CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material

The fruits of *P. emblica* Linn. were collected from the forest of Nan province, Thailand. The plant material was identified by Associate Professor Dr. Noppamas Soonthornchareonnon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The voucher specimen (PBM 01402) has been kept at the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

2.2 Extract from the fruits of *P. emblica* Linn.

The standardized extract of *P. emblica* fruits was kindly provided by Assoc. Prof. Dr. Noppamas Soonthornchareonnon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. A decoction of dry *P. emblica* fruit was developed in the laboratory since a water decoction of herb is one of the effective methods used in Ayurvedic and Thai traditional medicine for treatment of the various diseases (Farnsworth and Bunyapraphatsara, 1992). The method of preparation was described briefly as follows: 194 kg of dry *P. emblica* fruits was immersed in 100 L of water for 30 min, then boiled for 1 h and filtered to remove the residue. Next, the aqueous extract was repeatedly boiled and filtered 2 times. The water extract was spray dried to remove trace of solvent. The *P. emblica* water extract was stored at -20 °C after preparation.

2.3 Standardization of plant extract

The quality control of raw materials and the extract followed the Thai Herbal Pharmacopoeia (THP) including organoleptic examination, % loss on drying, extractive values, total ash and acid insoluble ash (Department of Medical Sciences, Ministry of Public Health, 2000). Chemical constituents including flavonoids, lactone, terpinoids and tannins, were also studied using thin layer chromatography (TLC) following the method of Farnsworth (Farnsworth, 1966) and percentage of

tannins, total polysaccharide, and uronic acid, type of monosaccharides, microbials, aflatoxin and heavy metals were examined as well. The extract was standardized by TLC and high performance liquid chromatography (HPLC) using the conditions listed in Table 4.

Table 4 TLC and HPLC conditions for analysis of *P. emblica* water extract

Operating parameter	Conditions
TLC	
Adsorbent	- Siliga gel GF_{254} (Merck® 1.05554)
Solvent system (mobile phase)	- Toluene : ethyl acetate : formic acid : methanol (30 : 30 : 8 : 2) - Ethyl acetate : formic acid : water (8 : 1 : 1)
Detector	- UV 254 and 366 nm - Anisaldehyde/sulfuric acid - Phosphomolybdic acid
Standard compound	- Ellagic acid, gallic acid and protocatechuic acid
HPLC	
Stationary state (column)	- Spheri-5 RP18, particle size 5 µm, 220 x 4.6 mm
Perkin Elmer and mobile phase	- Acetonitrile + 1mM H_3PO_4 (80 : 20) : 1mM H_3PO_4 = 8 : 92
Wavelength	- 216 nm
Injection volume	- 20 µl
Internal standard	- Protocatechuic acid
Standard compound	- Gallic acid

The percentage yield was 8.76 of raw materials. Glucose was the main monosaccharide, however, arabinose, galactose, glucuronic acid/galacturonic acid were also found. The values of quality control and quantity of chemical compounds of the raw material and extract were remained within the normal ranges, which are

shown in Tables 5 and 6, respectively. Moreover, the water extract was standardized by HPLC to contain 20.48% of gallic acid as shown in Figure 4.

2.4 Experimental animals

Male Sprague Dawley rats weighing 40-60 g (21 rats), 100-120 g (30 rats), 180-220 g (60 rats), 200-250 g (60 rats) and 250-300 g (126 rats) as well as female Sprague Dawley rats weighing 180-220 g (60 rats) and male ICR mice weighing 30-40 g (72 mice) 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 ± 1 °C, relative humidity 50 ± 10% and 12 h light-12 h dark cycle. All animals had free access to drinking water and standard pellet diet (082 C.P. MICE FEED, S.W.T. Co., Ltd., Samut Prakan, Thailand). They were acclimatized at least one week before starting the experiments. The Animal Ethics Committee of Faculty of Medicine, Thammasat University has approved all experimental protocols (No.0001/2006 and No.0001/2007).

2.5 Preparation of test substances

Distilled water were used as vehicle for all test substances, except in the ear edema model that the test substances were dissolved in dimethylsulfoxide (DMSO) and acetone (1:1).

2.6 Test substance administration

All test substances were orally administered in an equivalent volume of 0.2 ml/100 g body weight of the rats and in volume of 0.1 ml/10 g body weight of the mice, except morphine which was given intraperitoneally to mice. In the ear edema model, the test substances were applied locally to outer and inner surfaces of the ear. Control groups received only vehicle in the same volume and same route of administration.

Table 5 Monograph of the raw material of *P. emblica*

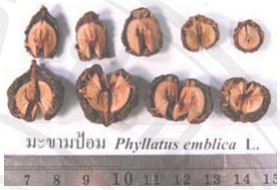
Physical appearance	Entire fruit is subspherical, wrinkle and about 2-3 cm in diameter 
% Loss on drying	6.6413
% Total ash	4.2345
% Acid insoluble ash	0.9050
% Extractive value	
Hexane extractive	6.2781
Dichloromethane extractive	7.4774
Ethanolic extractive	15.9893
Water extractive	37.6007
Chemical compounds screening	Flavonoids, hydrolysable tannin, terpenes, and green color of luminescence
Tannin (% w/w)	24.32
Gallic acid (% w/w)	2.11
Microbial test	Total aerobic count $<5.1x10^3$ (Regulation $<5.0x10^7$)
	Yeast and mold count $<9.0x10^2$ (Regulation $<5.0x10^4$)
	Enterobacteriaceae <10 (Regulation $<5.0x10^4$)
	Escherichia coli <10 (Regulation $<5.0x10^2$)
	Salmonella sp., *Clostridium* sp., *Staphylococcus aureus* not found
Aflatoxin test	Aflatoxin not found (detection limit of aflatoxin B1 = 5 ng)
Heavy metal test	As 0.21; Cd 0.07; Hg 0.02; Pb 0.17 ppm: less than regulation

Table 6 Monograph of *P. emblica* water extract

Physical appearance	The powder is brown, odor aromatic and sour, and astringent.
	
% Loss on drying	3.7919
% Total ash	6.0888
% Acid insoluble ash	0.8448
UV spectrum	λmax 217, 273 nm
IR spectrum	3296, 1715, 1612, 1536, 1447, 1331, 1212, 1141, 1036
Chemical compounds screening	Flavonoids, hydrolysable tannin, saponin, terpenes, and green color of luminescence
Tannins (% w/w)	42.51
Total carbohydrate (% w/w)	22.59
Uronic acid (% w/w)	10.99
Gallic acid (% w/w)	20.48
Microbial test	Total aerobic count $<1.9x10^2$ (Regulation $<5.0x10^7$)
	Yeast and mold count <1.5x10 (Regulation $<5.0x10^4$)
	Enterobacteriaceae <10 (Regulation $<5.0x10^4$)
	Escherichia coli <10 (Regulation $<5.0x10^2$)
	Salmonella sp., *Clostridium* sp., *Staphylococcus aureus* not found
Aflatoxin test	Aflatoxin not found (detection limit of aflatoxin B1 = 5 ng)
Heavy metal test	As 0.20; Cd 0.02; Hg 0.13; Pb 0.19 ppm: less than regulation

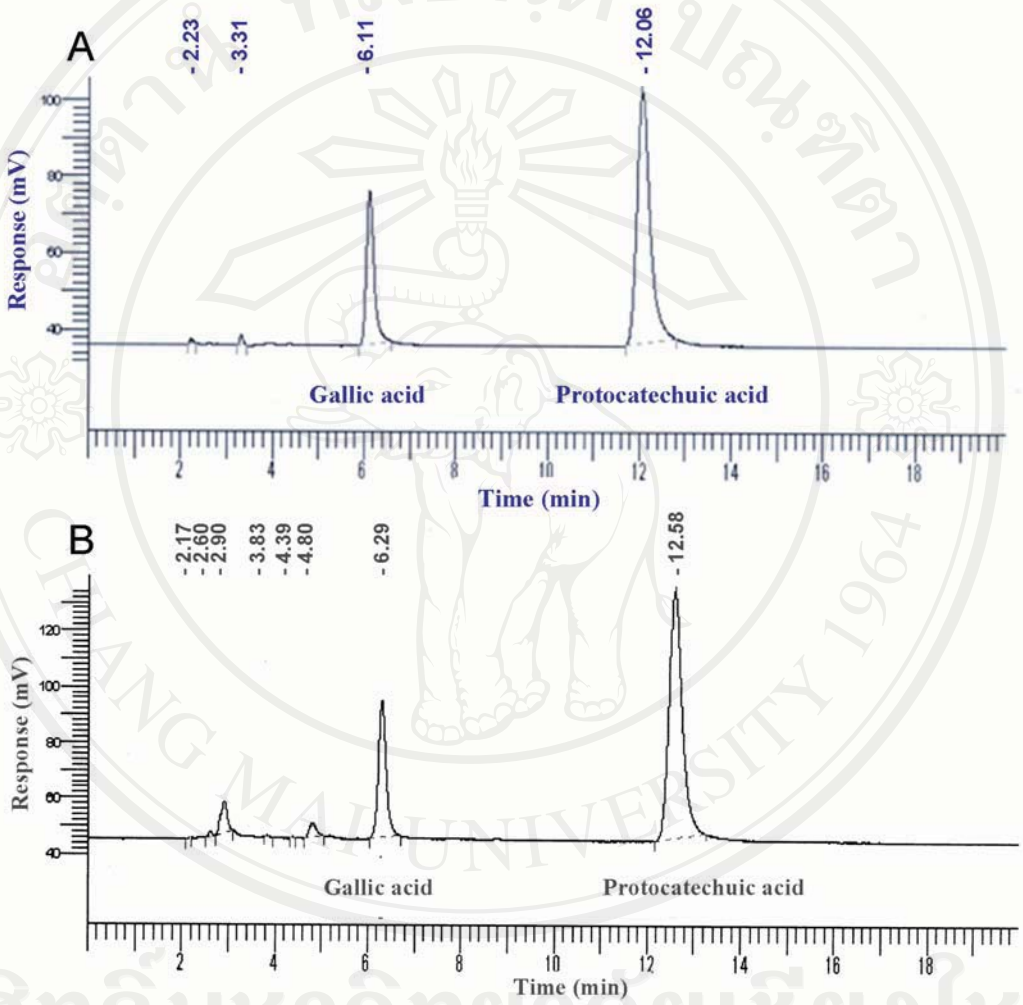


Figure 4 HPLC chromatogram of (A) standard gallic acid and protocatechuic acid and (B) *P. emblica* water extract compared to internal standard (protocatechuic acid).

2.7 Experimental protocol

2.7.1 Anti-inflammatory study

2.7.1.1 EPP-induced ear edema in rats (Brattsand *et al*., 1982)

This experiment was performed for investigation of the ability of an agent to inhibit increased vascular permeability leading to edema in inflammatory process.

Male rats weighing 40-60 g were used and divided into 3 groups of 3 animals each.

Group 1 control group, received DMSO and acetone (1:1) 20 µl/ear

Group 2 reference group, received phenylbutazone 1 mg/20 µl/ear

Group 3 test group, received *P. emblica* water extract 1 mg/20 µl/ear

Ear edema was induced by topical application of EPP 50 mg in 1 ml of acetone. EPP 1 mg/20 µl/ear was applied locally using an automatic microliter pipet to the inner and outer surfaces of both ears of each rat. *P. emblica* water extract, phenylbutazone and vehicle were applied in the same manner in a volume of 20 µl just before the irritants. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear was measured with digital vernier calipers. The protocol of experiment is shown in Figure 5.

The increase in ear thickness was compared with that of the vehicle-treated group and the percent inhibition was calculated as follows:

$$ED_x = ET_x - ET_o$$

$$\% ED = \frac{ED_c - ED_t}{ED_c} \times 100$$

where; ED_x = edema thickness at time x

ET_x = ear thickness (µm) at time x

ET_o = ear thickness (µm) before application of EPP or AA

ED_c = edema thickness (µm) of control group at time x

ED_t = edema thickness (µm) of test group at time x

% ED = percent edema inhibition of test substance at time x

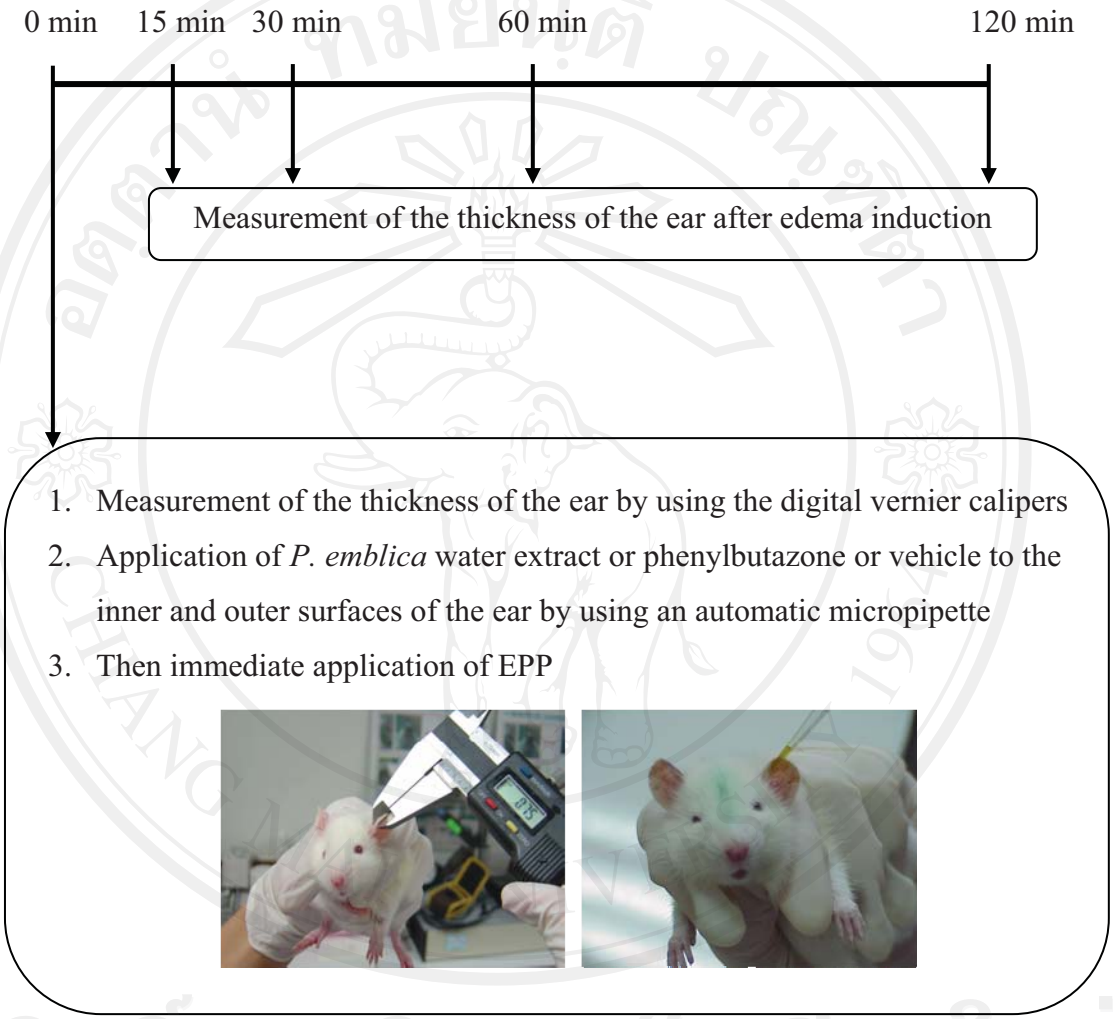


Figure 5 Diagram illustrating the procedure of the EPP-induced ear edema in rats.

7.1.2 AA-induced ear edema in rats (Young *et al*., 1984)

Male rats weighing 40-60 g were used and divided into 4 groups of 3 animals each.

Group 1 control group, received DMSO and acetone (1:1) 20 µl/ear

Group 2 reference group, received phenylbutazone 1 mg/20 µl/ear

Group 3 reference group, received phenidone 2 mg/20 µl/ear

Group 4 test group, received *P. emblica* water extract 1 mg/20 µl/ear

Ear edema was induced by topical application of AA 100 mg in 1 ml of acetone at a dose of 2 mg/20 µl/ear. Before and at 60 min after edema induction, the thickness of each ear was measured with digital vernier calipers. The protocol of experiment is shown in Figure 6.

7.1.3 Carrageenan-induced hind paw edema in rats (Winter *et al*., 1962)

This experiment was performed for investigation of the ability of the inhibitory effect of test agents on the hind paw edema formation induced by carrageenan. Male rats of 100-120 g body weight were divided into 5 groups of 6 animals each.

Group 1 control group, received distilled water

Group 2 reference group, received aspirin 300 mg/kg

Groups 3-5 test groups, received *P. emblica* water extract 150, 300 and 600 mg/kg, respectively

P. emblica extract, aspirin and distilled water were orally given 1 h prior to carrageenan injection. A volume of 0.05 ml of 1% carrageenan in sterile normal saline solution (NSS) was injected intradermally into the plantar of the right side of hind paw of an unanesthetized rat restrained in a plastic cage.

Foot volume of animal was determined by means of a volume displacement technique using a plethysmometer (model 7140, Ugo Basile, Italy). The right hind paw was immersed into the measuring chamber containing 0.05% sodium chloride in distilled water, exactly to an ink mark at anatomical hair line. Each paw volume was obtained from the average of 3 readings. The paw volume was measured prior to and

at 1, 3 and 5 h after carrageenan injection. The protocol of experiment is shown in Figure 7.

The edema volume of the paw and the percent edema inhibition of each test compound were obtained by the following calculation:

$$EV_x = PV_x - PV_o$$

$$\% EI_x = \frac{EV_x \text{ of control group} - EV_x \text{ of test group}}{EV_x \text{ of control group}} \times 100$$

where; EV_x = edema volume (ml) at time x

PV_x = paw volume (ml) at time x

PV_o = paw volume (ml) measured before carrageenan injection

$\% EI_x$ = percent edema inhibition of test substance at time x

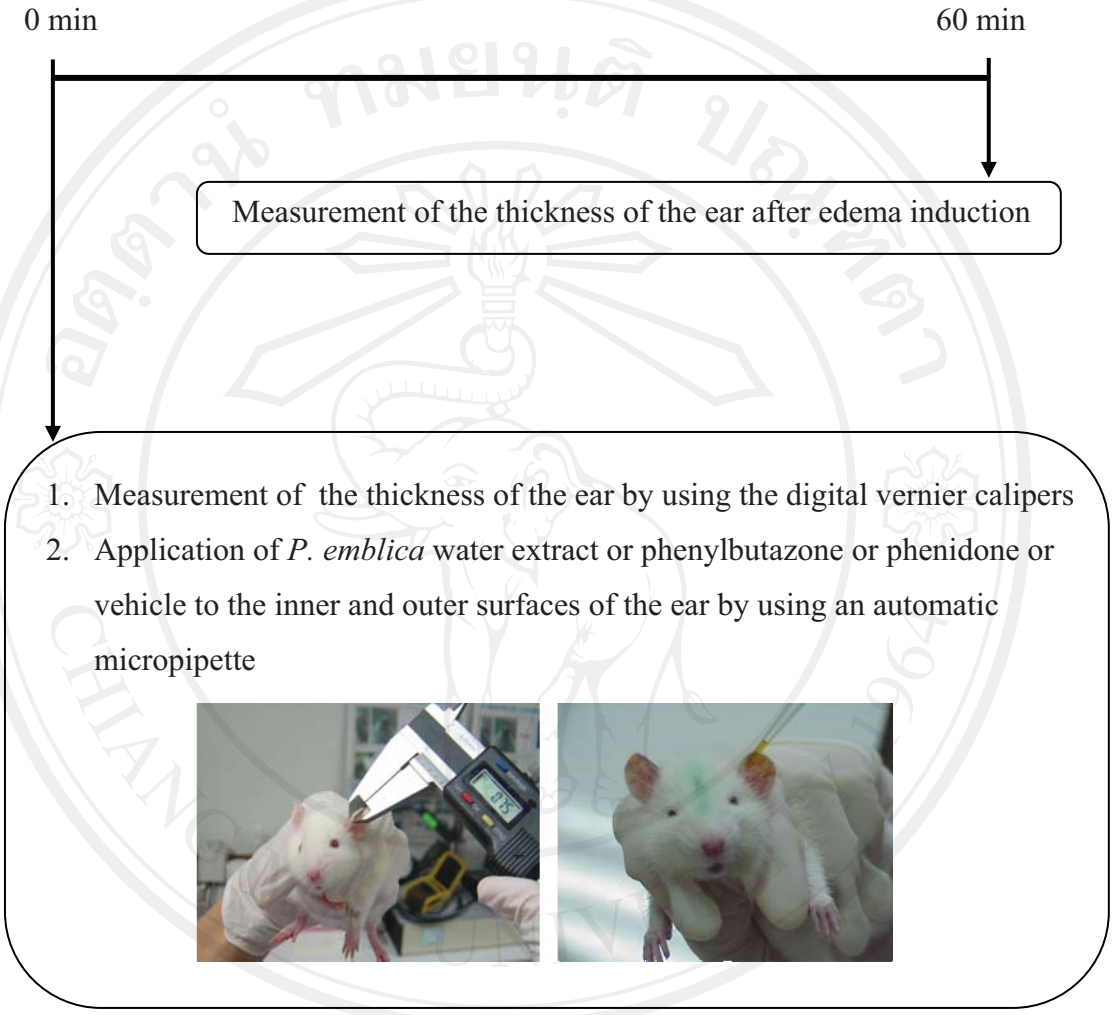


Figure 6 Diagram illustrating the procedure of the AA-induced ear edema in rats.

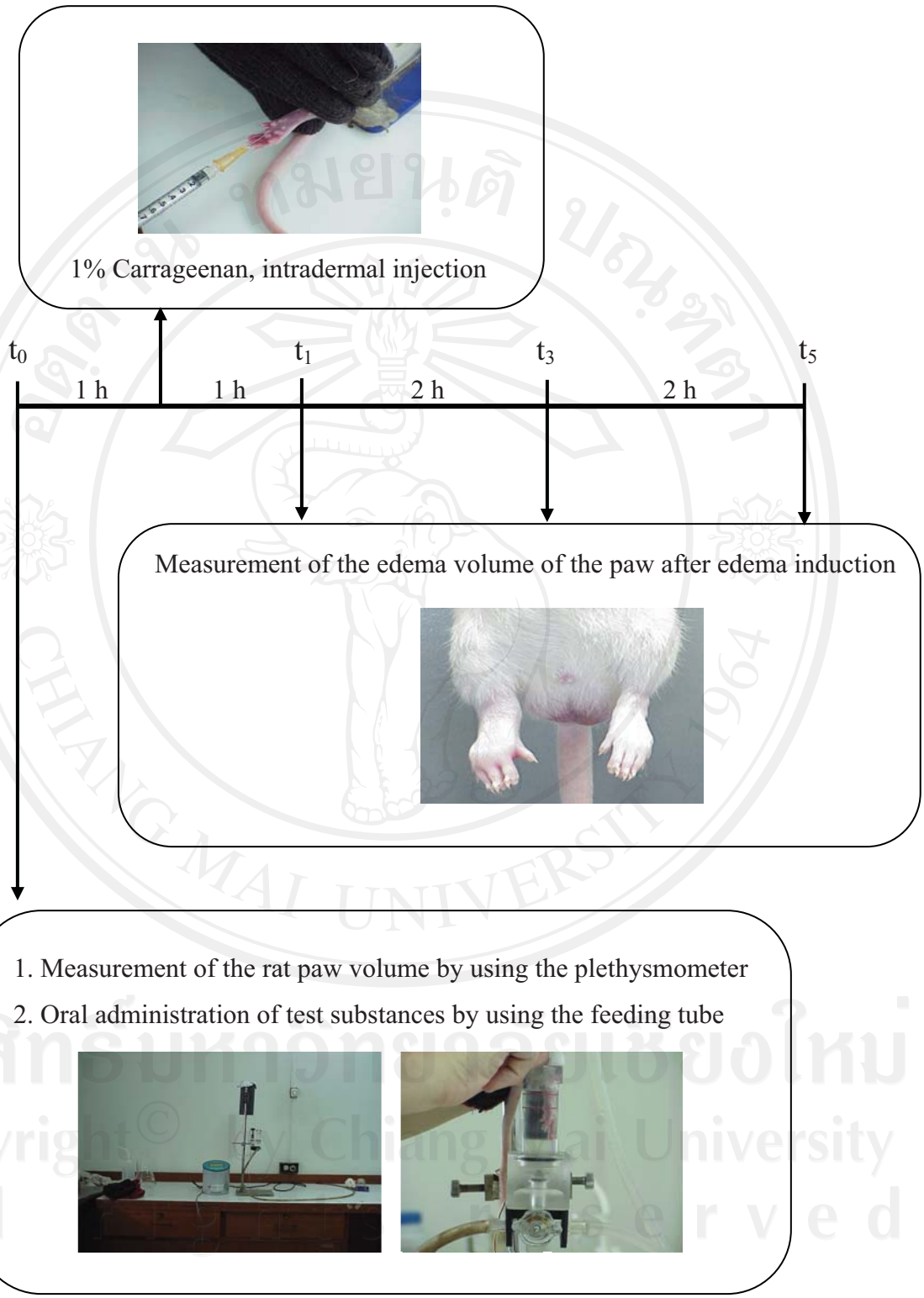


Figure 7 Diagram illustrating the procedure of the carrageenan-induced paw edema in rats.

7.1.4 Cotton pellet-induced granuloma formation in rats (Swingle and Shideman, 1972)

This experiment was performed for investigation of the ability of an agent to inhibit the proliferative component of the subchronic and chronic inflammatory process. The method was slightly modified as follows: adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made into a pellet. The pellets were sterilized in a hot air oven (model 600, Memmert, Germany) at 120 °C for 2 h. Male rats of 200-250 g body weight were used and divided into 5 groups of 6 animals each.

Group 1 normal group, received distilled water
Group 2 control group, received distilled water
Group 3 reference group, received aspirin 300 mg/kg
Group 4 reference group, received prednisolone 5 mg/kg
Group 5 test group, received *P. emblica* water extract 600 mg/kg

In groups 2-5, the abdominal skin of rats was shaped and disinfected with 70% alcohol. After that, two pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether anesthesia and sterile technique. The suture was then made and the animal was allowed to recover. The scheme of experiment is shown in Figure 8.

P. emblica water extract and prednisolone as well as aspirin were administered orally in a once daily dosage regimen throughout the experimental period of 7 days whereas the control group received distilled water only. On the eighth day after cotton pellets implantation, rats were anaesthetized with thiopental sodium (50 mg/kg, intraperitoneally). A cannula was inserted into the common carotid artery and blood was collected into a non-heparinized tube for determination of the amount of alkaline phosphatase and total protein. The enzyme activity was expressed as units of enzyme/mg of serum protein.

After collection of the blood, the rat was sacrificed and the abdominal skin was then opened. The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. Moreover, the thymus was also dissected out. Both the cotton pellets and the thymus were dried at 60 °C for 18 h and their dry weights were determined. The change in body weight from the first and the last day of experiment was also recorded.

The body weight gain, thymus dry weight, granuloma weight and transudative weight of test group were compared with those of the control group and reference groups. The percent granuloma inhibition of the test substance was calculated according to the following formula:

$$\text{Transudative weight} = Wt_w - Wt_d$$

$$\text{GW (mg/mg cotton)} = \frac{Wt_d - Wt_i}{Wt_i}$$

$$\%\ GI = \frac{\text{GW of control group} - \text{GW of test group}}{\text{GW of control group}} \times 100$$

where; Wt_w = wet weight of granuloma pellet (mg)

Wt_d = dry weight of granuloma pellet (mg)

Wt_i = initial dry weight of cotton pellet determined before implantation (mg)

GW = granuloma weight (mg)

% GI = percent granuloma inhibition

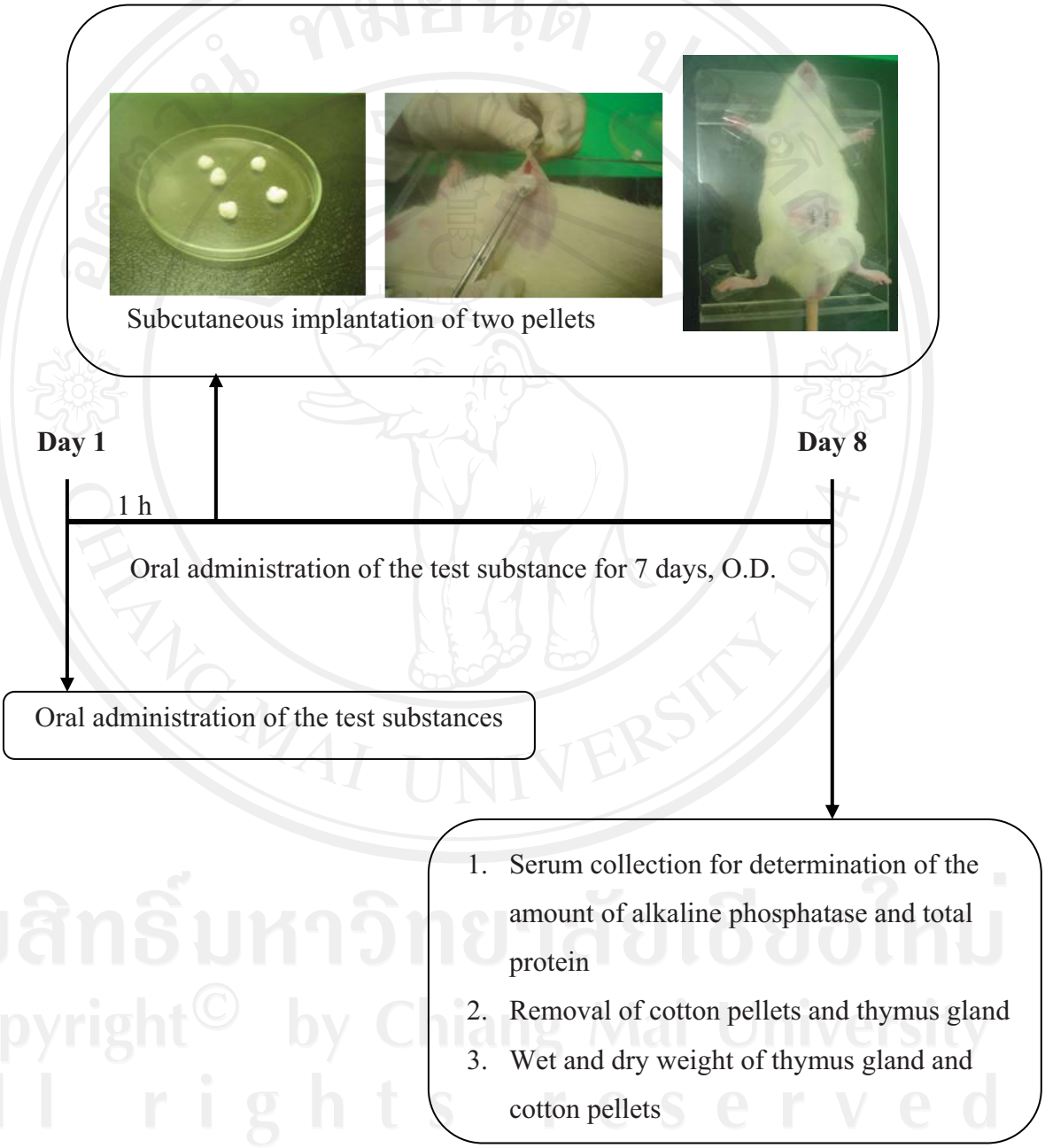


Figure 8 Diagram illustrating the method for cotton pellet-induced granuloma formation in rats.

2.7.2 Analgesic study

2.7.2.1 Formalin test in mice (Hunskaar and Hole, 1987)

The formalin test comprises the early phase and the late phase assessment of the analgesic effect of test substances. Male ICR mice weighing 30-40 g were used and divided into 6 groups of 6 animals each.

Group 1	control group, received distilled water
Group 2	reference group, received aspirin 300 mg/kg
Group 3	reference group, received morphine 10 mg/kg
Groups 4-6	test groups, received *P. emblica* water extract 150, 300 and 600 mg/kg

7.2.1.1 Early phase

P. emblica water extract and aspirin in a volume of 0.1 ml/10 g body weight were administered orally 1 h before formalin injection except morphine was administered by intraperitoneal injection 30 min before formalin injection. In the early phase assessment, 20 µl of 1% formalin in NSS was injected subcutaneously into the left dorsal hind paw after test substances administration. Then, between 0-5 min after formalin injection, the time in seconds the mice spent for intensive licking the left dorsal hind paw was determined. The scheme of experiment is shown in Figures 9.

7.2.2.2 Late phase

In the late phase assessment, another set of mice as above was used. The formalin was injected 40 min after oral administration of test drugs except morphine was administered by intraperitoneal injection 10 min before formalin injection and the licking time was determined between 20-30 min after formalin injection (Figure 10).

The licking time and the percent pain inhibition of each test compound were obtained by the following calculation:

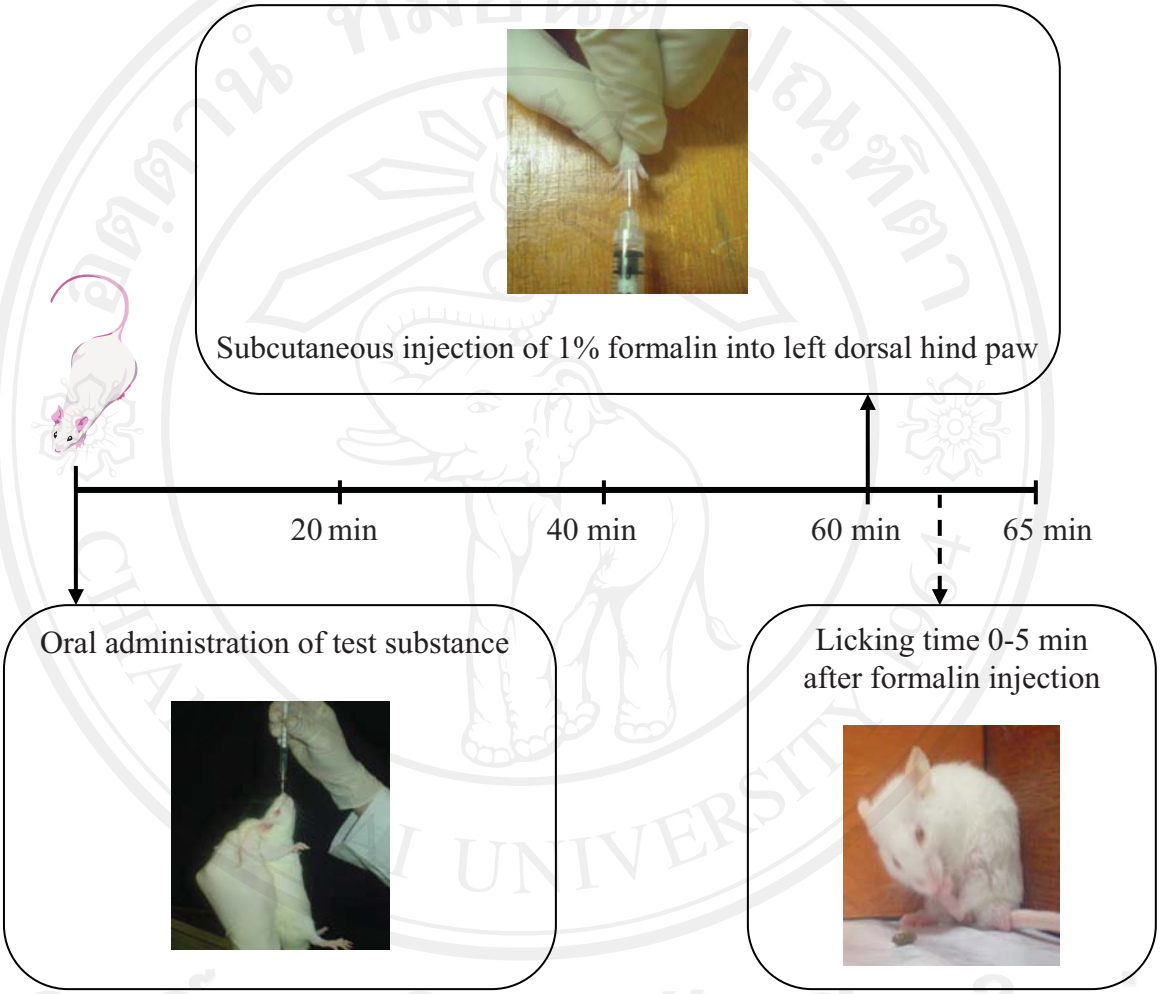


Figure 9 Diagram illustrating the procedure of the formalin test (early phase) in mice.

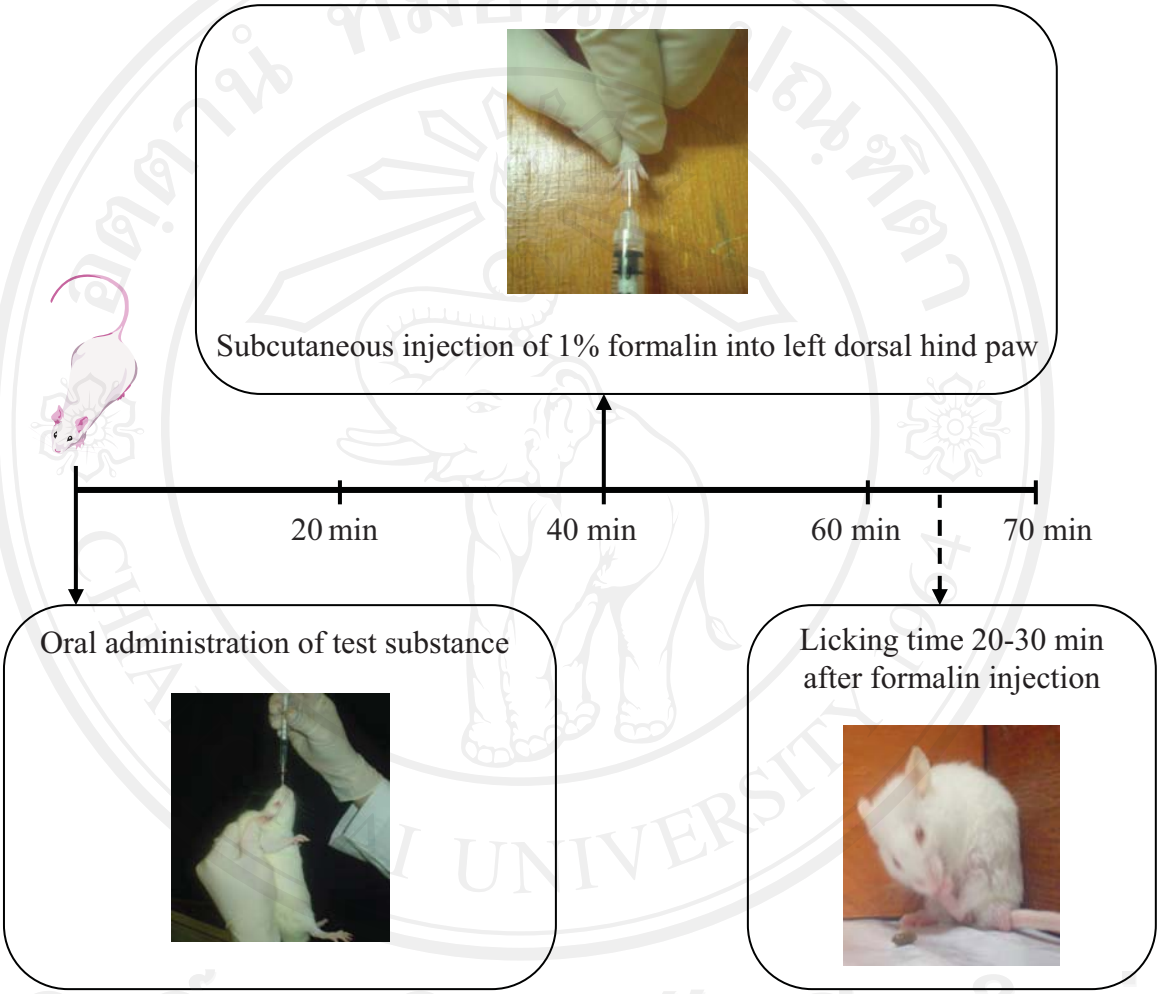


Figure 10 Diagram illustrating the procedure of the formalin test (late phase) in mice.

$$\% \text{ inhibition} = \frac{L_c - L_t}{L_c} \times 100$$

where; L_c = Licking time (sec) of control group

L_t = Licking time (sec) of test group

2.7.3 Antipyretic study

2.7.3.1 Yeast-induced hyperthermia in rats (Teotino *et al.*, 1963)

The antipyretic activity of *P. emblica* water extract was tested and compared with aspirin. Male rats weighing 200-250 g were used and divided into 5 groups of 6 animals each.

Group 1	control group, received distilled water
Group 2	reference group, received aspirin 300 mg/kg
Groups 3-5	test groups, received *P. emblica* water extract 150, 300 and 600 mg/kg, respectively

The rats were housed and maintained under uniform environmental conditions. Disturbances likely to disturb them were avoided. Before pyrexia was induced, the animals were restrained in plastic cages and the initial rectal temperatures were recorded using a twelve-channel electric thermometer (LETICA, model TMP 812 RS, Panlab S.L., Spain) connected with the probes which were inserted into the rat rectums to about 5 cm deep. In order to adapt the rat to the handling procedure for probe insertion, the basal rectal temperature was taken 1 h after probe insertion. Thereafter hyperthermia was induced in rats by subcutaneous injection of 1 ml/100 g body weight of 25% brewer's yeast in NSS. When the temperature was at a peak, 18 h after yeast injection, the rectal temperature was again recorded. Those animals which showed a rise in rectal temperature of more than 1 °C were used. Test substances were then administered orally and the rectal temperature of animals was recorded at 30 min interval for 2 h following drug treatment. The protocol is diagrammatically shown in Figure 11.

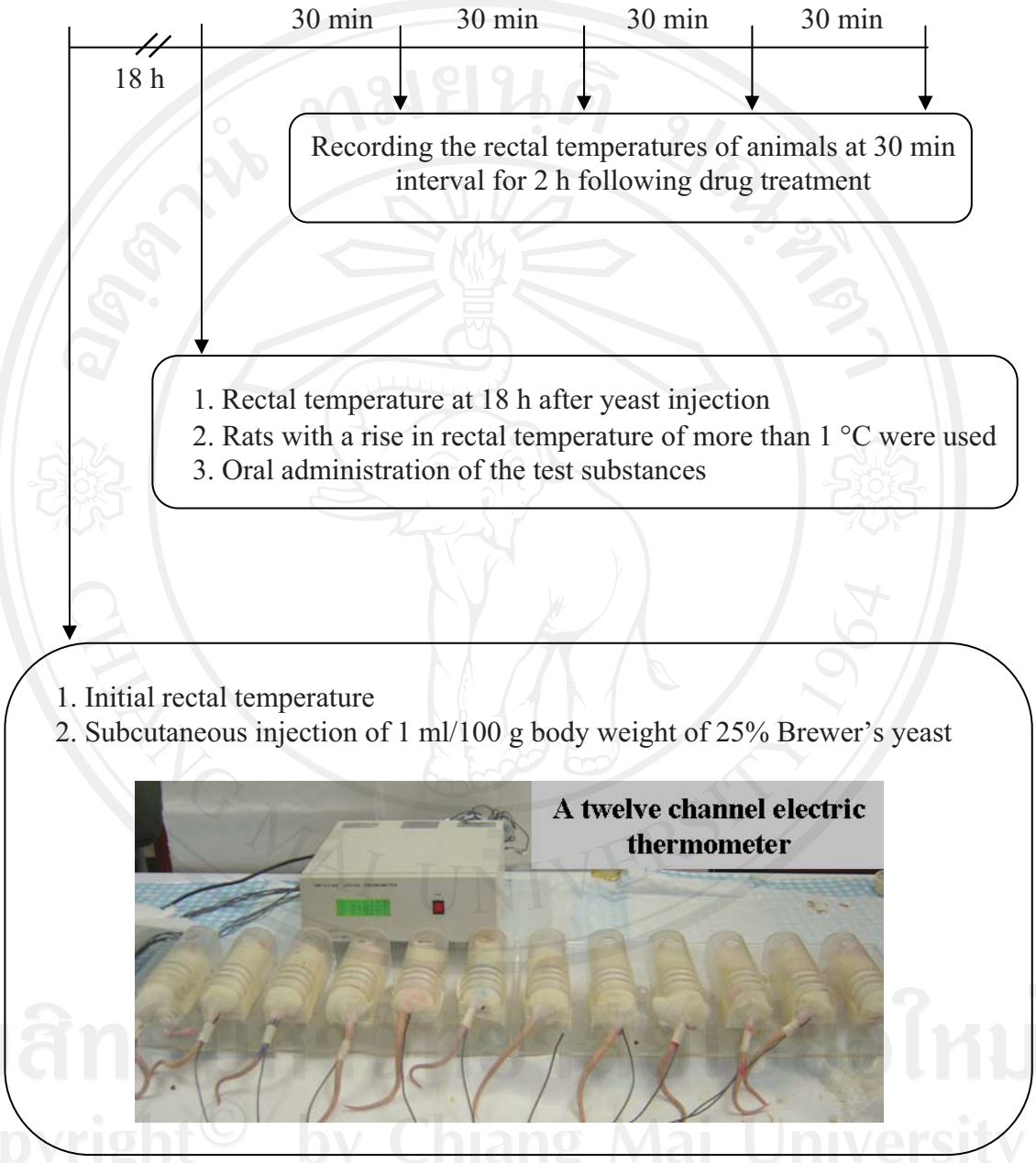


Figure 11 Diagram illustrating the procedure of the yeast-induced hyperthermia in rats.

2.7.4 COX inhibitor screening assay

The Cayman COX inhibitor screening assay kit was used in this study. The COX inhibitor screening assay directly measures $PGF_{2\alpha}$ produced by stannous chloride ($SnCl_2$) reduction of COX-derived PGH_2. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major PG compounds. The Cayman COX assay is more accurate and reliable than an assay based on peroxidase inhibition. The Cayman COX inhibitor screening assay includes both ovine COX-1 and human recombinant COX-2 enzymes in order to screen isozyme-specific inhibitors. This assay is an excellent tool which can be used for general inhibitor screening, or to eliminate false positive leads generated by less specific methods.

2.7.4.1 Performing COX reaction

1. Background tubes

   COX-1 and COX-2 were inactivated by transferring 20 µl of each enzyme to a 500 µl microfuge tube and placed in boiling water for 3 min. The inactivated enzymes were used to generate the background values. The following reagents were added to two test tubes: 970 µl of reaction buffer, 10 µl of heme, and 10 µl of inactive COX-1 or inactive COX-2 (Figure 12A).

2. COX-1 or COX-2 100% initial activity tubes

   Nine hundred fifty microliter of reaction buffer, 10 µl of heme, 10 µl of COX-1 or COX-2 were added to 2 test tubes. Then, 20 µl of buffer or solvent vehicle was added to the 100% initial activity tubes (Figure 12B).

3. COX-1 or COX-2 inhibitor tubes

   Nine hundred fifty microliter of reaction buffer, 10 µl of heme, 10 µl of COX-1 or COX-2 were added to 6 test tubes. Then, 20 µl of test substance in buffer or solvent vehicle (aspirin, celecoxib, *P. emblica* water extract) was added to the COX-1 and COX-2 inhibitor tubes (Figure 12C).

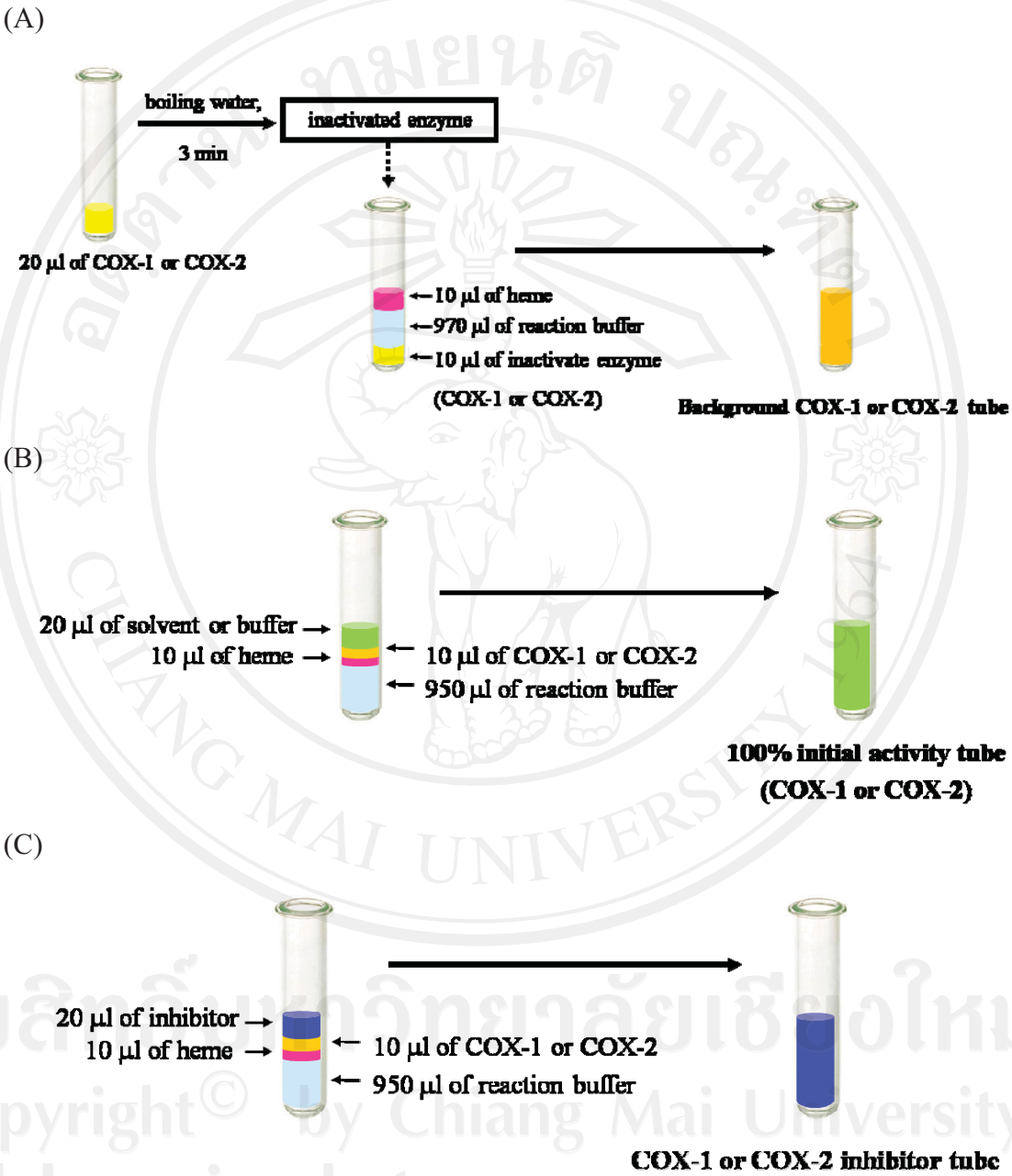


Figure 12 Diagram illustrating the procedure for preparing background tube (A), 100% initial activity tube (B) and inhibitor tube (C).

4. All test tubes were incubated for 10 min at 37 °C. The reaction was initiated by adding 10 µl of AA to all the test tubes. Then, the test tubes were vortexed and incubated for another 2 min at 37 °C. Fifty microliter of 1 M HCl was added to each test tube to stop enzyme catalysis. The test tubes were removed from the water bath and added 100 µl of the saturated stannous chloride solution to each test tube and vortexed. The test tubes were incubated for 5 min at room temperature. The protocol of performing COX reaction is shown in Figure 13. The PGs were quantified by EIA.

2.7.4.2 EIA pre-assay preparation

1. PG standard

The lyophilized PG standard was dissolved in 1 ml of EIA buffer. The concentration of this solution (the bulk standard) was 10 ng/ml. The preparation of the PG standard by two-fold serial dilutions (tube S1-S8) is shown in Figure 14.

2. COX reaction dilution

a. Background sample (inactive enzyme tubes)

Two clean test tubes were obtained and labeled BC1 and BC2. The scheme of preparation is shown in Figure 15. Nine hundred ninety microliter of EIA buffer was added to each test tube. Ten microliter of background COX-1 and COX-2 were added to the tube labeled BC1 and COX-2, respectively. Then, the test tubes were mixed thoroughly. Each test tube contains a 1:100 dilution of the original sample.

b. 100% Initial activity samples

Three clean test tubes per sample were obtained and numbered them IA1 through IA3. The protocol of preparation is shown in Figure 16. Nine hundred ninety microliter of EIA buffer and 10 µl of 100% initial activity sample were added to tube IA1 and mixed thoroughly. Nine hundred fifty microliter of EIA buffer and 50 µl of tube IA1 were added to tube IA2 and mixed thoroughly. Tube IA2 contained a 1:2,000 dilution of the original sample. Five hundred microliter of EIA buffer and 500 µl of tube IA2 were added to tube IA3 and mixed

thoroughly. Tube IA3 contained a 1:4,000 dilution of the original sample. Tubes IA2 and IA3 were run in the EIA.

c. COX inhibitor samples

Three clean test tubes per sample were obtained and numbered them C1 through C3. The protocol is diagrammatically shown in Figure 17. Nine hundred ninety microliter of EIA buffer and 10 µl of COX inhibitor sample were added to tube C1 and mixed thoroughly. Nine hundred fifty microliter of EIA buffer and 50 µl of tube C1 were added to tube C2 and mixed thoroughly. Tube C2 contains a 1:2,000 dilution of the original sample. Five hundred microliter of EIA buffer and 500 µl of tube C2 were added to tube C3 and mixed thoroughly. Tube C3 contains a 1:4,000 dilution of the original sample. Tubes C2 and C3 were run in the EIA.

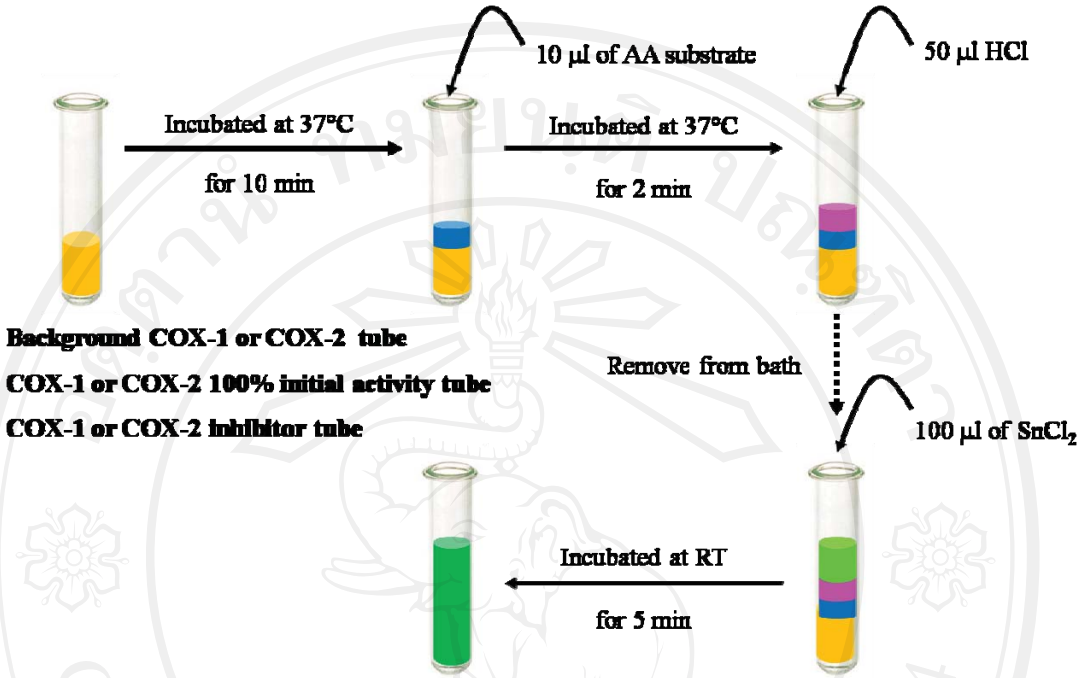


Figure 13 Diagram illustrating the procedure for performing COX reaction.

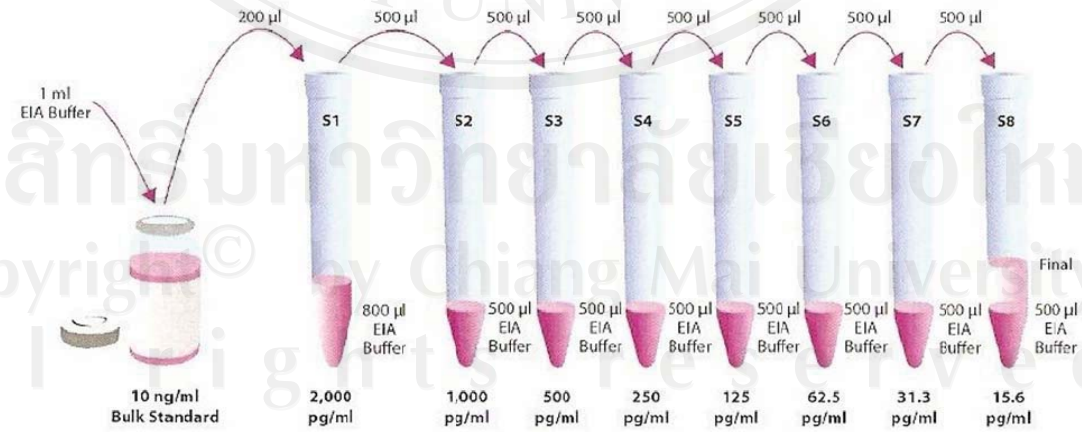


Figure 14 The preparation of the PG standard for EIA.

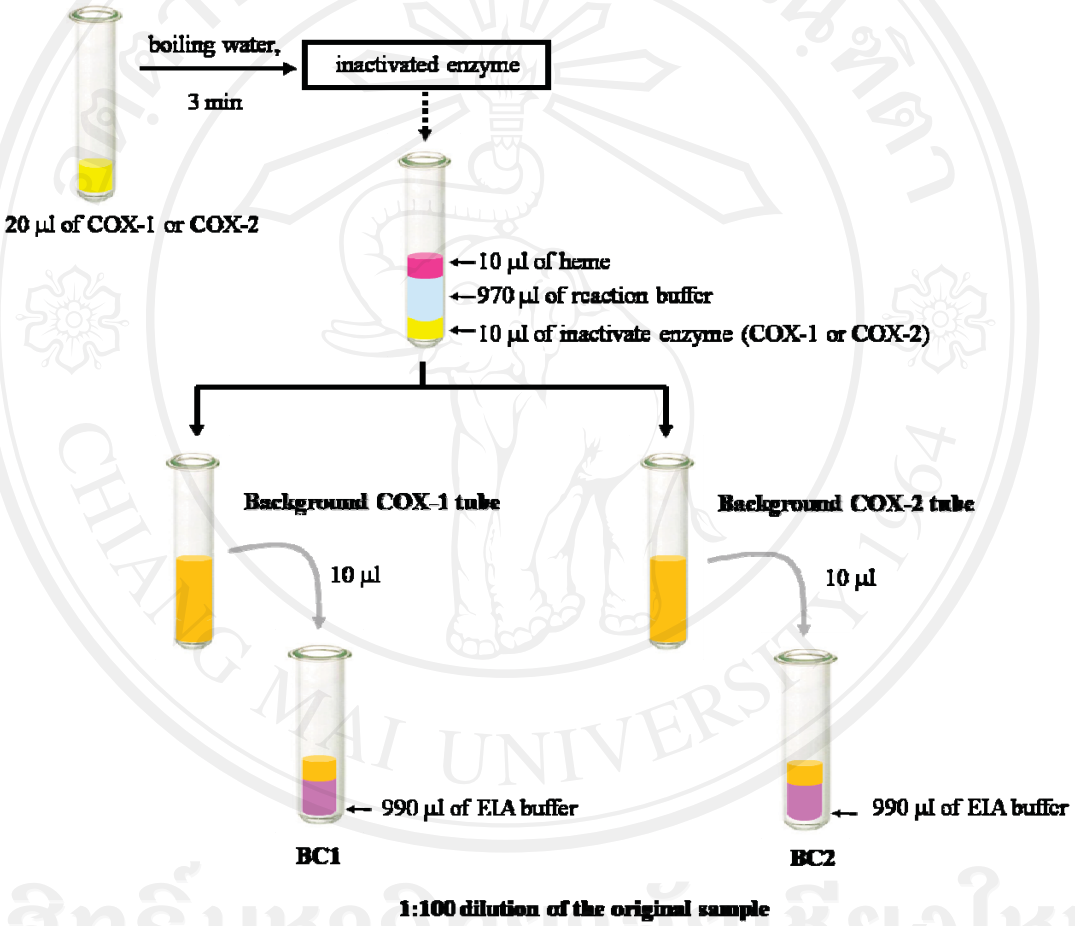


Figure 15 The preparation of background sample for EIA.

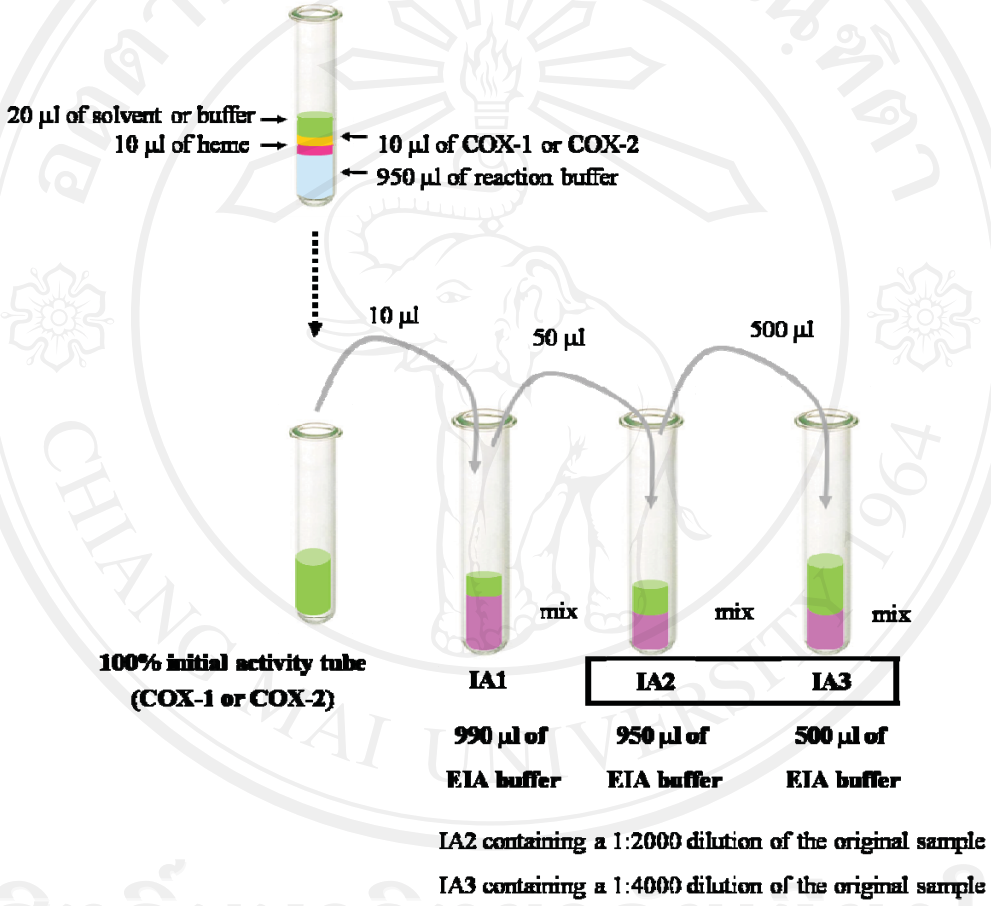


Figure 16 The preparation of 100% Initial activity samples for EIA.

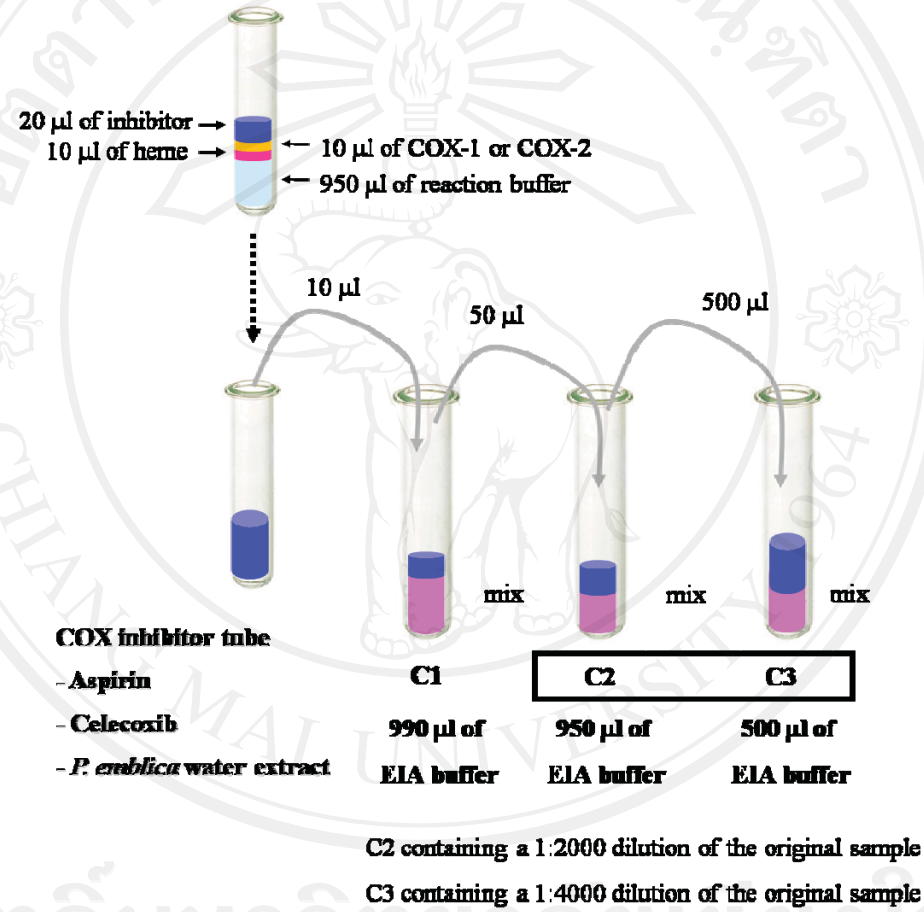


Figure 17 The preparation of COX inhibitor samples for EIA.

2.7.4.3 Performing the assay

EIA assay is based on the competition between PGE_2 and PG acetylcholinesterase (AChE) tracer (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibody. Because the concentration of the PGE_2 tracer is held constant while the concentration of PGE_2 varies, the amount of PGE_2 tracer that is able to bind to the PG screening antiserum (primary antibody) will be inversely proportional to the concentration of PGE_2 in the well. This antibody PGE_2 complex binds to mouse anti-rabbit IgG (secondary antibody) that has been previously attached to the well.

Plate set up

The 96 well plate included with this kit was supplied ready to use. It was not necessary to rinse the plate prior to adding the reagents.

Pipetting the reagents (Figure 18)

1. EIA buffer

   One hundred microliter of EIA buffer was added to non-specific binding (NSB) wells, and 50 µl of EIA buffer was added to maximum binding (B_0) wells.

2. PG standard (Test tube S1-S8)

   Fifty microliter of tube S8 was added to both of the lowest standard wells, and 50 µl of tube S7 to each of the next two standard wells. This procedure was continued until all the standards were aliquoted.

3. Background samples (test tubes BC1 and BC2)

   Fifty microliter of sample per well was added. Each sample was assayed in duplicate.

4. COX 100% initial activity samples (test tube IA2 and IA3)

   Fifty microliter of sample per well was added. Each sample was assayed in duplicate.

5. COX inhibitor samples (test tubes C2 and C3)

   Fifty microliter of sample per well was added. Each sample was assayed in duplicate.

6. PG screening AChE tracer

Fifty microliter of PG screening AChE tracer was added to each well except for the total activity (TA) and the blank (Blk) wells.

7. PG screening antiserum

Fifty microliter of PG screening antiserum was added to each well except for the TA, the NSB, and the Blk wells.

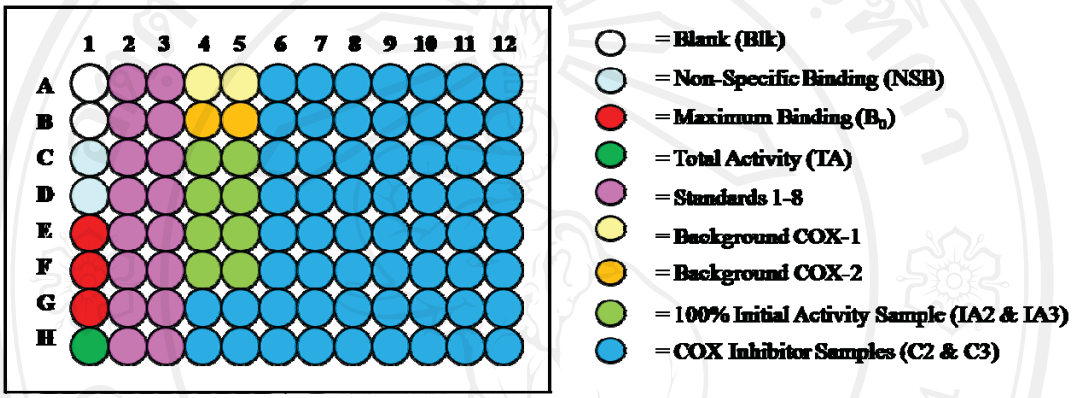


Figure 18 Suggested plate format.

Incubation the plate

The plate was covered with plastic film and incubated for 18 h at room temperature.

Development the plate

When ready to develop the plate, Ellman's reagent was reconstituted with 20 ml of UltraPure water. The wells were emptied and rinsed five times with wash buffer. Two hundred microliter of Ellman's reagent was added to each well and 5 µl of tracer to the TA well. The plate was covered with plastic film. Optimum development was obtained by using an orbital shaker equipped with a large, flat cover to allow the plate to develop in the dark. This assay typically developed in 60-90 min. The product of this enzymatic reaction had a distinct yellow color. The intensity of this color, determined spectrophotometrically, was proportion to the amount of PGE_2 tracer bound to the well (Figure 19).

Read the plate

The plate was read at 410 nm wavelength using a microplate reader. The plate was checked periodically until the B_0 wells had reached a minimum of 0.3 A.U. The plate was read when the absorbance of the B_0 wells was in the range of 0.3-0.8 A.U.

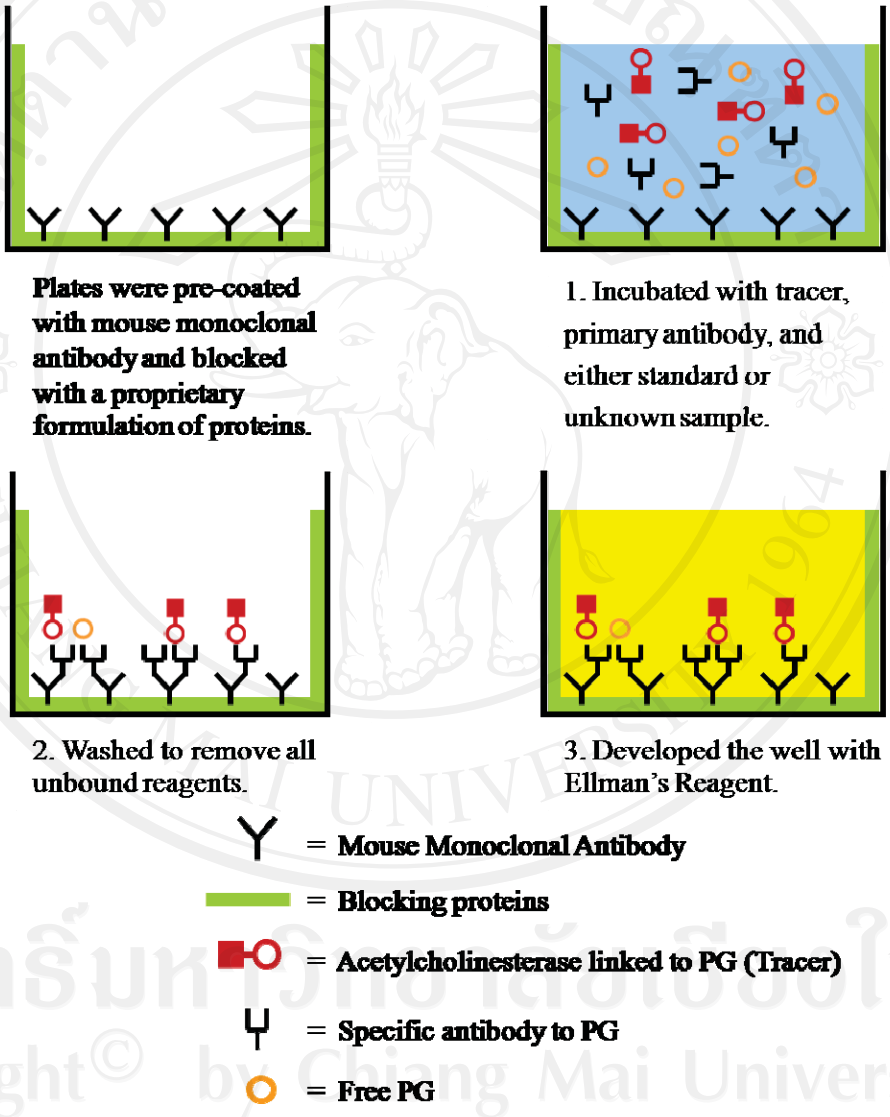


Figure 19 Enzyme immunoassay (EIA) for determination of the prostanoid products.

2.7.4.4 Calculating the results

Preparation of the data

The following procedure was used for preparation of the data prior to graphical analysis.

1. The absorbance reading from the NSB and B_0 wells was averaged.
2. The NSB average was subtracted from the B_0 average. This was the corrected B_0 or corrected maximum binging.
3. The % B/B_0 (% sample or standard bound/maximum bound) for the remaining wells was calculated according to the following formula:

$$\% B/B_0 = \frac{\text{Standard or sample absorbance} - \overline{\text{NSB}}}{\text{Corrected } B_0} \times 100$$

Determining the concentration of PG in the samples

1. The % B/B_0 value for each sample was calculated
2. The PG concentration of each sample was determined by identifying the % B/B_0 on the standard curve and reading the corresponding values on the x-axis. The COX samples were multiplied by the appropriate dilution factor (BC1 and BC2 = 100; IA2 and C2 = 2,000; IA3 and C3 = 4,000).
3. The background value (BC1 and BC2) was subtracted from the 100% initial activity and COX inhibitor samples.
4. Each inhibitor sample was subtracted from the 100% initial activity sample, then divided by the 100% initial activity sample, and multiplied by 100 to give the percent inhibition.
5. The percent inhibition was graphed with the inhibitor concentration to determine the IC_{50} value (concentration at which there is 50% inhibition).

2.7.5 Chondroprotective test

2.7.5.1 Cartilage explants culture

Articular cartilage was dissected from the metacarpophalangeal joints of pigs aged 20-24 weeks. Cartilage discs (3 mm^2) was biopsied from the weight-bearing region of the articular surface. Randomly selected explant discs (3 per well, approximately 30 mg total) were cultured in a 24-well tissue culture plate with serum-free medium (Dulbecco's modified Eagle's medium, DMEM) containing 200 units/ml penicillin and 200 μg/ml streptomycin. The explants were maintained in a humidified incubator with 5% CO_2 at 37 °C for 24 h and the media were collected (day 0 media) prior to the three days of treatment. Recombinant human IL-1β at 5 ng/ml was added to induce cartilage degradation in culture medium with or without *P. emblica* water extract. As a positive control, culture medium supplemented with diacerein was used. Treatments were performed in triplicated wells using tissue from the same animal donor. The protocol of experiment is shown in Figure 20. Conditioned media were collected on day 3 of culture, stored at -20 °C until analysis for indicators of degradation. Extracellular matrix (ECM) biomolecules released from cartilage induced by IL-1β were glycosaminoglycan (GAG) and hyaluronan (HA). The release of ECM biomolecules was estimated by calculating:

$$\% \text{ change} = \{(\text{Day 3 medium} - \text{Day 0 medium})/ \text{Day 0 medium}\} \times 100$$

2.7.5.2 Measurement of GAG levels (Farndale *et al*., 1986)

The sulfated glycosaminoglycan (S-GAG) concentrations were determined using a colorimetric dye binding assay. The maximum absorbance of the dye solution was at 620 nm. Chondroitin 6-sulfate (CS-C) standards (0-40 μg/ml, 50 μl) or samples (50 μl) were transferred to a microplate. The dye solution (200 μl) was added to each well and absorbance was immediately measured at 620 nm since precipitation might form on standing. A standard curve of CS-C concentration and absorbance at 620 nm was plotted. The concentration of CS-C in the samples was calculated from the standard curve. The protocol of experiment is shown in Figure 21.

Figure 20 Chondroprotective test: cartilage explants culture.

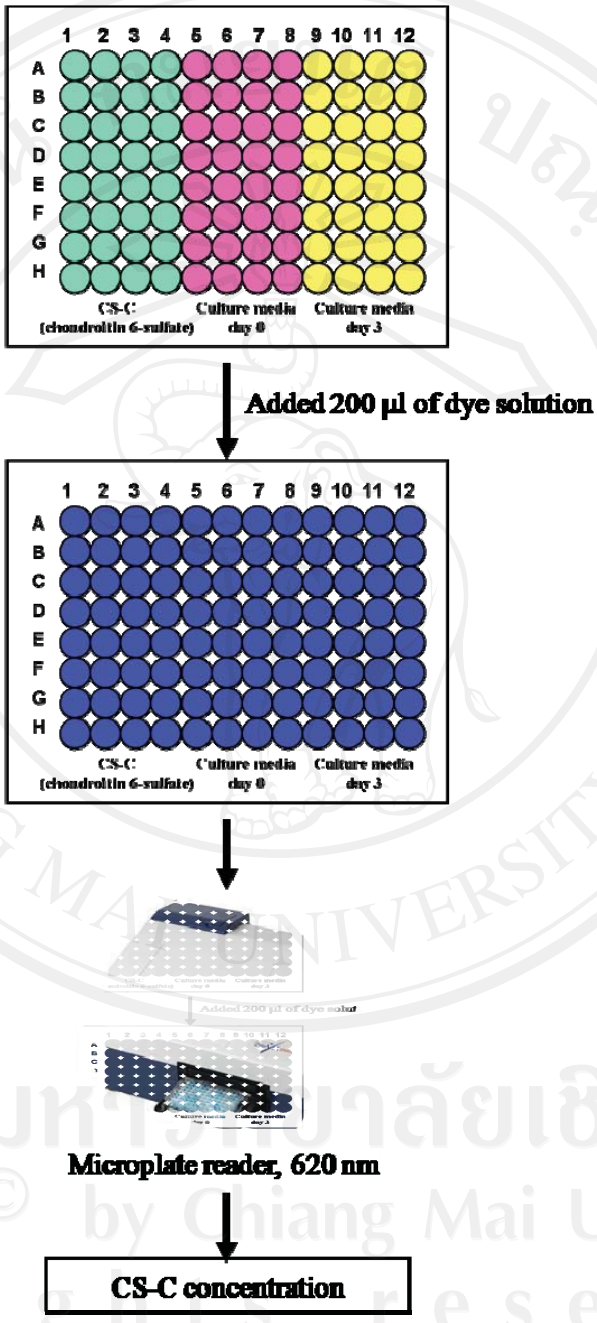


Figure 21 A colorimetric dye binding assay: measurement of GAG levels.

2.7.5.3 Measurement of HA levels (Kongtawelert and Ghosh, 1990)

HA level was measured using a competitive inhibition based enzyme-linked immunosorbent assay (ELISA) as previously described, with modification. Briefly, culture media samples containing unknown amounts of HA (175 µl), as well as standard samples containing known concentrations of highly purified HA were placed in small polypropylene tubes of appropriate concentrations of biotinylated-HA binding proteins (B-HABP) (175 µl) and incubated at room temperature (25 °C) for 1 h. Aliquots (100 µl) of this reaction mixture were applied to umbilical cord HA-coated and bovine serum albumin (BSA)-blocked microplates and incubated at 25 °C for 1 h. The wells were then washed with phosphate buffered saline (PBS) solution, and the appropriate dilution of anti-biotin peroxidase conjugate (1:2000 in PBS) was added to each well, incubated at 25 °C for 1 h and washed. Peroxidase substrate (OPD, o-phenylenediamine) was then added. After incubation at 25 °C for 20 min, the reaction was stopped by the addition of 4 M H_2SO_4 (50 µl). The absorbance ratio at 492/690 nm was measured using a microplate reader. HA concentrations in the culture media samples were calculated relative to a standard curve generated from the purified HA preparation. The protocol is diagrammatically shown in Figure 22.

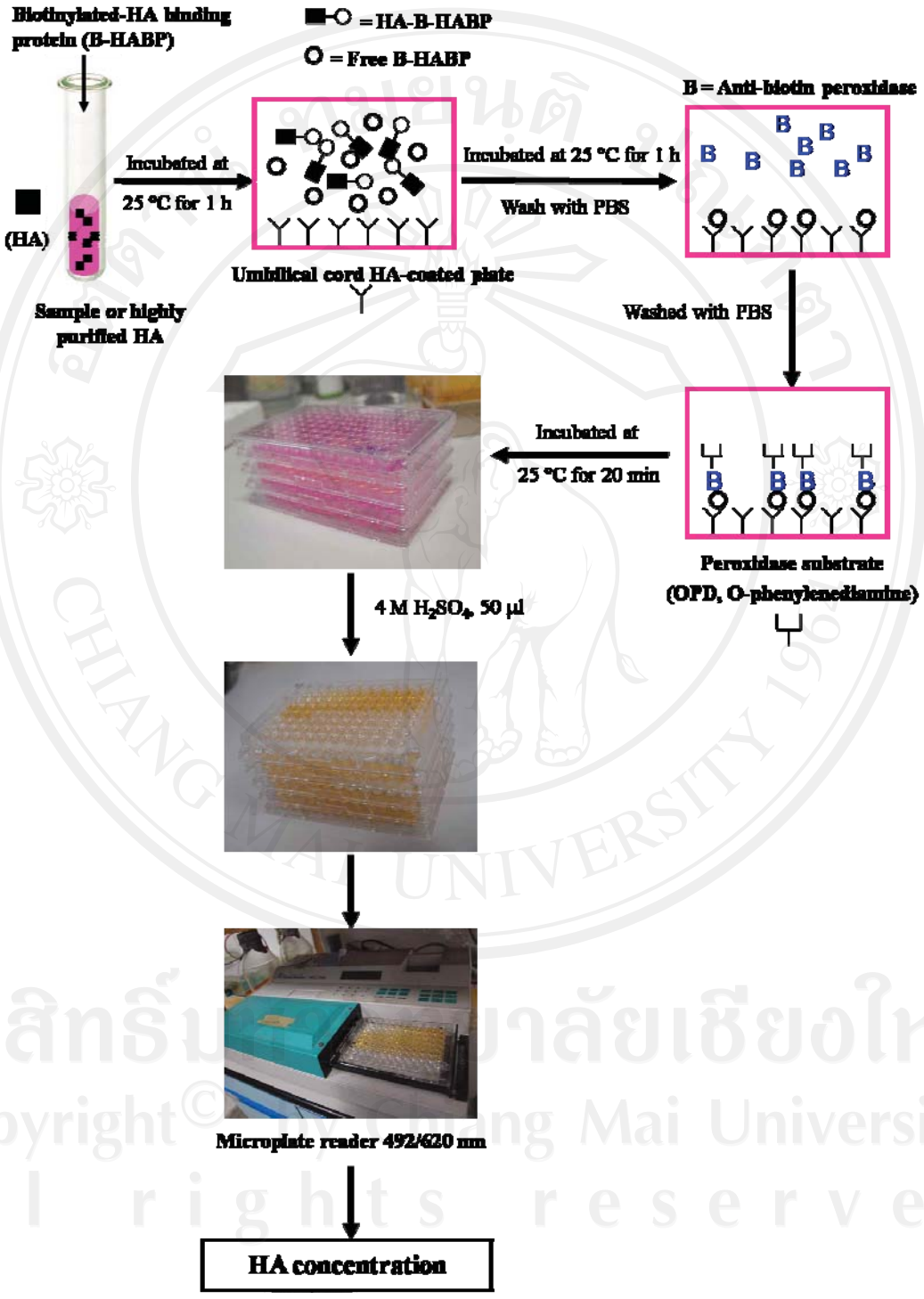


Figure 22 A competitive inhibition based ELISA: measurement of HA levels.

2.7.5.4 Determination of uronic acid content (Blumenkrantz and Asboe-Hansen, 1973; Taylor, 1992)

Uronic acid is widely determined as the representative of GAGs in biological substances. This assay measures uronic acid by releasing the monosaccharide using acid hydrolysis. Cartilage was collected on day 21 of culture, and then digested with 200 µl of papain solution (2 U/ml) at 60 °C for 48 h. A portion of papain-digested cartilage was used for determining uronic acid. For a standard curve, 0 to 2.4 µg of glucuronic acid lactone or sample (5 µl of dilution; 1:10) in up to 60 µl of water was added to a test tube. Concentrated sulfuric acid-borate reagent (300 µl) was added to the tube and mixed. The tubes were then incubated at 100 °C in a water bath for 10 min and cooled to room temperature in ice bath. The solution of carbazole at the concentration of 50 mg/40 ml in absolute ethanol (12 µl) was added and mixed. The uronic acid reaction was incubated at 100 °C in a water bath for 15 min. The absorbance of the pink to red color was read in a spectrophotometer at 540 nm against distilled water blank. The protocol of experiment is shown in Figure 23.

7.5.5 Collagen content measured by hydroxyproline (HPR) assay (Hoemann *et al*., 2002)

The collagen content in the samples (cartilage) was calculated based on hydroxyproline determinations. Cartilage was collected on day 21 of culture. The cartilages were then digested with 200 µl of papain solution (2 U/ml) at 60 °C for 48 h. A portion of papain-digested cartilage (100 µl) was hydrolyzed with HCl (100 µl) for 8 h at 100 °C and at 60 °C overnight. After that, the pH of sample was adjusted to 6.0 with 6 N NaOH. Samples (50 µl) were combined with 100 µl of freshly prepared oxidizing solution (chloramines-T) and incubated for 5 min at room temperature. Samples were mixed with 100 µl of Ehrlich's reagent, incubated at 60 °C for 45 min, and absorbance at 570 nm was measured with a spectrophotometer. Samples were extrapolated against hydroxyproline standards between 0 and 10 µg/ml. The protocol is diagrammatically shown in Figure 24.

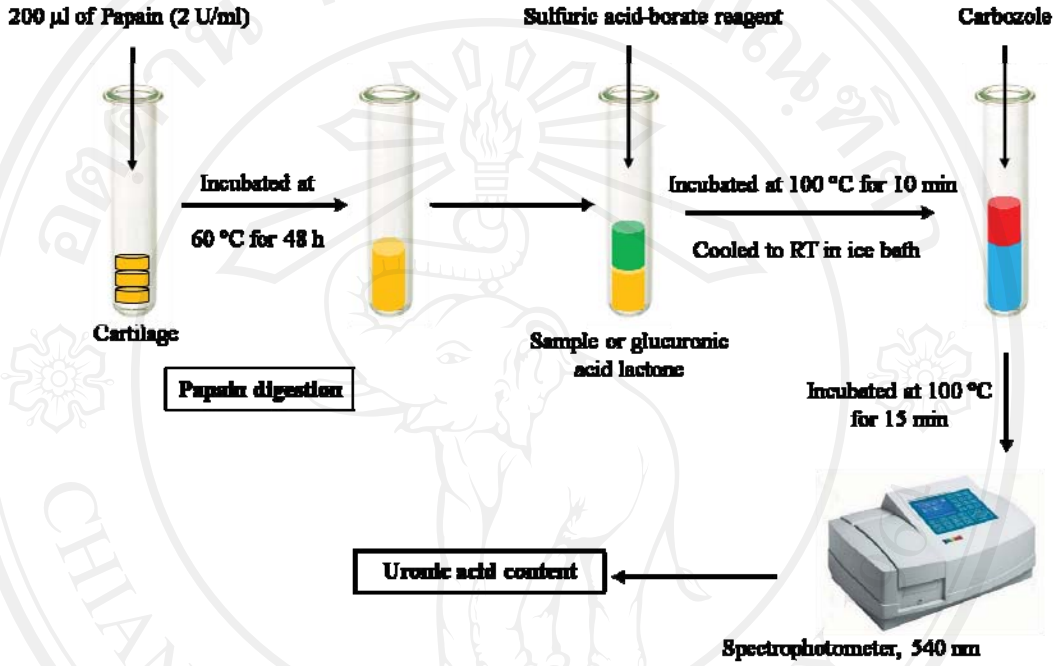


Figure 23 Determination of uronic acid content.

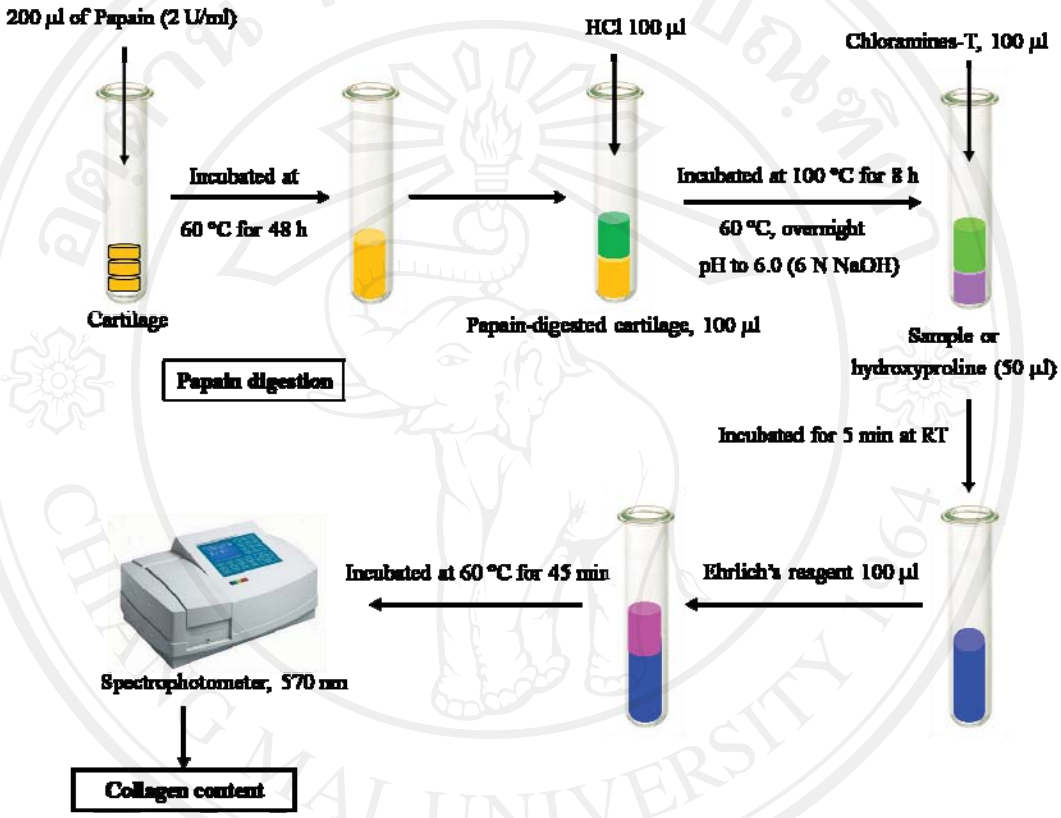


Figure 24 Hydroxyproline assay: measurement of collagen content.

2.7.6 Anti-ulcerogenic study

2.7.6.1 Preparation of rats for anti-ulcerogenic activity study

Male rats weighing 250-300 g were fasted 48 h, but had free access to water. The water was withdrawn 1 h before starting the experiment. In each method of ulcer induction, the rats were divided into 7 groups of 6 animals each.

Group 1	normal group, received distilled water
Group 2	negative control group, received *P. emblica* water extract 600 mg/kg
Group 3	positive control group, received distilled water
Group 4	reference group, received cimetidine 100 mg/kg
Groups 5-7	test groups, received *P. emblica* water extract 150, 300 and 600 mg/kg, respectively

P. emblica water extract, cimetidine or distilled water was given orally to the rats 1 h before induction of gastric lesions (Groups 3-7).

2.7.6.2 Methods used to induce gastric lesions

2.7.6.2.1 Ethanol/hydrochloric acid (EtOH/HCl) (Mizui and Doteuchi, 1983)

Each rat was administered 1 ml of EtOH/HCl (absolute ethanol 60 ml + HCl 1.7 ml + water 38.3 ml) orally. One hour later, the rats were sacrificed for determination of gastric lesions (Figure 25).

2.7.6.2.2 Indomethacin (Nwafor *et al*., 2000 with modification)

Suspension of indomethacin in 5% carboxymethycellulose was administered orally with a single dose of 100 mg/kg to the rats. Five hours later, the rats were sacrificed for determination of gastric lesions (Figure 26).

2.7.6.2.3 Restraint water immersion stress (Takagi and Okabe, 1968)

Rats were restrained in stainless steel cages and immersed up to their xiphoid in a water bath maintained at 20 ± 2 °C. Five hours after restraint in cool water, the rats were sacrificed for determination of gastric lesions (Figure 27).

After the rats were sacrificed, the stomachs were removed and opened along the greater curvature, rinsed with isotonic saline and pinned out on a wax plate. The glandular portion of the stomach was then examined for lesions. The length (mm) of each lesion was measured under a dissecting microscope (10x). The sum of the total length of lesions in each group divided by the number of rats in that group was expressed as the gastric lesions.

The percent inhibition of gastric lesion formation was calculated as follows:

$$\% \text{ Inhibition} = \frac{G_c - G_t}{G_c} \times 100$$

where;

G_c = gastric lesions of control group (mm)

G_t = gastric lesions of test group (mm)

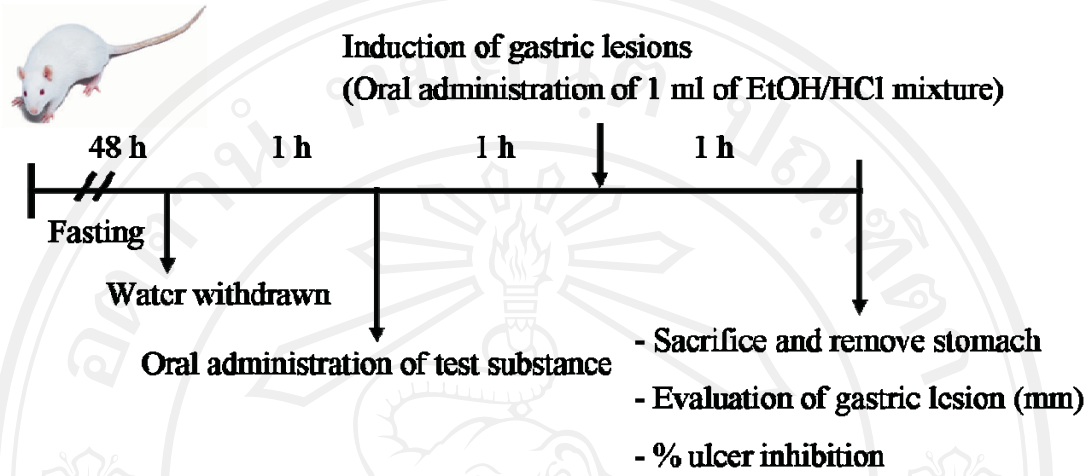


Figure 25 Diagram illustrating the procedure of the EtOH/HCl-induced gastric lesions in rats.

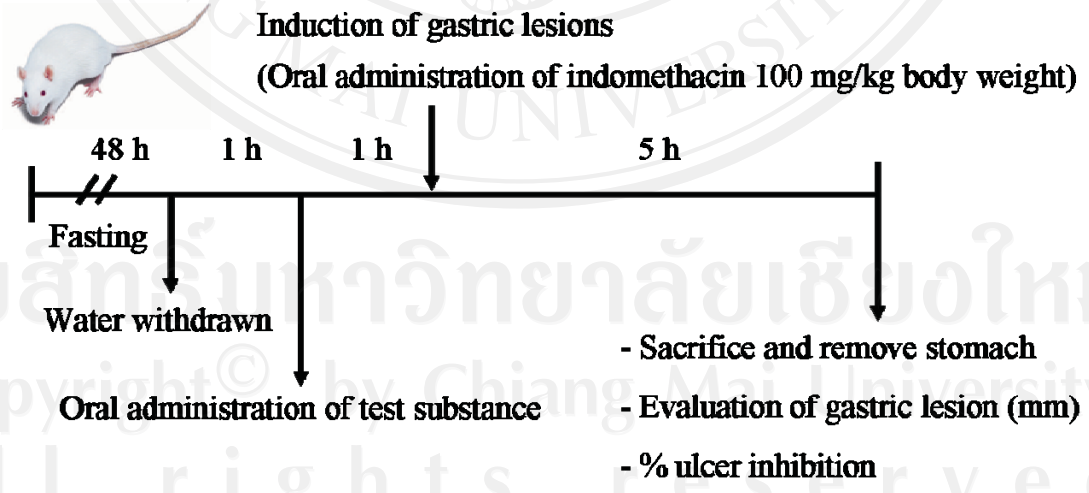


Figure 26 Diagram illustrating the procedure of the indomethacin-induced gastric lesions in rats.

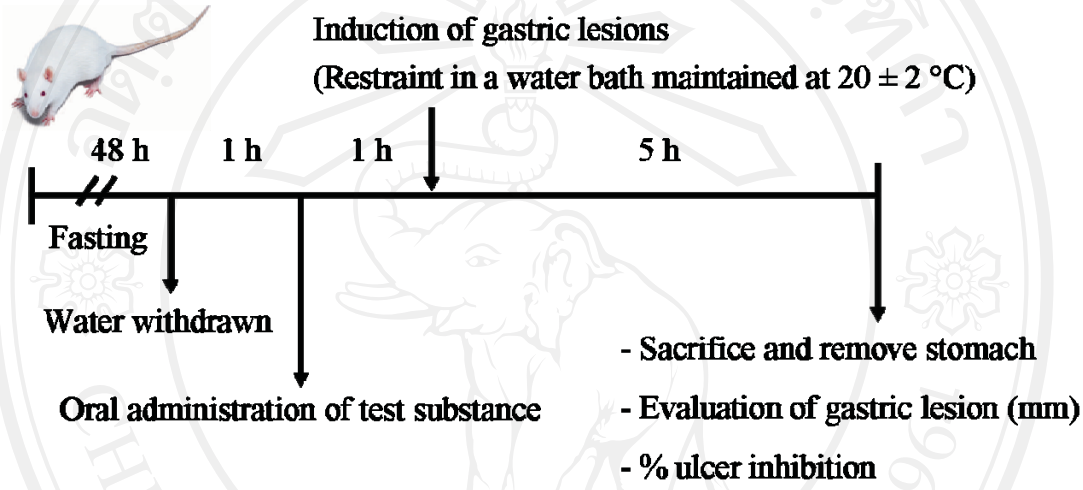


Figure 27 Diagram illustrating the procedure of the restraint water immersion stress-induced gastric lesions in rats.

2.7.7 Toxicity study

2.7.7.1 Acute oral toxicity

The acute oral toxicity was tested according to the WHO guideline (WHO, 2000) and OECD guideline for testing of chemicals (OECD, 2001) as follow:

Adult Sprague Dawley rats (5 weeks) of both sexes with 140-160 g body weight were used and divided into 2 groups of 5 male and 5 female.

Group 1 control group, received distilled water

Group 2 test group, received *P. emblica* water extract 5,000 mg/kg

The test substances were given orally by gavage using a ball-tipped stainless steel feeding needle. Rats were deprived of food but not water 16-18 h prior to administration of test substances. After the test substance had been administered, food was withheld for a further 3-4 h. The protocol is diagrammatically shown in Figure 28.

Toxic signs and the severity, onset, progression and reversibility of the signs were observed and recorded in relation to the dose and time after test substance administration at the 1st, 2nd, 4th and 6th h and once daily for 14 days. The number of survivors were noted after 24 h and these animals were then maintained for a further 14 days with a once daily observation. The observations included changes in fur texture, skin, eyes, mucous membranes, orifices, and also clinical signs of the respiratory, circulatory, autonomic and central nervous system as well as somato-motor activity, and behavioral changes. Any toxic signs such as tremors, convulsions, salivation, diarrhea, lethargy, sleepiness, morbidity, fasciculation, mydriasis, miosis, droppings, discharges, hypotonia, etc. were recorded. The most common toxic signs that may provide valuable clues to target organ or system of toxicity of test substance and autonomic signs are listed in Tables 7 and 8, respectively (Chan *et al*., 1982). At the conclusion of the experiment on day 15, all rats were fasted for 16-18 h, and then sacrificed for necropsy examination. The internal organs, such as brain, heart, lungs, livers, kidneys, spleen, adrenals, and sex organs were excised and weighed. The gross pathological observations of the tissues were performed in histopathological examination.

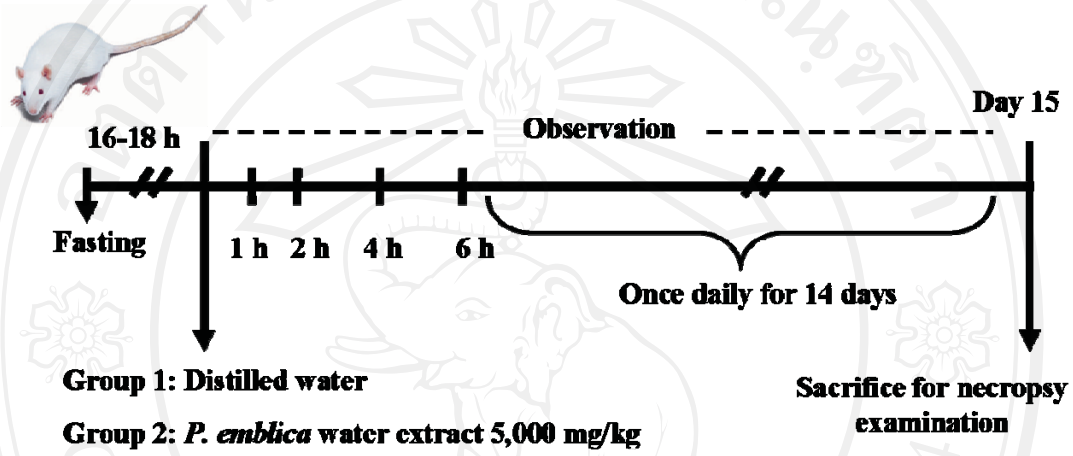


Figure 28 Acute oral toxicity study in rats.

Table 7 Common signs and observation in toxicity test

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
1. Respiratory: blockages in the nostrils, changes in rate and depth of breathing, changes in color of body surfaces	A. Dyspnea: difficult or labored breathing, essentially gasping for air, respiratory rate usually show	
	I. Abdominal breathing: breathing by diaphragm, greater deflection of abdomen upon inspiration	CNS respiratory center, paralysis of costal muscles, cholinergic inhibition
	II. Gasping: deep labored inspiration, accompanied by a wheezing sound	CNS respiratory center, pulmonary edema, secretion accumulation in airways (increase cholinergic)
	B. Apnea: a transient cessation of breathing following a forced respiration	CNS respiratory center, pulmonary cardiac insufficiency
	C. Cyanosis: bluish appearance of tail, mouth, foot pads	Pulmonary cardiac insufficiency, pulmonary edema
	D. Tachypnea: quick and usually shallow respiration	Stimulation of respiratory center, pulmonary cardiac insufficiency
	E. Nostril discharges: red or colorless	Pulmonary edema, hemorrhage
2. Motor activities: changes in frequency and nature of movements	A. Decrease or increase in spontaneous motor activities, curiosity, preening, or locomotions	Somato-motor, CNS

Table 7 (continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
	B. Somnolence: animal appears drowsy, but can be aroused by prodding and resumes normal activities	CNS sleep center
	C. Loss of righting reflex: loss of reflex to maintain normal upright posture when placed on the back	CNS, sensory, neuromuscular
	D. Anesthesia: loss of righting reflex and pain response (animal will not respond to tail and toe pinch)	CNS, sensory
	E. Catalepsy: animal tends to remain in any position in which it is placed	CNS, sensory, neuromuscular, autonomic
	F. Ataxia: inability to control and coordinate movement while animal is walking with no spasticity, epraxia, paresis, or rigidity	CNS, sensory, autonomic
	G. Unusual locomotion: spastic, toe walking, pedaling, hopping, and body posture	CNS, sensory, neuromuscular
	H. Prostration: immobile and rests on belly	CNS, sensory, neuromuscular
	I. Tremors: involving trembling-quivering of the limbs or entire body	Neuromuscular, CNS

Table 7 (continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
	J. Fasciculation: involving movements of muscles, seen on the back, shoulders, hind limbs, and digits of the paws	Neuromuscular, CNS, autonomic
3. Convulsions (seizures): marked involuntary contraction or seizures of contraction of voluntary muscle	A. Chronic convulsions: convulsive alternating contraction and relaxation of muscles	CNS, respiratory failure, neuromuscular, autonomic
	B. Tonic convulsions: persistent contraction of muscles, attended by rigid extension of hind limbs	CNS, respiratory failure, neuromuscular, autonomic
	C. Tonic-clonic convulsions: both types may appear consecutively	
	D. Asphyxial convulsions: usually of clonic type, but accompanied by gasping and cyanosis	
	E. Opisthotonos: titanic spasm in which the back is arched and the head is pulled towards the dorsal position	
4. Reflexes	A. Corneal (eyelid closure): touching of the cornea causes eye lids to close	Sensory, neuromuscular

Table 7 (continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
	B. Pirmal: twitch of external ear elicited by light stroking of inside surface of ear	Sensory, neuromuscular, autonomic
	C. Righting	CNS, sensory, neuromuscular
	D. Myotact: ability of animal to retract its hind limb when limb is pulled down over the edge of a surface	Sensory, neuromuscular
	E. Light (pupillatory): constriction of pupil in the presence of light	Sensory, neuromuscular, autonomic
	F. Startle reflex: response to external stimuli such as touch, noise	Sensory, neuromuscular
5. Ocular signs	A. Lacrimation: excessive tearing, clear or colored	Autonomic
	B. Miosis: constriction of pupil regardless of the presence or absence of light	Autonomic
	C. Mydriasis: dilation of pupils regardless of the presence or absence of light	Autonomic
	D. Exophthalmos: abnormal retraction of eye in orbit	Autonomic
	E. Ptosis: dropping of upper eyelids, not reversed by prodding animal	Autonomic

Table 7 (continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
	F. Chromadacryorrhea (red lacrimation)	Autonomic, hemorrhage, infection
	G. Relaxation of nictitating membrane	Autonomic
	H. Cornea opacity, iritis, conjunctivitis	Autonomic
6. Cardiovascular signs	A. Bradycardia: decreased heart rate	Autonomic, pulmonary-cardiac insufficiency
	B. Tachycardia: increased heart rate	Autonomic, pulmonary-cardiac insufficiency
	C. Vasodilation: redness of skin, tail, tongue, ear, foot pad, conjunctivae sac and warm body	Autonomic, CNS, increase cardiac output, hot environment
	D. Vasoconstriction: blanching or whitening of skin, cold body	Autonomic, CNS, cold environment, cardiac output decrease
	E. Arrhythmia: abnormal cardiac rhythm	CNS, autonomic, pulmonary cardiac insufficiency, myocardiac infarction
7. Salivation	A. Excessive secretion of saliva: hair around mouth becomes wet	Autonomic
8. Piloerection	A. Contraction of erectile tissue of hair follicles resulting in rough hair	Autonomic

Table 7 (continued)

Clinical observation	Observed signs	Organs, tissues, or systems most likely to be involved
9. Analgesia	A. Decrease in reaction to induced pain (e.g. hot plate)	Sensory, CNS
10. Muscle tone	A. Hypotonia: generalized decrease in muscle tone	Autonomic
	B. Hypertonia: generalized increase in muscle tone	Autonomic
11. Gastrointestinal signs: dropping (feces)	A. Solid, dried and scant	Autonomic, constipation, GI motility
	B. Loss of fluid, watery stool	Autonomic, diarrhea, GI motility
Emesis	A. Vomiting and retching	Sensory, CNS, autonomic (in rat, emesis is absent)
Diuresis	A. Red urine (rhinorrhea)	Damage in kidney
	B. Involuntary urination	Autonomic, sensory
12. Skin	A. Edema: swelling of tissue filled with fluid	Irritation, renal failure, tissue damage, long term immobility
	B. Erythema: redness of skin	Irritation, inflammation, sensitization

Table 8 Autonomic signs in toxicity test

Sympathomimetic	Piloerection
	Partial mydriasis
Sympathetic block	Ptosis
	Diagnostic if associated with sedation
Parasympathomimetic	Salivation (examined by holding blotting paper)
	Myosis
	Diarrhea
	Chromodacryorrhea in rats
Parasympathomimetic block	Mydriasis (maximal)
	Excessive dryness of mouth (detect with blotting paper)

2.7.7.2 Chronic oral toxicity

According to WHO guideline (2000) and OECD guideline (1981), adult male and female rats (7 weeks), weighing 180-220 g were used. Rats were divided into 5 groups of 20 rats (10 male and 10 female).

Group 1 control group, received distilled water for 270 days

Groups 2-4 test groups, received three doses of *P. emblica* water extract of 300, 600 and 1,200 mg/kg, respectively for 270 days

Group 5 satellite group, received the highest dose of *P. emblica* water extract (1,200 mg/kg) for 270 days and kept for another 28 days post-treatment in order to observe for reversible, persistent or delayed occurrence of toxicity

During the period of administration, all experimental animals were weighed and observed daily to detect signs of toxicity. Daily visual observations included scrutiny for changes in fur texture, skin, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as somato-motor activity and behavioral changes (Table 7 and 8). All signs were recorded and if any rat which died during the test period was examined pathologically. The protocol is diagrammatically shown in Figure 30.

At the end of 270 days (satellite group 298 day), all surviving animals were fasted overnight but with free access to water and afterwards were anesthetized for collection of blood from a common carotid artery. Blood samples were collected into heparinized and dry non-heparinized centrifuge tubes. Blood analysis (both hematology and chemistry) was carried out. The heparinized blood (2.5 ml) was used for hematological study including white blood cell and differential leukocyte count, platelet, hematocrit and hemoglobin estimation, using Sysmex K-1000 fully automated hematology analyzer. The non-heparinized blood (5 ml) was allowed to coagulate, then centrifuged and the serum was separated. Serum was assayed for glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total birilubin, direct bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and ALP (Auletta, 2002; Levine, 2002). These levels were determined automatically by using the COBAS INTEGRA System.

After blood collection, the animals were sacrificed for tissue studies. The positions, shapes, sizes and colors of internal organs were evaluated. The organs such as brain, lungs, heart, livers, pancreas, spleen, kidneys, adrenals, ovaries, uterus, testes and epididymis were removed, blotted free of blood and weighed immediately on an electronic balance for subsequent analysis. Eyes, thymus, stomach, duodenum, intestine, thoracic spines and muscle with sciatic nerve were also observed (Auletta, 2002). All tissues were preserved in 10% neutral buffered formaldehyde solution for pathological examination. After routine processing, the paraffin sections of each tissue was cut in 5 μm thickness and stained with haematoxylin and eosin (H&E) for a microscopic examination. The results of histopathological examination were performed by pathologist.

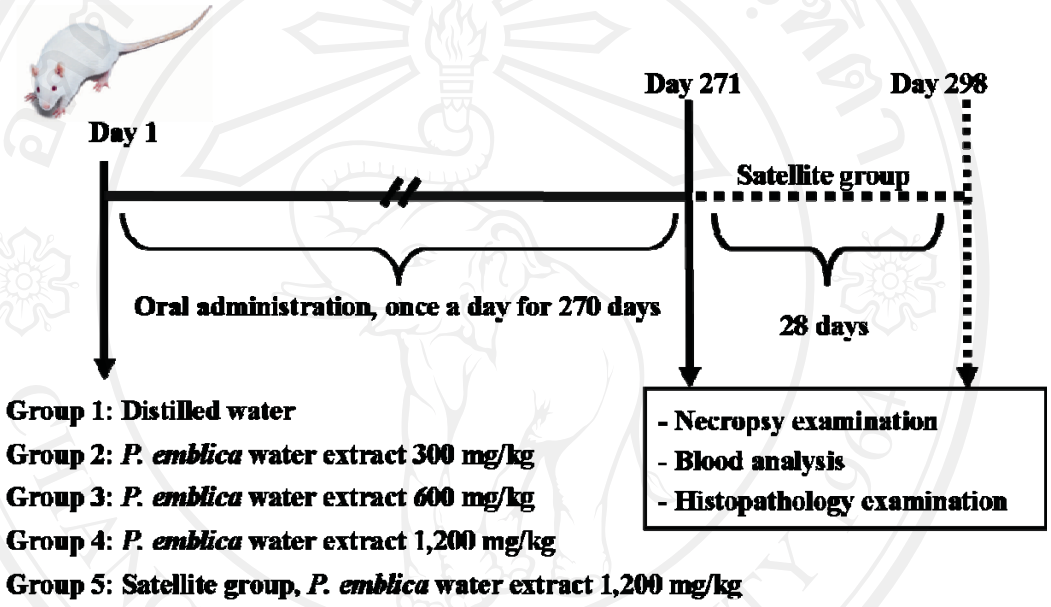


Figure 29 Chronic oral toxicity study in rats.

2.8 Statistical analysis

The data from the experiments were expressed as mean ± standard error of mean (S.E.M.). Statistical comparison between groups was analyzed by using one-way analysis of variance (ANOVA), post hoc least-significant difference (LSD). Student *t*-test was analyzed in acute toxicity and chondroprotective tests. *P* values less than 0.05 were considered significant.

2.9 Drugs and chemicals

Drugs

1.1 Aspirin (Acetylsalicicylic acid, Sigma Chemical Company, St. Louis, U.S.A)

1.2 Indomethacin (Sigma Chemical Company, St. Louis, U.S.A.)

1.3 Morphine sulphate injection U.S.P. (The Government Pharmaceutical Organization, Bangkok, Thailand)

1.4 Phenidone (Riedel-de Haen AG, D-3016 Seelze 1, Germany)

1.5 Phenylbutazone (Sigma Chemical Company, St. Louis, U.S.A.)

1.6 Prednisolone (Scherisone®, Schering Bangkok Ltd., Nonthaburi, Thailand)

1.7 Thiopental sodium for injection BP (Anesthal®, Jagsonpal Pharmaceuticals Ltd., Haryana, India)

Chemicals

2.1 Absolute ethanol (Merck, Darmstadt, Germany)

2.2 Acetone (Merck, Darmstadt, Germany)

2.3 Arachidonic acid (Sigma Chemical Company, St. Louis, U.S.A.)

2.4 Dimethylsulfoxide (Merck Schuchardt OHG, Hohenbrunn, Germany)

2.5 Ethyl phenylpropiolate (Fluka Chemicals Co., Ltd., U.S.A.)

2.6 Formaldehyde (Merck, Darmstadt, Germany)

2.7 Hydrochloric acid (Merck, Darmstadt, Germany)

2.8 Lambda carrageenan (Fluka Chemical Co., Ltd., U.S.A.)

2.9 Brewer's Yeast (Fluka Chemicals Co., Ltd., U.S.A.)