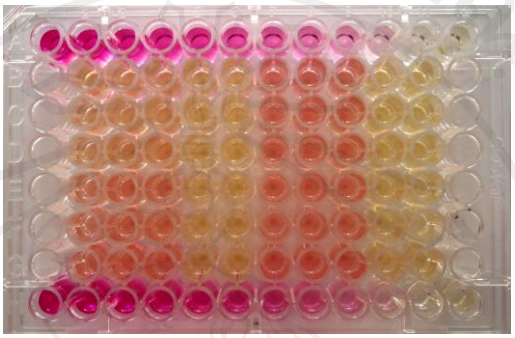


Figure 2.15 NO post-treatment assay

100 µL of each supernatant from LPS or sample-treated cells

100 µL of Griess reagent



The absorbance was measured at 540 nm

Figure 2.16 Nitrite assay using Griess reagent

2.7.4 Treatment protocols for quantitative reverse transcription polymerasechain reaction (RT-qPCR)

The treatment protocol for RT-qPCR assay is illustrated in Figure 2.17. RAW 264.7 cells were seeded in 35 mm petri dish in the concentration of $2x10^5$ cells/mL and cultured for 24 h.

RAW264.7 cells were treated with various concentrations of ML extract for 1 h and then 1 µg/mL LPS was added. The mixture was further incubated for 6 h. At the end of incubation time, the media was removed and the treated-cells were used for RNA extraction.

2.7.5 RNA extraction method

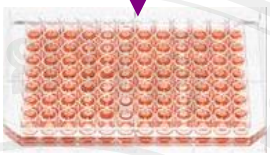
Total RNA was isolated from the cells using Trizol reagent (Invitrogen, USA), according to manufacturer's instructions (Li *et al*., 2009). As shown in Figure 2.18, RAW264.7 cells were washed twice with cold PBS. The cells were lysed directly in a culture dish by adding 500 µL of TRIZOL reagent and scraping with cell scraper. Cell suspension was then transfered to a new microcentrifuge tube. A hundred microlitres of chloroform was added, mixed by vortex and incubated at room temperature for 5 minutes. The samples were centrifuged at 12,000 g for 15 min at 2 to 8 ^oC. After centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. A colorless upper aqueous phase removed to new microcentrifuge tube and precipitated the RNA from the aqueous phase by adding with 500 µL isopropyl alcohol, then mixed and incubated RNA tube at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 2 to 8 ^oC. The RNA precipitates forms a gel-like pellet on the side and bottom of the tube, and then remove the supernatant completely. RNA pellet was washed twice with 75% ethanol 500 µL. The samples were mixed by vortex and then centrifuged at 7,500 g for 5 min at 2 to 8 ^oC, and was removed all leftover ethanol. The RNA pellet was dried at room temperature for 5-10 min.

To determine sample concentration and purity of RNA, RNA pellet was dissolved in DEPC-treated water and mixed by passing solution a few times through a

pipette tip. One microlitre of RNA was diluted in 49 µL of DEPC-treated water (1:50 dilution) before measuring the absorbance at 260 and 280 nm by GeneQuant pro (Amersham Biosciences, Germany). The OD 260 and 280 are frequently used to measure RNA and protein concentration, respectively. The ratio of OD 260/OD 280 values should be above 1.6. Smaller ratio usually indicates contamination of protein or organic chemicals. Diagram illustrating total RNA determination method is shown in Figure 2.18. An OD 1.0 at 260 nm equals to 40 µg /mL RNA. The concentration of RNA was calculated by using the formula below:

Concentration of RNA (µg/mL) = OD260 x 40 x dilution factor

RAW cells were seeded in 96 well plates



Incubated for 24 h

Pre-treatment

ML extracts (50 µg/mL)

Incubated for 1h

LPS (1 µg/mL)

Incubated for 6h

RNA extraction

RT-PCR

Figure 2.17 Treatment protocol for RT-qPCR assay

Removed supernate

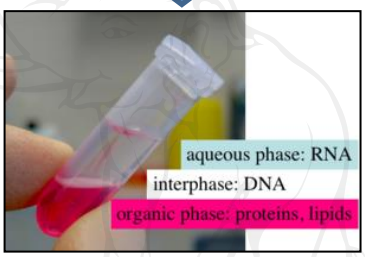
500 µL Trizol reagent was added, scraping with cell scraper

Transfered to microcentrifuge tube, RT 5 min

100 µL Chloroform (Trizol 5 : Chloroform 1)

Vortex, RT 5 min

Centrifuged at 12,000 g, 2-8 °C, 15 min



Removed aqueous phase to new microcentrifuge tube

500 µL isopropyl alcohol

Mixed by inverse, RT 10 min or -20 °C overnight

Centrifuged at 12,000 g, 2-8 °C, 10 min

RNA pellet precipitation

Figure 2.18 RNA extraction method using Trizol reagent

Washed RNA pellet with 75% ethanol in DEPC water

Mixed by vortex

Centrifuged at 7,500 g, 2-8 °C, 15 min

Removed all leftover ethanol

Air-dried RNA pellet for 5-10 min

Dissolved RNA in 50 µl DEPC-treated water by passing solution a few times through a pipette tip

Spin down, RT 5-10 min

Diluted 4 µL of RNA with 196 µL of DEPC-treated water (1:50 dilution)

Determined sample concentration and purity at 260 nm and 280 nm

Figure 2.18 RNA extraction method using Trizol reagent (continued)

2.7.6 cDNA synthesis by reverse transcription method

First strand cDNA was synthesized using RevertAid M-MulV reverse transcriptase (Fermentas). Brifely, 1.0 μg of total RNA was reverse-transcribed by oligo-(dT)18 primer and AMV reverse transcriptase using RevertAid TM First Strand cDNA synthesis kit (Fermentas, Germany), according to the manufacturer's instructions. The reaction mixtures are shown in Table 2.1 and 2.2. M1 buffer contained 1 μg of extracted RNA, 0.5 μg of oligo-(dT) 18 and 2 μL of DEPC water. Reagents in M1 buffer were incubated at 70 °C, 5 min, after cooling down 1 min, the M2 buffer was added. M2 buffer contained 5x reaction buffer, 1 mM dNTPs, 20 units of ribonuclease inhibitor, and 200 units of reverse transcriptase, final volume of this reaction was 20 μL. The reaction mixture was incubated at 70 ^{o}C for 5 min, 4 ^{o}C for 1 min, and then 42 ^{o}C for 60 min. The resulting cDNA was diluted five times and used as a template for RT-PCR reaction.

2.7.7 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The GeNei Red Dye PCR Master Mix (2x) kit was used for PCR amplification. A 20 μL PCR reaction mixture consisted of 2.0 μL of cDNA, 10 μL of red dye PCR master mix [(final concentration: 1X contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 2.0 mM $MgCl_2$), 0.2 mM dNTPs, 2 units of *Taq* polymerase)], 7.5 μL of dd H_2O, and 0.25 μL GAPDH primers (Table 2.3).

Each reaction mixture was then placed on an iCycler thermal cycle (Bio-Rad, USA). The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 28 cycles of the amplification process, which were denatured at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 45 sec, and the final extension step at 72 °C for 7 min.

Table 2.1 M1 reaction buffer

Reagent	1 Reaction
Template RNA (0.1 µg/µl, total 1 µg)	10 µL
Oligo dT18 (0.5 µg/µl)	1 µL
DEPC water	2 µL
Total volume	**13 µL**

Table 2.2 M2 reaction buffer

Reagent	1 Reaction
5x reaction buffer	4 µL
Ribonuclease inhibitor	0.5 µL
10 mM dNTP mix	2 µL
Reverse transcriptase	0.5 µL
Total volume	**7** µL

Table 2.3 PCR reaction mixture

Reagent	1 Reaction
GeNei Red Dye PCR Master Mix (2x)	10 µL
20 µM of Forword primer GAPDH (murine)	0.25 µL
20 µM of Reverse primer GAPDH (murine)	0.25 µL
dd H_2O	7.5 µL
cDNA	2 µL
Total volume	**20** µL

2.7.8 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay

There are many methods for quantify gene expression such as Northern bloting and RT-PCR. Quantitative RT-PCR (RT-qPCR) is a method used to quantify the mRNA of cytokines, which are expressed at low levels. In this study, the expression of TNF-α, IL-1β, IL-6, iNOS and COX-2 mRNA were determined by RT-qPCR technique. The mRNA expression levels were normalized with GAPDH, a housekeeping gene. The primers used in this study were chosen from on-line primer design program. The primer sequences are presented in Table 2.4.

The SYBR green-based quantitative PCR amplification was performed in triplicate using a PCR thermal cycler (7500 Real-time PCR System). As shown in Table 2.5, the 20 µL reaction mixture contained 5 µL of cDNA (dilute 1:5), 10 µL of Thunderbird^{IM}SYBR® qPCR Mix (final conc. 1X), 0.04 µL of ROX solution (final conc. 0.1X), 0.6 µL of forward and reverse primers (final conc.10 µM), and 1.76 µL of ddH_2O. Each reaction mixture was then placed on an ABI 7500 machine. Cycling for PCR amplification was performed as follows: initial denaturation at 95 °C for 10 min, follow by 40 cycles at 95 °C for 15 sec, at 60 °C for 30 sec and at 72 °C for 30 sec.

Table 2.4 Primer sequences

Target gene	Sense (5'-3')	antisense (5'-3')
TNF-α	TCT CAT GCA CCA CCA TCA AGG ACT	ACC ACT CTC CCT TTG CAG AAC TCA
IL-1β	AAG GGC TGC TTC CAA ACC TTT GAC	ATA CTG CCT GCC TGA AGC TCT TGT
IL-6	ATC CAG TTG CCT TCT TGG GAC TGA	TAA GCC TCC GAC TTG TGA AGT GGT
iNOS	TCT TTG ACG CTC GGA ACT GTA GCA	ACC TGA TGT TGC CAT TGT TGG TGG
COX-2	TAC TAC ACC AGG GCC CTT CC	CAT ATT TGA GCC TTG GGG GT
GAPDH	AGG TGGTCT CCT CTG ACT TC	TAC CAG GAA ATG AGC TTG AC

Table 2.5 RT-PCR reaction mixture

Reagent	Reaction volume	Final concentration
DW	3.76 µL	
ThunderbirdIMSYBR® qPCR Mix	10 µL	1X
Forward Primer	0.6 µL	0.3 µM
Reverse Primer	0.6 µL	0.3 µM
50X ROX reference dye	0.04 µL	0.1X
cDNA (dilute 1:5)	5 µL	
Total volume	20 µL	

2.8 *In vivo* experimental models

2.8.1 Experimental animals

Male Sprague-Dawley rats weighing between 130-150 g and 250-300 g were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, Thailand. All animals were kept in an animal room maintained under environmentally controlled conditions of 24 $\pm$ 1˚C and 12 h light - 12 h dark cycle. They were had free access to drinking water and standard pelleted diet (082 C.P. MICE FEED, S.W.T. Co., Ltd., Samut Prakan, Thailand). The rats were acclimatized at least one week before starting the experiments. All experimental procedures were approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (NO. 08/2556).

2.8.2 Preparation of test substances and test substances administration

All test substances were suspended in 5% polysorbate 80 U.S.P. (Tween 80). All rats were orally administered test substances in an equivalent volume of 5 mL/kg body weight. Control group received only vehicle in the same volume by the same route of administration.

2.8.3 Anti-arthritic activity study

Anti-arthritic activity study was performed using complete Freund's adjuvant (CFA)-induced arthritis model (Newbould, 1963; 1969). The rats weighing between 130-150 g were used in the study. Before starting the experiment, both sides of the paw volume of the rats were measured by plethysmometer (model 7140, Ugo Basile, Italy). Each paw volume was obtained from the average of 3 readings.

After anesthetized with diethyl ether, the rats were injected with 0.1 mL of CFA into the plantar surface of the right paw. Fourteen days later the rats were measured paw edema volume. They were randomly divided into five groups of six rats as follows:

Group 1: Control group, received 5% tween 80
Group 2: Reference group, received indomethacin at the dose of 1 mg/kg
Group 3-5: Test groups, received ML extract at doses of 100-400 mg/kg

The treatments initiated from day 0 (fourteen days after arthritic induction) and continued up to day 28th. The paw edemas were measured by plethysmometer every seven day. At the end of the experiment, all rats were sacrificed with an overdose of ether and the legs were collected for histological examination. The protocol of CFA-induced arthritis is shown in Figure 2.19.

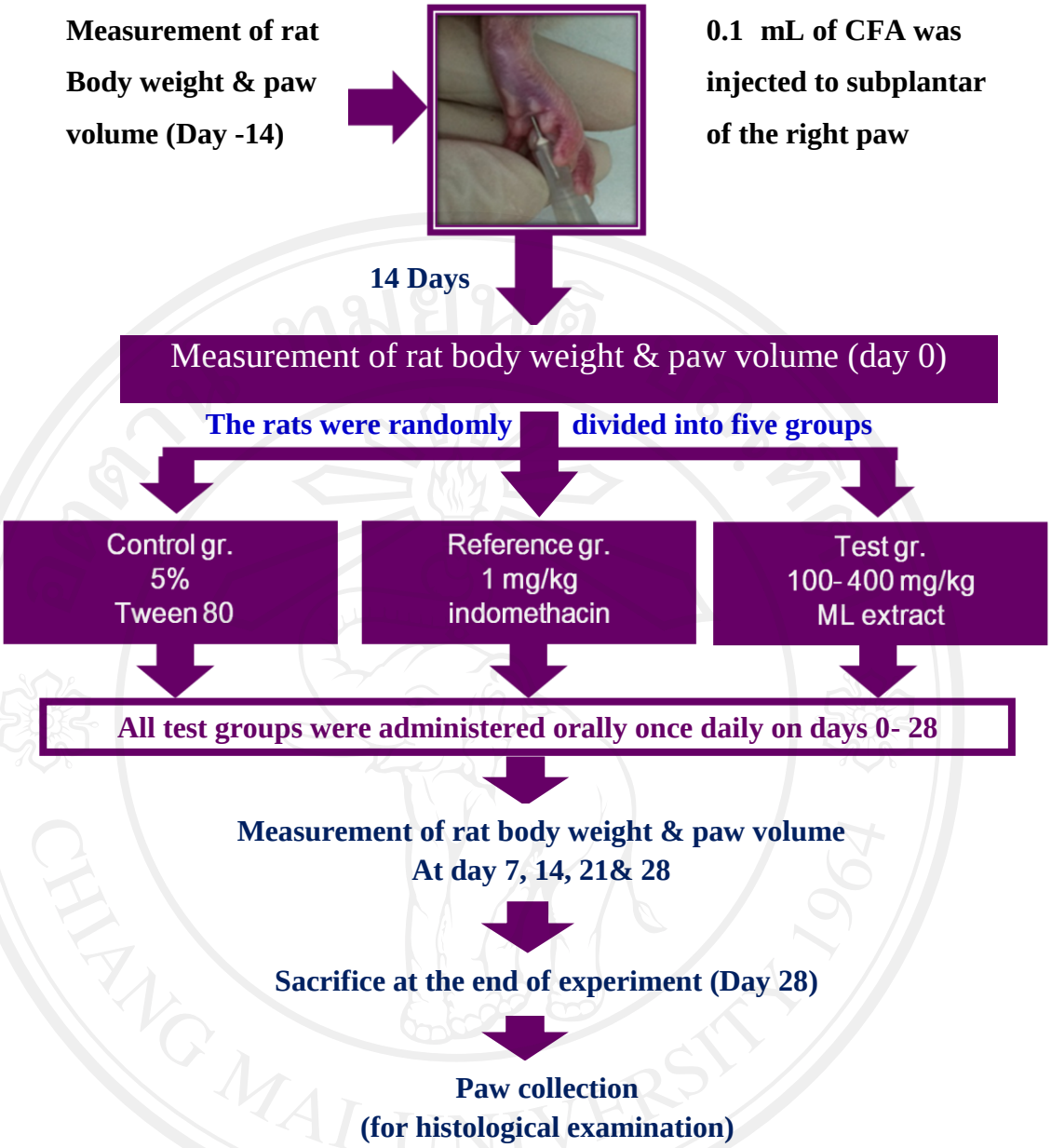


Figure 2.19 The procedure of CFA-induced arthritis in rats

2.8.3.1 Assessment of arthritis

To assess anti-arthritic activity of ML extract, the primary and secondary lesions were observed. The volumes of injected and non-injected paws were measured using a plethysmometer (model 7140, Ugo Basile, Italy) (Figure 2.20).

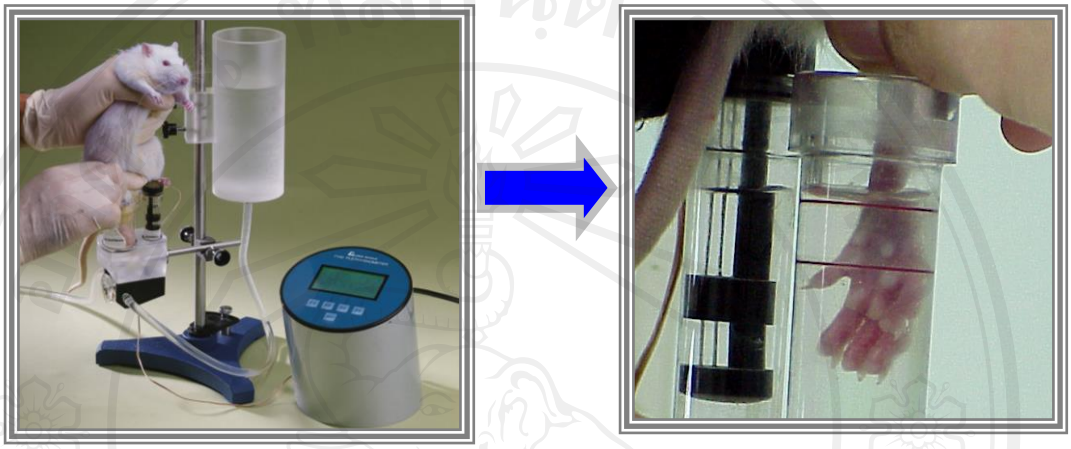


Figure 2.20 Measurement of paw edema volume using plethysmometer

2.8.4 Gastroprotective activity studies

2.8.4.1 Preparation of rats for gastroprotective study

The rats weighing 250-300 g were used in this experiment. For each gastric ulcer prevention model, the rats were fasted for 48 h (Matsumoto *et al*., 1989; Rujjanawate *et al*., 2004) and water withdrawn for 1 h before starting the experiment.

2.8.4.2 Ethanol/Hydrochloric acid (EtOH/HCl)-induced gastric ulcer

The rats were divided into 6 groups of 6 rats as follows:

Group 1: Control group, received 5% Tween 80

Group 2: Reference group, received cimetidine at the dose of 100 mg/kg

Group 3: Reference group, received misoprostol at the dose of 0.1 mg/kg

Group 4-6: Treated groups, received ML extract at the doses of 100-400 mg/kg

One hour after drug administration, the rats were administrated orally with 1.0 mL EtOH/HCl (60 mL EtOH + 1.7 mL HCl + 38.3 mL H_2O) (Mizui *et al.*, 1983). One hour later, all animals were sacrificed for determination of gastric ulcer (Figure 2.21).

2.8.4.3 Indomethacin-induced gastric ulcer

The rats were divided into 6 groups of 6 rats as follows:

Group 1: Control group, received 5% Tween 80

Group 2: Reference group, received cimetidine at the dose of 100 mg/kg

Group 3: Reference group, received misoprostol at the dose of 0.1 mg/kg

Group 4-6: Treated groups, received ML extract at the doses of 100-400 mg/kg

One hour after drug administration, the rats were administrated orally with indomethacin [suspended in 0.5% carboxymethylcellulose (CMC)] at the dose of 100 mg/kg (Nwafor *et al*., 2000). After 5 h, the rats were sacrificed with an overdose of ether. The stomachs were removed and opened for determination of gastric ulcer (Figure 2.22).

2.8.4.4 Restraint water immersion stress-induced gastric ulcer

The rats were divided into 5 groups of 6 rats as follows:

Group 1: Control group, received 5% Tween 80

Group 2: Reference group, received cimetidine at the dose of 100 mg/kg

Group 3-5: Treated groups, received ML extract at the doses of 100-400 mg/kg

As shown in Figure 2.23, 1 hour after drug administration, the rats were restrained individually in stainless steel cages and immersed up to their xiphoid in a water bath maintained at 22 ± 2 °C. After 5 h of this exposure, the rats were sacrificed and examined for gastric ulcer (Takagi *et al*., 1964).

2.8.4.5 Evaluation of gastric ulcer

Each rat was sacrificed with an overdose of ether. The abdomen was excised and opened along the greater curvature. After washing with normal saline, the gastric lesion was counted using a binocular magnifier (10X). The lesion index was assessed using the following methods:

A. Length of lesion

The length in millimeter of lesion was determined by measuring each lesion along its greater diameter (Chattopadhyay *et al*., 2006). The sum of the total length in each group divided by the number of rats in that group was expressed as an ulcer index.

$$\text{Ulcer index} = \frac{\Sigma \text{ Total lengths of lesion in each group}}{\text{Number of rats in that group}}$$

B. Percent inhibition of gastric ulcer

Percent curation of gastric ulcer was measured as described by Takagi *et al*. using the following formula:

$$\% \text{ Inhibition} = \frac{(\text{Ulcer index}_{\text{control}} - \text{Ulcer index}_{\text{treated}}) \times 100}{\text{Ulcer index}_{\text{control}}}$$

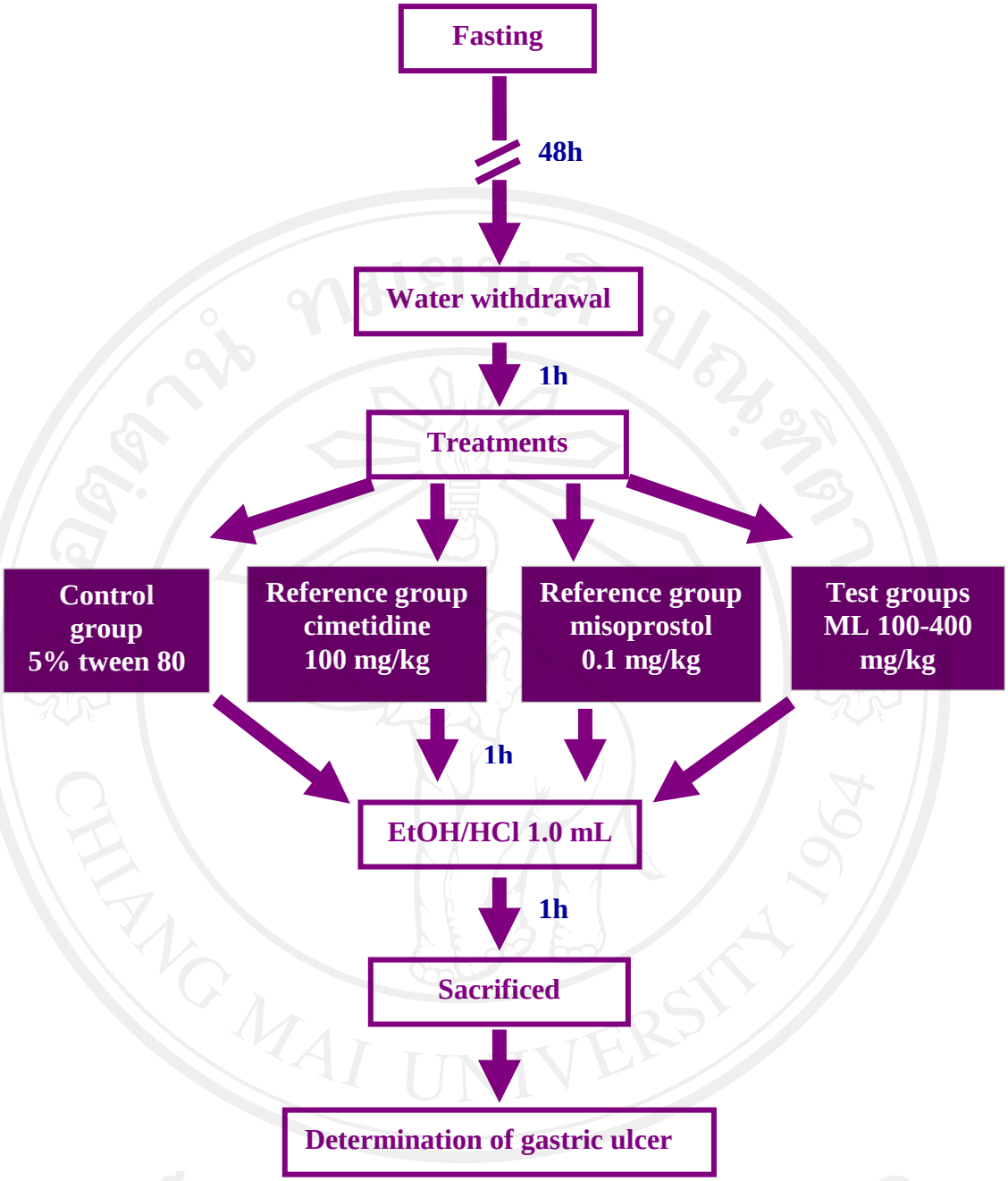


Figure 2.21 Method of EtOH/HCl-induced gastric ulcer in rats

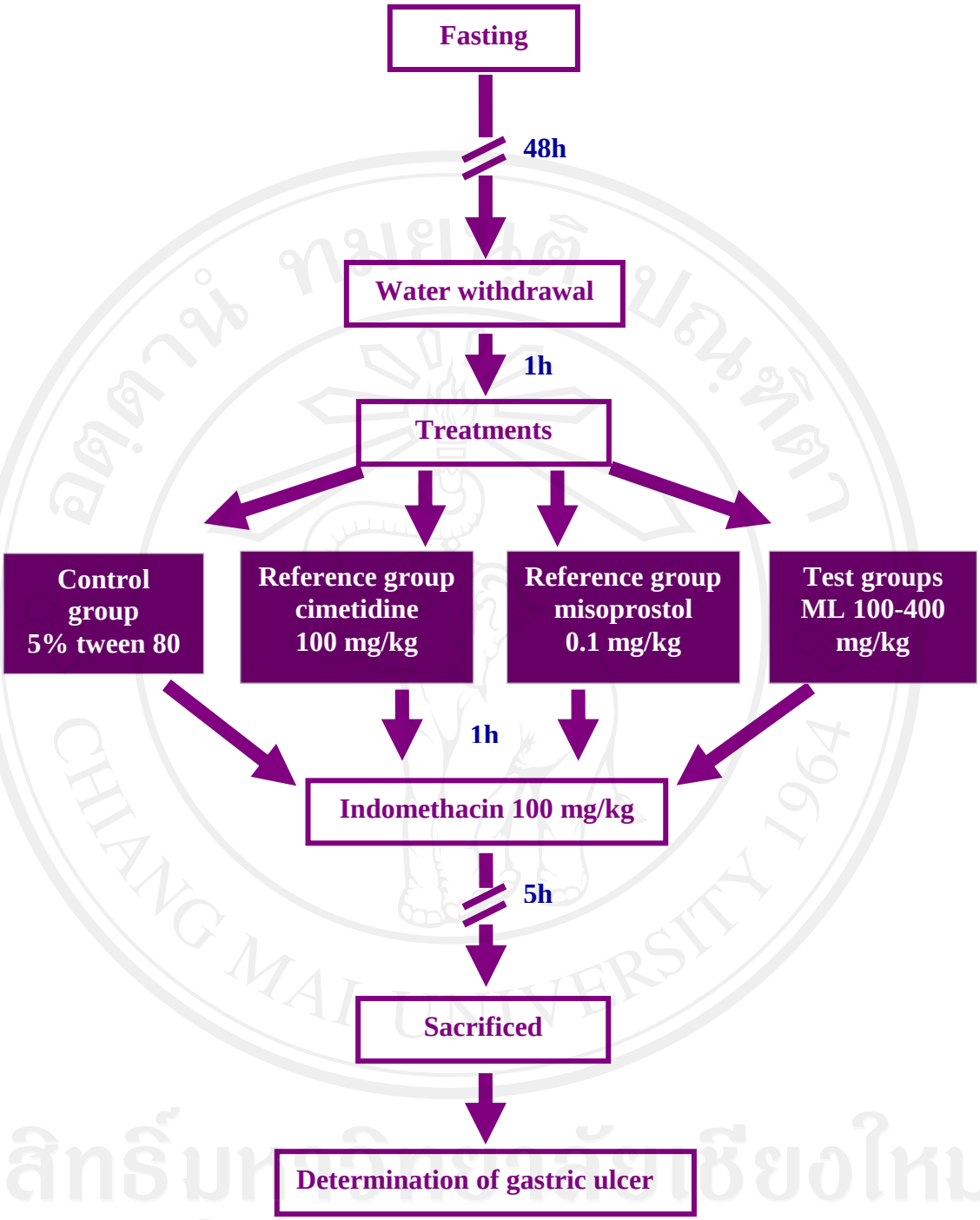


Figure 2.22 Method of indomethacin-induced gastric ulcer in rats

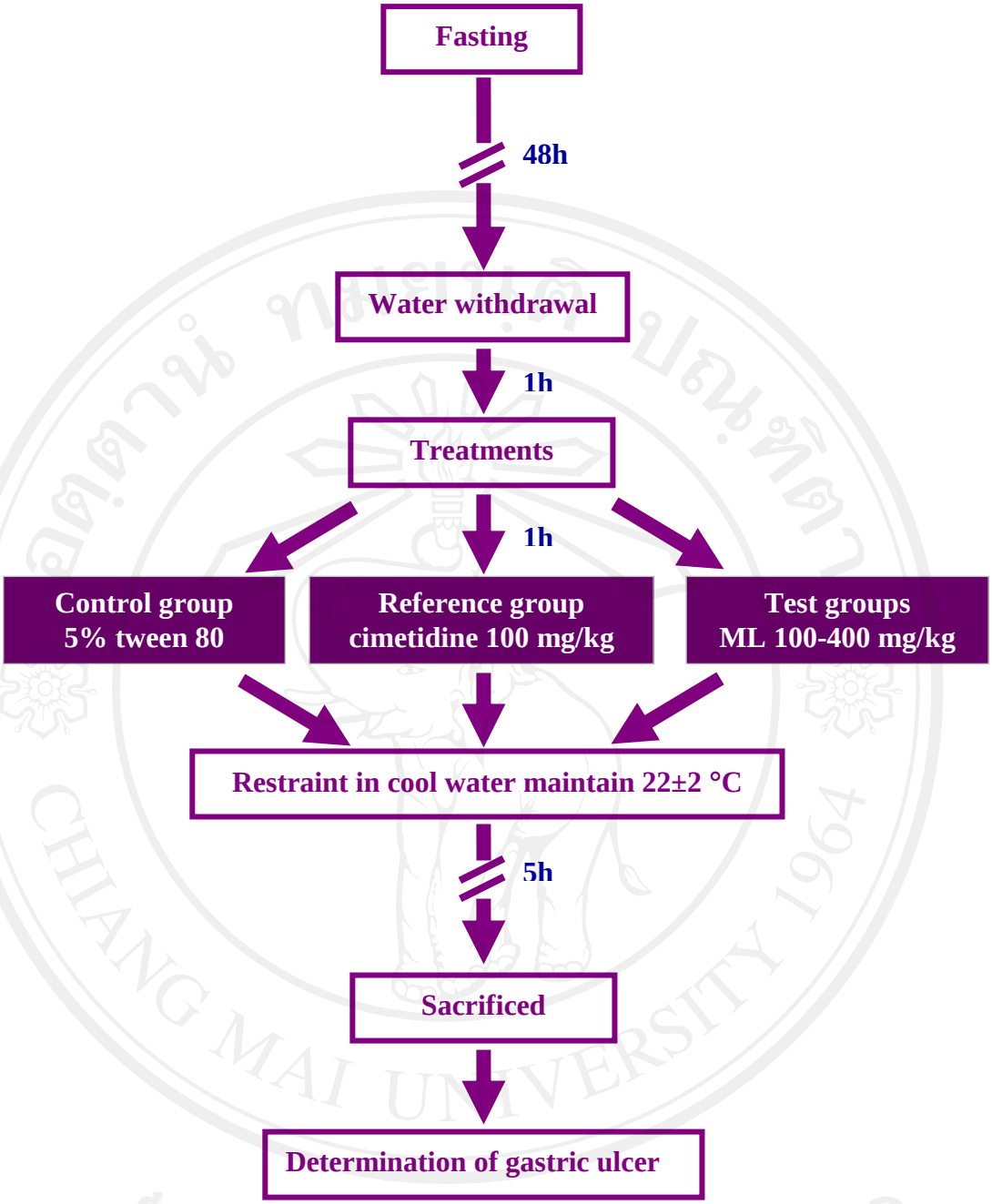


Figure 2.23 Method of restraint water immersion stress-induced gastric ulcer in rats

2.8.5 Investigation of the mechanism of gastroprotective activity

2.8.5.1 Gastric visible mucus secretion

Gastric wall mucus was determined using the Alcian blue method (Corne *et al*., 1974). The rats were fasted 48 h and the water were withdrawn 1 h before starting the experiment. The rats were divided into 6 groups of 6 rats as follows:

Group 1: Normal rat group, received 5% Tween 80
Group 2: ML group, received ML extract at the dose of 400 mg/kg
Group 3: Control group, received 5% Tween 80
Group 4: Reference group, received misoprostol at the dose of 0.1 mg/kg
Group 5: Reference group, received cimetidine at the dose of 100 mg/kg
Group 6: Treated group, received ML extract at the dose of 400 mg/kg

One hour after drugs administration, the rats in groups 3-6 received EtOH/HCl to induce gastric ulcer (Mizui and Doteuchi, 1983), whereas the rats in group 1 and 2 received distill water. One hour later, the rats were sacrificed with an overdose of ether (Figure 2.24). The abdomen was opened and the stomach was removed and cut along the lesser curvature. After rinse with normal saline, the stomach was weighed and immersed in 0.1% Alcian blue solution dissolved in 0.16 M sucrose buffered for 2 h. The excess of uncomplexed dye was rinsed from the stomach twice with 0.25 M sucrose for 15 and 45 min, respectively. The dye-gastric mucus complex was extracted in 0.5 M magnesium chloride for 2 h. The blue extract was shaken vigorously with an equal volume of diethyl ether and then centrifuged at 4,500 rpm for 10 min. The aqueous layer was separated and measured the absorbance at 580 nm by a spectrophotometer (Figure 2.25). The Alcian blue dissolved in 0.5 M magnesium chloride, at the concentrations of 10, 20, 40, 80 and 100 µg/mL were used as a standard agent. The amount of gastric visible mucus was expressed as µg alcian blue/g stomach.

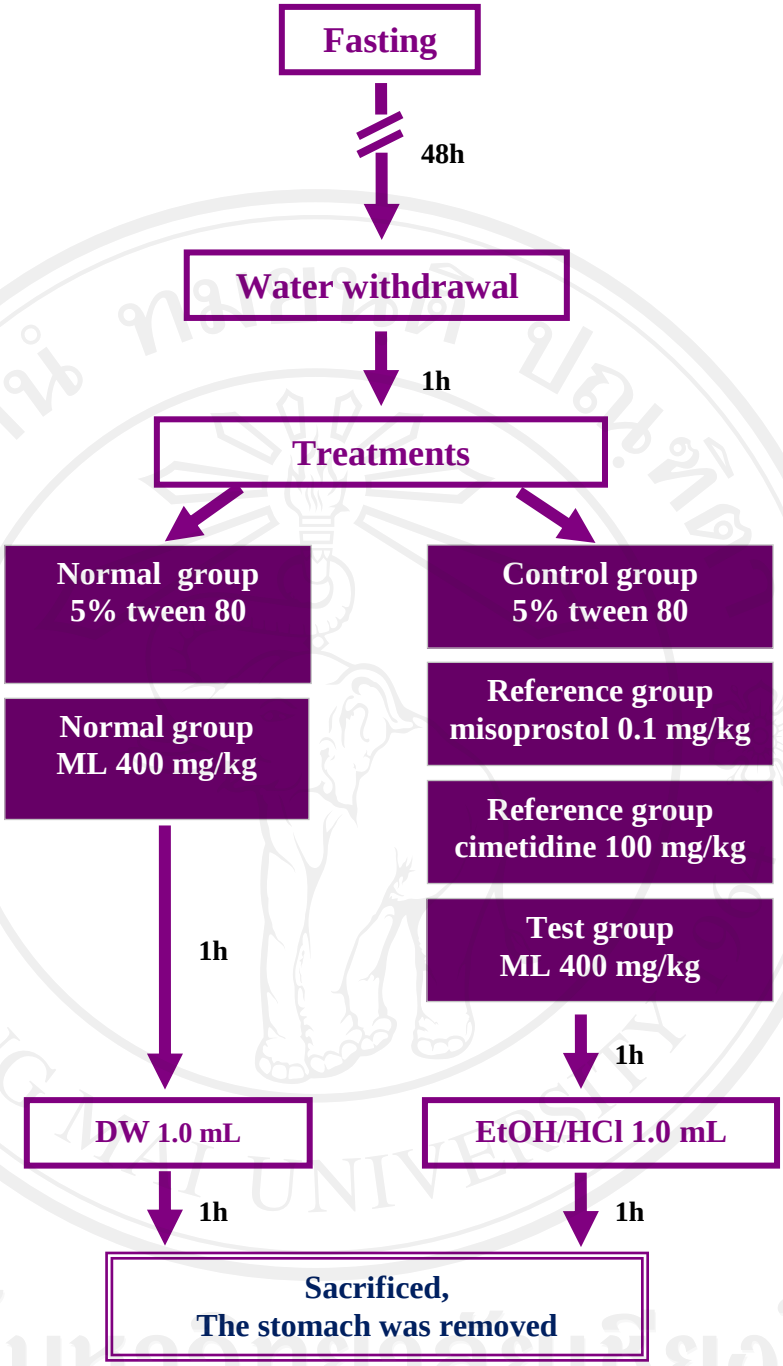


Figure 2.24 Method of gastric visible mucus secretion

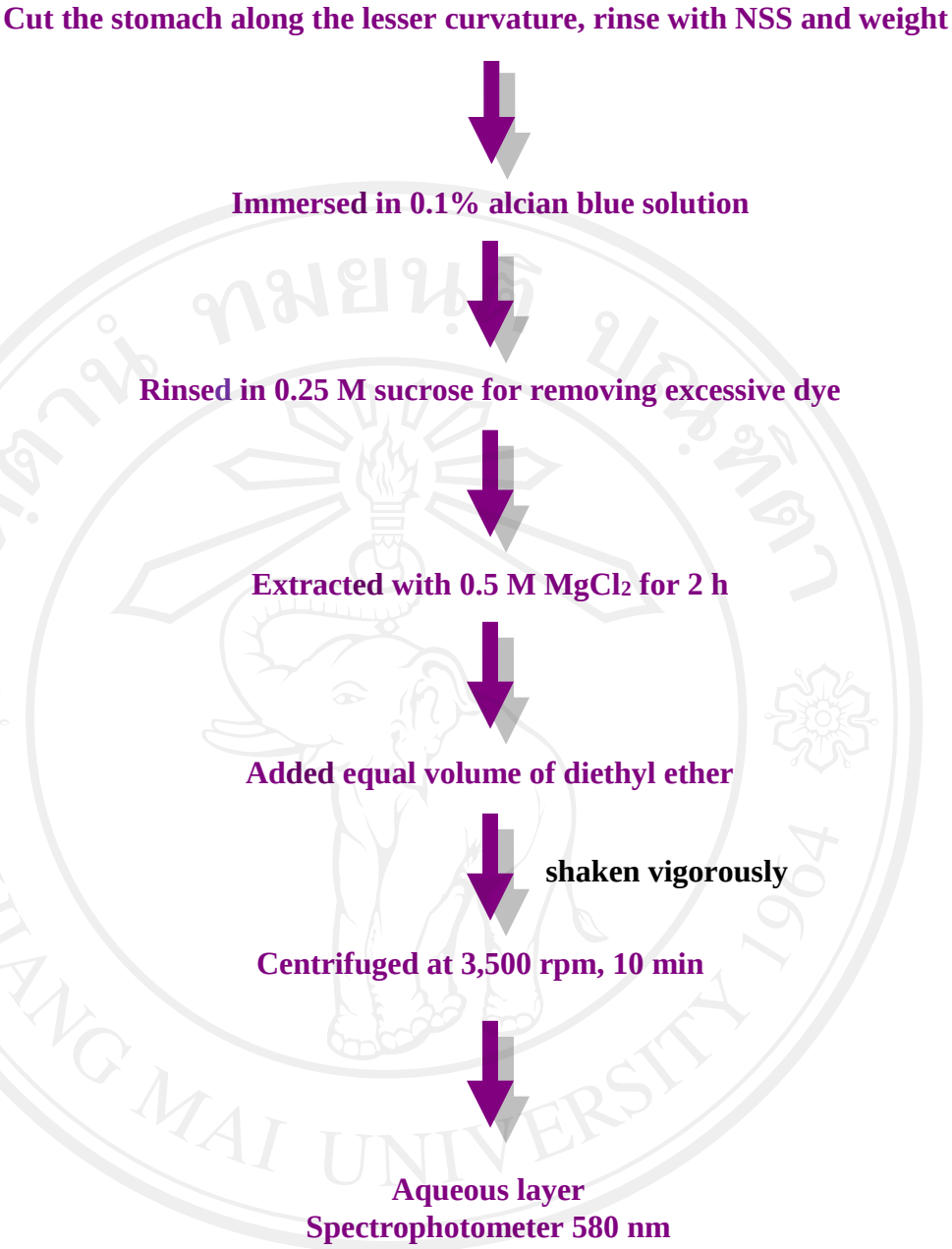


Figure 2.25 The alcian blue method

2.8.5.2 Pylorus ligation

Pylorus ligation experiment was performed using the method described by Shay *et al* (Shay, 1945). The rats were divided into 3 groups of 6 rats as follows:

Group 1: Control group, received 5% Tween 80
Group 2: Reference group, received cimetidine at the dose of 100 mg/kg
Group 3: Treated group, received ML extract at the dose of 400 mg/kg

As presented in Figure 2.26, the rats were fasted for 48 h before started the experiment. One hour after drug administration, the rats were lightly anesthetized by ether. The abdomen was opened and the pylorus was ligated. The abdomen was closed by suturing. Five hours later, the rats were sacrificed by an overdose of ether. The stomach was removed and the gastric juice was collected in 15 mL centrifuge tube and then it was centrifuged at 3,500 rpm for 10 min. The supernatant was measured for total gastric juice volume, pH and total acid output. The pH of gastric juice was measured by pH meter. The total acidity was determined by titration with 0.1 N NaOH using phenolphthalein as an indicator. Total acidity of gastric juice was calculated as follow:

$$N_1V_1 = N_2V_2$$

where;

N_1 = Normality of gastric juice
N_2 = Normality of NaOH
V_1 = volume of gastric juice (mL)
V_2 = volume of NaOH (mL)

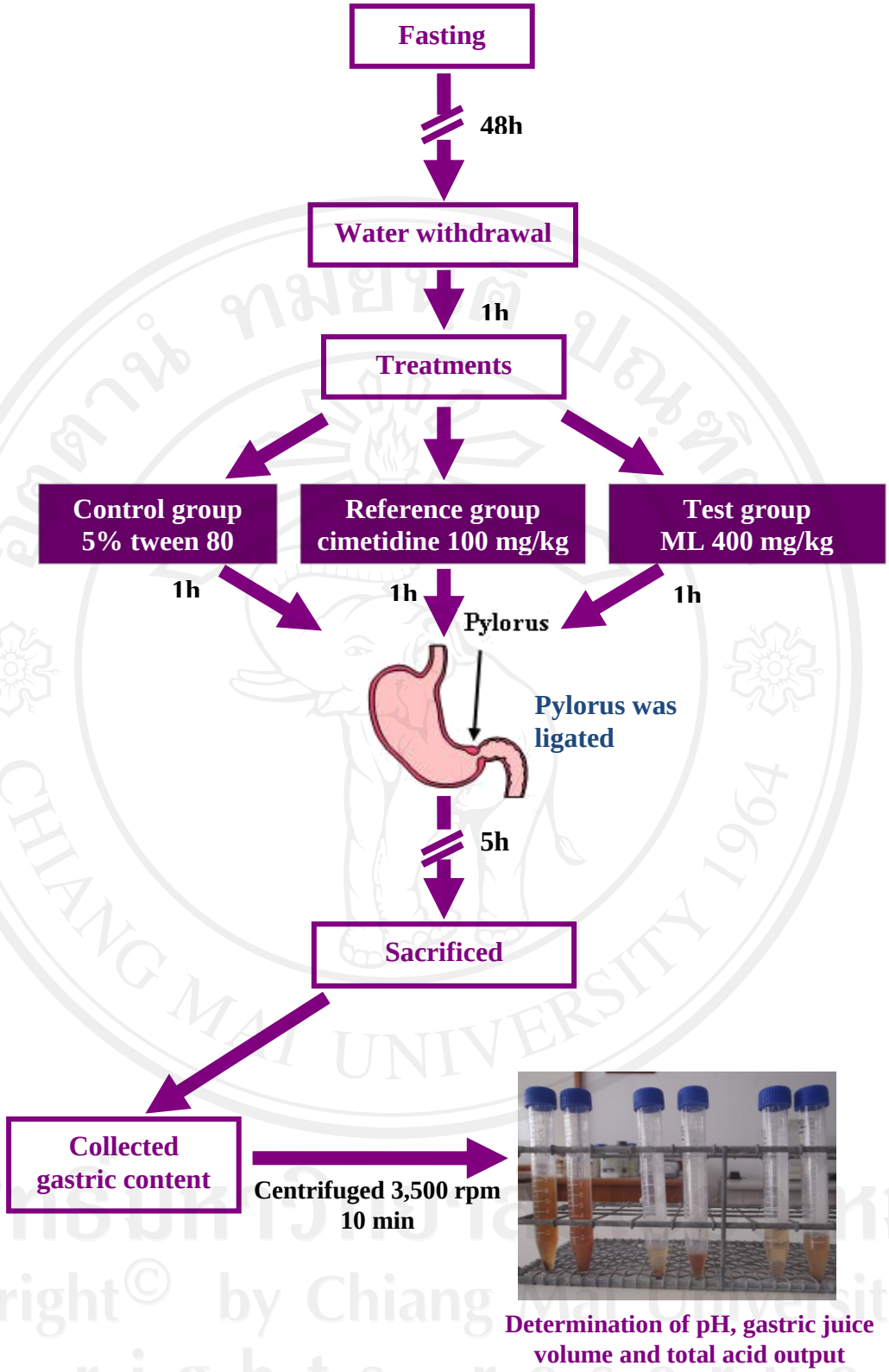


Figure 2.26 Method of pylorus ligation

2.9 Data analysis and statistical methods

Data from the experiments were expressed as mean ± standard error of mean (S.E.M). Statistical comparison between groups were analyzed by using one way ANOVA and post hoc least-significant difference (LSD) test and *p* values less than 0.05 were considered significant.