CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

Ethyl acetate extract of the leaves of *T. integrifolia*, designated as TI extract, has been prepared and kindly provided by Professor Dr. Vichai Reutrakul, Director of the Center for Innovation in Chemistry (PERCH-CIC).

T. integrifolia was collected in December 2007 from Khaosok National Park, Surathani, Thailand and was identified by Narong Nanthasean. A voucher specimen No. BKF 14812 has been deposited at The Herbarium, The Royal Forestry Department, Bangkok. The air-dried and powdered of leaves (3.7 kg) of *T. integrifolia* was sequentially extracted with hexane, ethyl acetate and methanol at room temperature, followed by filtration and evaporated to dryness with a rotary evaporator under reduced pressure. The extracts were finally freeze-dried to give hexane, ethyl acetate (TI extract) and methanol extracts of 40.6, 70.9 and 150.8 g, respectively. The TI extract was reconstituted in vehicle (5% Tween80) to the required concentrations for the experiments, except for the EPP-induced ear edema model the TI extract was dissolved in acetone.

2.2 Phytochemical screening test (60, 61)

Chemical constituents were screened by phytochemical tests for alkaloid, tannin, and glycoside (e.g. antraquinone, cumarin, flavonol, flavones, saponin, cardiac glycoside, steroid, terpene, and anthocyanin).

2.3 Experimental animals

Male Sprague Dawley rats weighing 40-300 g as well as female Sprague Dawley rats weighing 180-220 g and male ICR mice weighing 30-40 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 control conditions of 24 ± 1 ºC, relative humidity 50 ± 10% and 12 h light-12 h dark cycle. All animals were 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.

2.4 Preparation of test substances

5% Tween80 was used as vehicle for all test substances, except in the ear edema model, the test substances were dissolved in acetone.

2.5 Test substance administration

All test substances were orally administered in an equivalent volume of 5 mL/kg body weight of the rats and mice. In the ear edema model, the test substances were applied locally on outer and inner surfaces of the ear. Control groups received only vehicle in the same volume and same route of administration.

2.6 Experimental protocol

2.6.1 Analgesic studies

2.6.1.1 Writhing response (62, 63)

Male mice weighing 30-40 g were divided into 5 groups of 6 mice each and administered orally of test drugs as follows:

Group 1	control group received vehicle (5% Tween80)
Group 2	reference group received diclofenac (10 mg/kg)
Group 3-5	test groups received TI extract (50, 100 and 200 mg/kg, respectively)

At 1 h after test drugs administration, mice were injected intraperitoneally in a volume of 0.1 mL/10 g body weight of 0.75% aqueous acetic acid. After acetic acid injection, mice were placed in a transparent plastic box for observation of the writhing responses for 15 min starting at 5 min after injection. The writhes consist of abdominal wall contraction, pelvic rotation and hind limb extension. Mean values of the treated groups were compared with that of the control group. The percent inhibition of writhing response was calculated according to the following formula:

$$\%WI = \frac{\text{NW of control group} - \text{NW of test group}}{\text{NW of control group}} \times 100$$

where; %WI = percent writhing response inhibition

NW = number of writhing

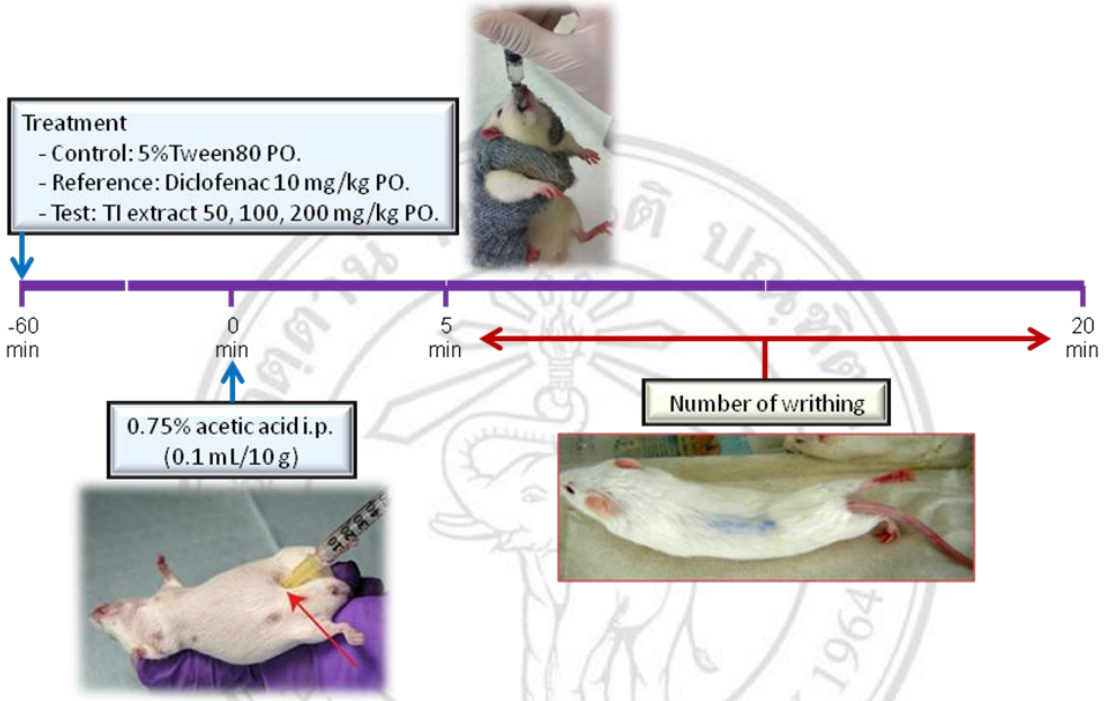

Figure 2.1 Diagram illustrating the procedure of writhing response.

2.6.1.2 Tail-flick test (64, 65)

Male rats weighing 180-200 g were used. They were screened for the basal reaction time and then divided into 6 groups of 6 rats each.

Group 1 control group received vehicle (5% Tween80)

Group 2 reference group received diclofenac (10 mg/kg)

Group 3 reference group received codeine (200 mg/kg)

Group 4-6 test groups received TI extract (50, 100 and 200 mg/kg, respectively)

Rat's tail was placed over a flush mounted photocell window of the Tail Flick Apparatus (Ugo Basile 7360, Italy). Heat was applied by infrared lamp (50 W blub) mounted on a reflector. The timer was activated when the lamp is turn on and stopped when rat flicked its tail away from the photocell window. The rat's tail was exposed to

the infrared lamp and the length of time taken for the rat to flick its tail away from the heat was recorded. The light intensity was adjusted to give a normal reaction time of 2-4 sec. The cut-off time of 10 sec was the maximum time which an unflick tail was allowed to expose to the heat without causing tissue damage. The reaction time was measured before and at 1, 2 and 3 h after drug administration. The analgesic response was calculated as a percentage maximum possible response time.

$$\%MT = \frac{T_t - T_b}{10 - T_b} \times 100$$

where; %MT = percent maximum possible response time

T_t = reaction time after received test drugs

T_b = baseline reaction time

10 = cut-off time of 10 sec

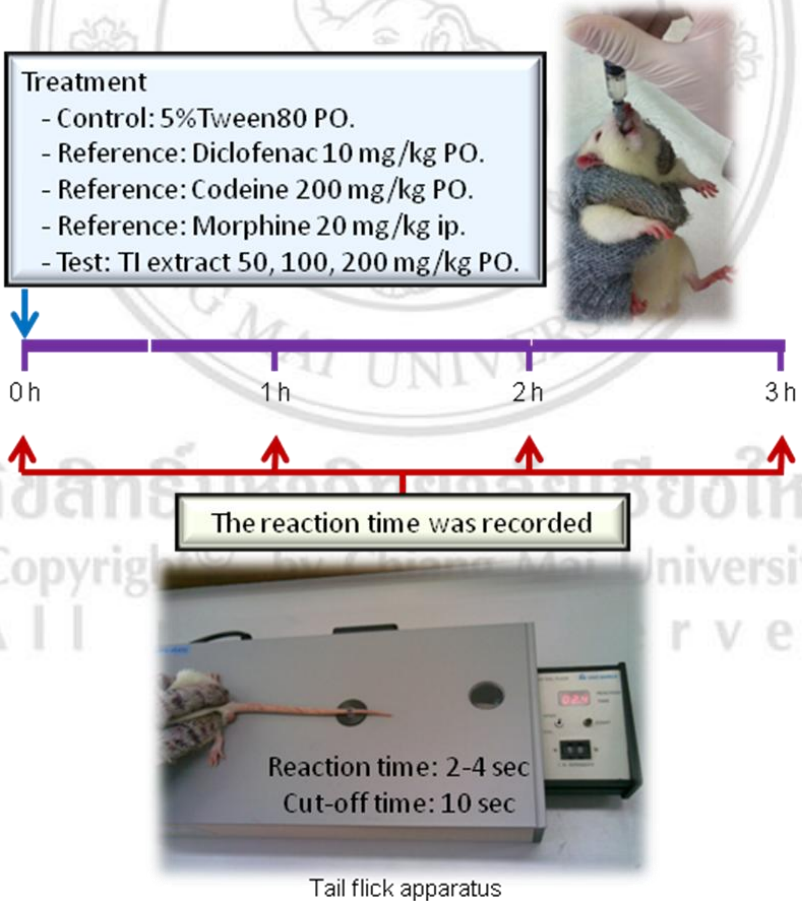

Figure 2.2 Diagram illustrating the procedure of tail-flick test.

2.6.2 Anti-inflammatory studies

2.6.2.1 EPP-induced ear edema in rats (66)

This experiment was performed to investigate the ability of an agent to inhibit an increase of 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 applied acetone (20 µL/ear)
Group 2	reference group applied diclofenac (3 mg/20 µLl/ear)
Group 3	test group applied TI extract (3 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 on the inner and outer surfaces of both ears of each rat. TI extract, diclofenac, 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 increase in ear thickness was compared with that of the vehicle-treated group and the percent inhibition was calculated as follows:

$$ED_x = ET_x - ET_0$$

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

where; ED_x = edema thickness (µm) at time x

ET_x = ear thickness (µm) at time x

ET_0 = ear thickness (µm) before application of EPP

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

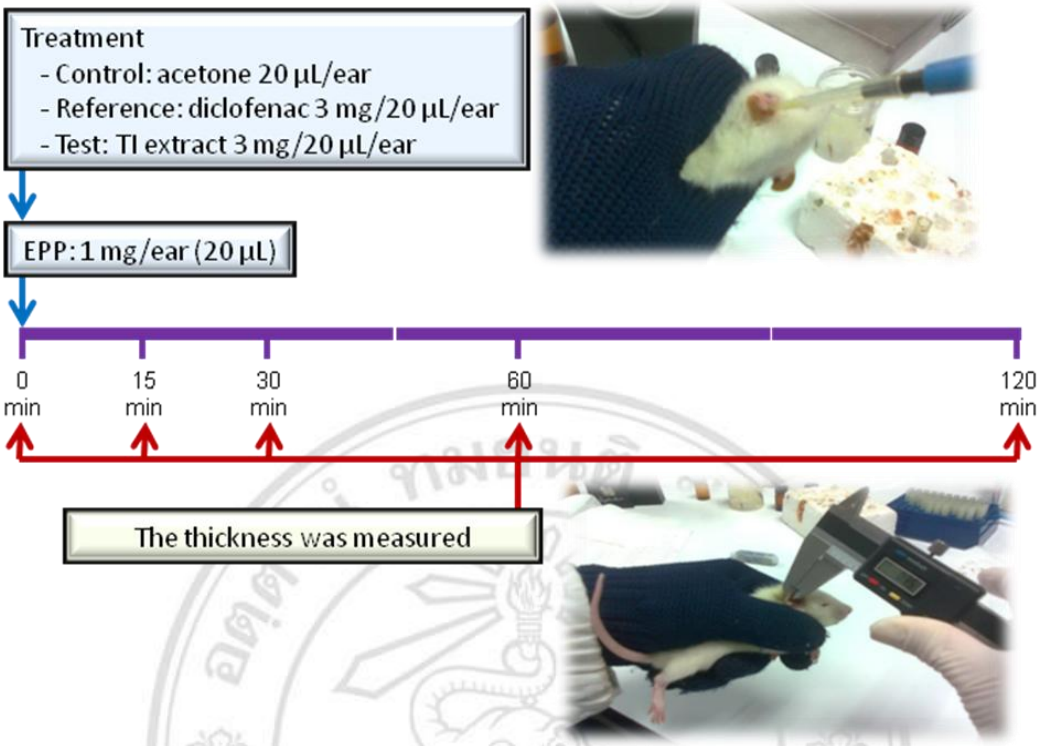

Figure 2.3 Diagram illustrating the procedure of EPP-induced ear edema in rats.

2.6.2.2 Carrageenan-induced hind paw edema in rats (67)

This experiment was performed to investigate 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 vehicle (5% Tween80)
Group 2	reference group received diclofenac (10 mg/kg)
Group 3-5	test groups received TI extract (50, 100 and 200 mg/kg, respectively)

TI extract, diclofenac and 5%Tween80 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 restrain 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% NaCl 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 edema volume of the paw and the percent edema inhibition of each test compound were obtained by the following calculations:

$$EV_x = PV_x - PV_0$$

$$\%EI_x = \frac{EV_c - EV_t}{EV_c} \times 100$$

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

PV_x = paw volume (mL) at time x

PV_0 = paw volume (mL) measured before carrageenan injection

EV_c = paw volume (mL) of control group at time x

EV_t = paw volume (mL) of test group at time x

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

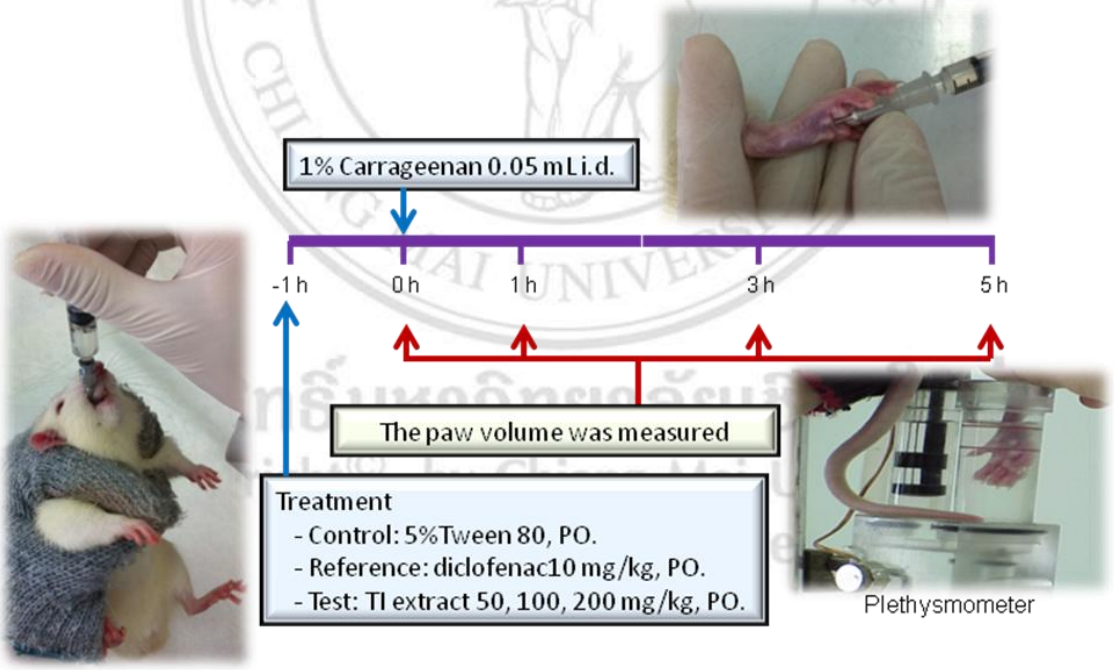

Figure 2.4 Diagram illustrating the procedure of carrageenan-induced hind paw edema in rats.

2.6.2.3 Arachidonic acid (AA)-induced hind paw edema in rats (68)

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

Group 1	control group received vehicle (5% Tween80)
Group 2	reference group received diclofenac (10 mg/kg)
Group 3	reference group received prednisolone (5 mg/kg)
Group 4-6	test groups received TI extract (50, 100 and 200 mg/kg, respectively)

TI extract, diclofenac, prednisolone and 5%Tween80 were orally given 1 h prior to AA injection. A volume of 0.1 mL of 0.5% AA in 0.2 M carbonate buffer (pH 8.4) 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, the edema volume of the paw and the percent edema inhibition of each group were determined as same as mention above. The paw volume was measured prior to and at 1 h after AA injection.

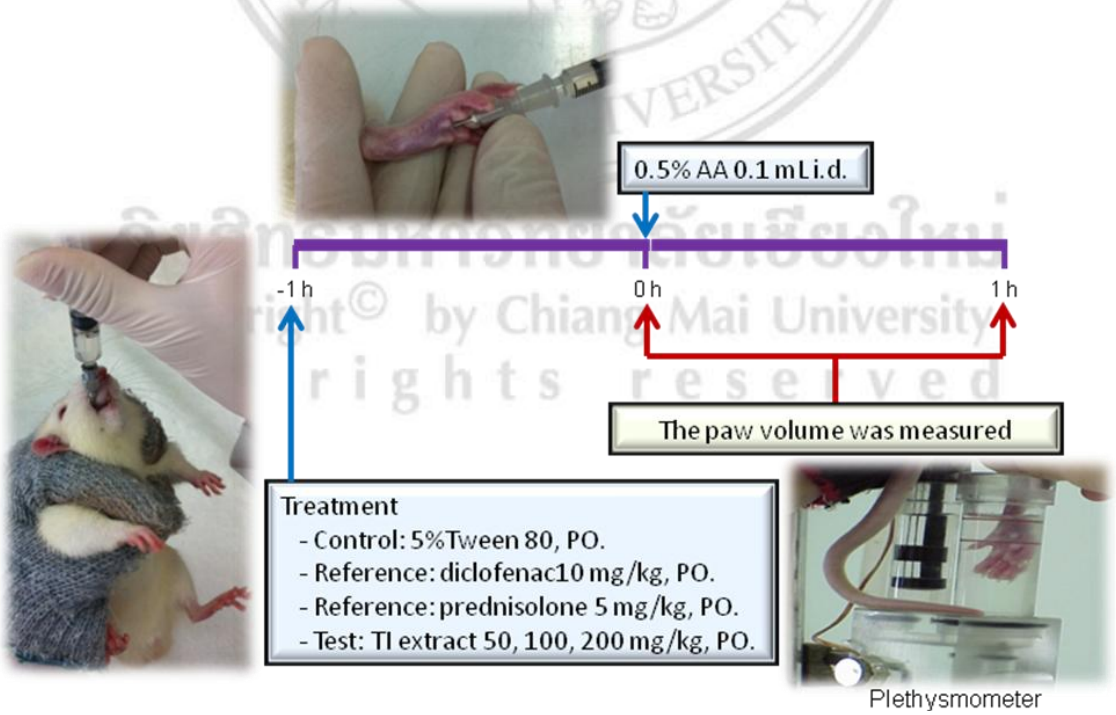

Figure 2.5 Diagram illustrating the procedure of AA-induced hind paw edema in rats.

2.6.2.4 Cotton pellet-induced granuloma formation in rats (69)

This experiment was performed to investigate 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 were cut into pieces weighing 20 $\pm$ 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 180-200 g body weight were used and divided into 5 groups of 6 animals each.

Group 1	normal group
Group 2	control group received 5% Tween80
Group 3	reference group received diclofenac (5 mg/kg)
Group 4	reference group received prednisolone (5 mg/kg)
Group 5	test group received TI extract (200 mg/kg)

In groups 2-5, 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.

TI extract and prednisolone as well as diclofenac were administered orally in a once daily dosage regimen throughout the experimental period of 7 days whereas the control group receives 5%Tween80 only. On the eighth day after cotton pellets implantation, rats were anaesthetized with thiopental sodium (50 mg/kg, intraperitoneal injection). The blood was collected for determination of the amount of alkaline phosphatase and total protein. After that, the rat was sacrificed and the abdominal skin was 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 the test group were compared with those of the control group and the reference groups. The percent granuloma inhibition of the test group and the reference group were calculated according to the following formulae:

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

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

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

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

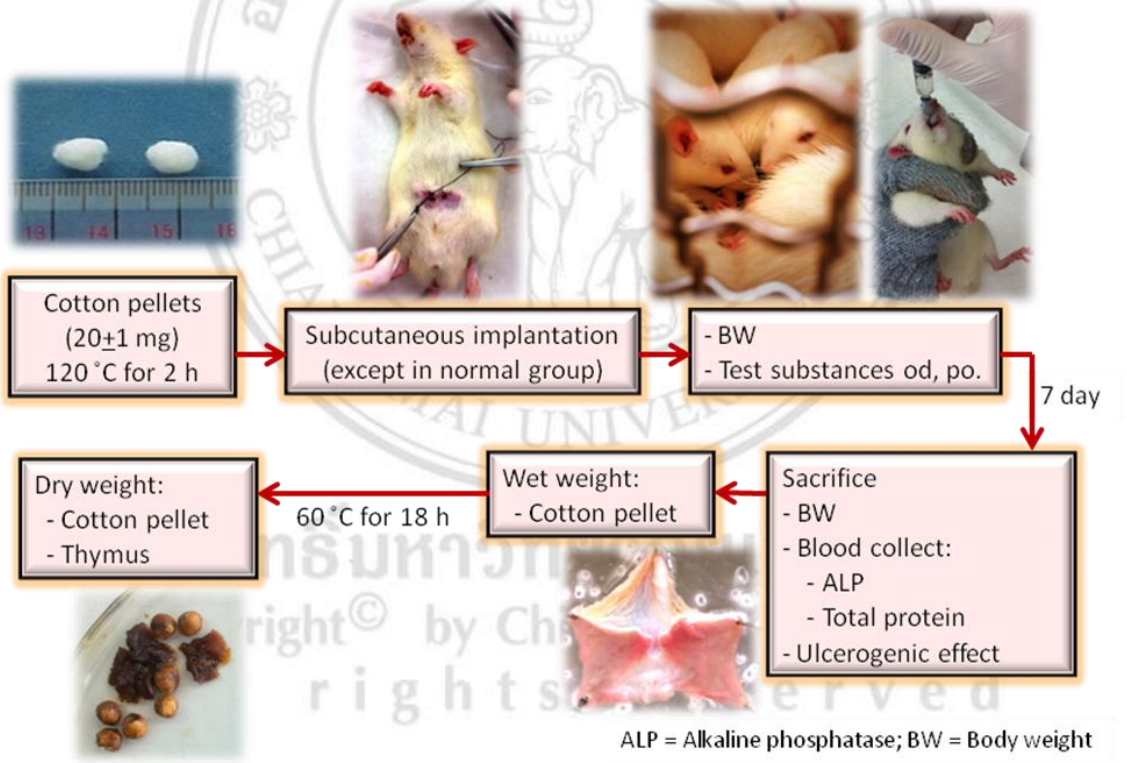

Figure 2.6 Diagram illustrating the method for cotton pellet-induced granuloma formation in rats.

2.6.3 Anti-ulcerogenic studies

2.6.3.1 Gastric ulcer prevention

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 5 groups of 6 animals each.

Group 1	control group received vehicle (5% Tween80)
Group 2	reference group received ranitidine (100 mg/kg)
Group 3-5	test groups received TI extract (50, 100 and 200 mg/kg, respectively)

TI extract, ranitidine or 5%Tween80 were given orally to the rats 1 h before induction of gastric lesions. The gastric ulcers were induced to the rats by 3 methods as follows:

2.6.3.1.1 Ethanol/hydrochloric acid (EtOH/HCl)-induced gastric lesions (70)

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

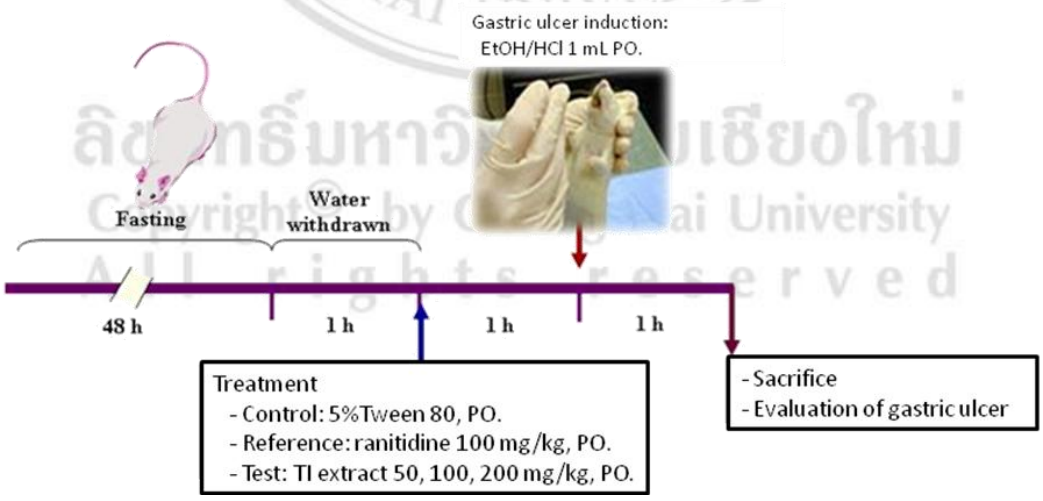

Figure 2.7 Diagram illustrating the procedure of EtOH/HCl-induced gastric lesions.

2.6.3.1.2 Indomethacin-induced gastric lesions (71)

Indomethacin was suspended in 5% Tween80 and administered orally with a single dose of 100 mg/kg. The rats were sacrificed for determination of gastric lesions for 5 h later.

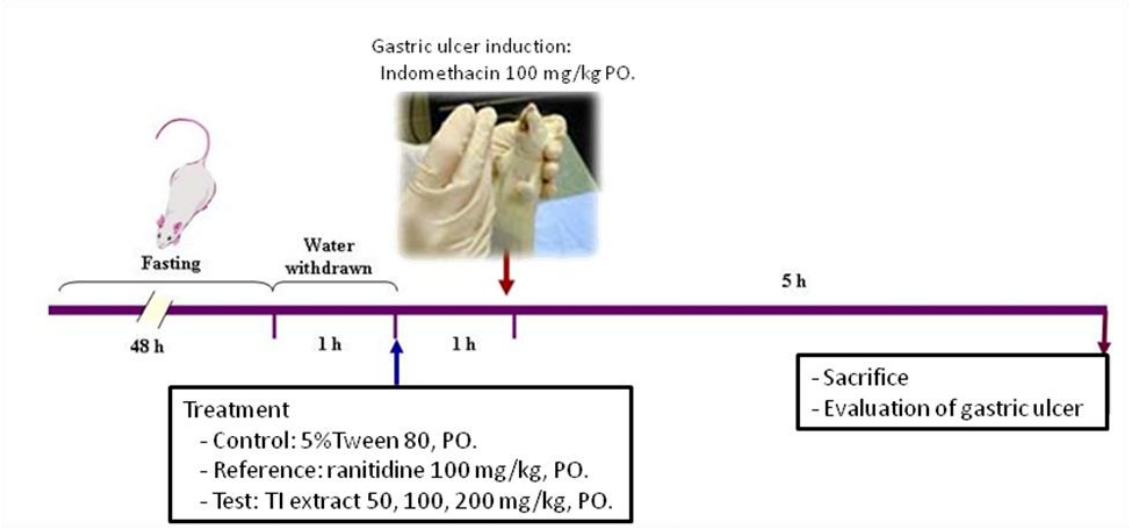

Figure 2.8 Diagram illustrating the procedure of indomethacin-induced gastric lesions.

2.6.3.1.3 Restraint water immersion stress-induced gastric lesions (72)

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

After sacrifice, 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 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 was divided by the number of rats in that group and expressed as the ulcer index. The percent inhibition of ulcer index was calculated as follows:

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

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

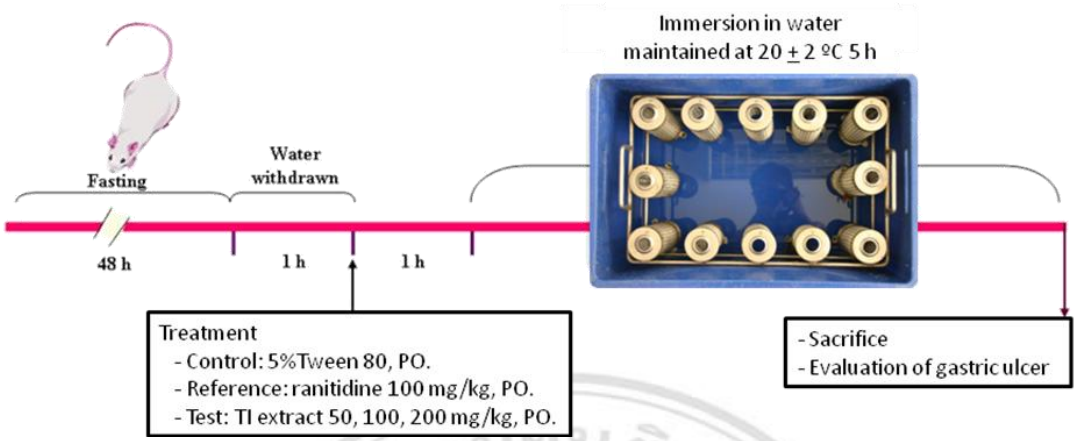

Figure 2.9 Diagram illustrating the procedure of restraint water immersion stress-induced gastric lesions.

2.6.3.2 Investigation the mechanisms of anti-gastric ulcer activity

2.6.3.2.1 Pylorus ligation (73)

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. The rats were divided into 5 groups of 6 animals each.

Group 1	control group received vehicle (5% Tween80)
Group 2	reference group received ranitidine (100 mg/kg)
Group 3-5	test groups received TI extract (50, 100 and 200 mg/kg, respectively)

One hour after drug administration, rats were lightly anesthetized by ether. The abdomen was opened and the pylorus was ligated. The abdomen was closed by suturing. The animals were sacrificed 5 h later by an overdose of ether. The stomach was removed and its content was subjected to measurement of volume, pH and total acid output.

The gastric juice was centrifuged and the total acidity of the supernatant was determined by titrating with 0.1 N NaOH to an end point of pH 7.4 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)

Gastric secretory rate and total acidity were expressed as mL and µEq per 100 g body weight of rat per hour, respectively.

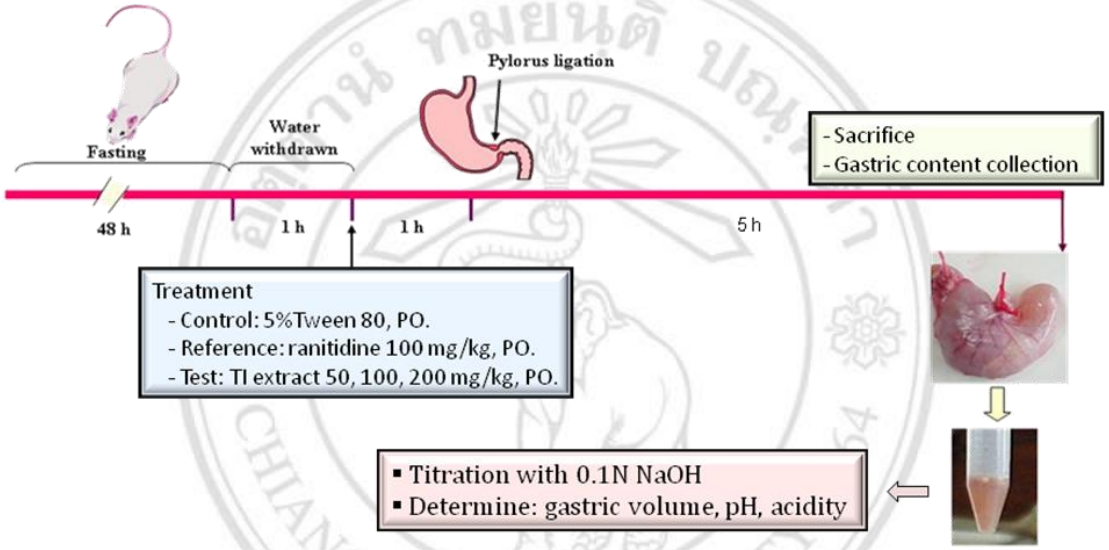

Figure 2.10 Diagram illustrating the procedure of pylorus ligation.

2.6.3.2.2 Gastric visible mucus secretion (74)

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. The rats were divided into 6 groups of 6 animals each.

Group 1 normal group received vehicle (5% Tween80)

Group 2 control group received vehicle (5% Tween80)

Group 3 reference group received ranitidine (100 mg/kg)

Group 4 reference group received misoprostal (100 µg/kg)

Group 5 test group received TI extract (200 mg/kg)

Group 6 test group received TI extract (200 mg/kg)

One hour after oral drug administration, only groups 2-5 were induced gastric ulcer by oral administration of 1 mL of EtOH/HCl. The rat was sacrificed and the

stomach was removed. Gastric wall mucus was determined using the Alcian blue method. The stomach was cut along the lesser curvature, rinsed with normal saline and blotted with filter paper. The stomach was weighed and immersed in 8 mL of 0.1% (w/v) alcian blue for 2 h. The excess of uncomplex dye was rinsed from the stomach twice with 0.25 M sucrose for 30 and 15 min, respectively. The dye-gastric mucus complex was extracted in 10 mL of 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 water layer was separated and measured the absorbance at 580 nm by a spectrophotometer (Milton Roy Company, U.S.A.). The quantity of alcian blue extract was calculated from standard curve of concentration and absorbance of alcian blue solution. The amount of gastric visible mucus was expressed as µg/mL of alcian blue/g of the stomach. Gastric wall mucus was calculated as follow:

$$\text{Gastric wall mucus} = \frac{\text{Concentration of Alcian blue (µg/mL)}}{\text{Weight of wet stomach (g)}}$$

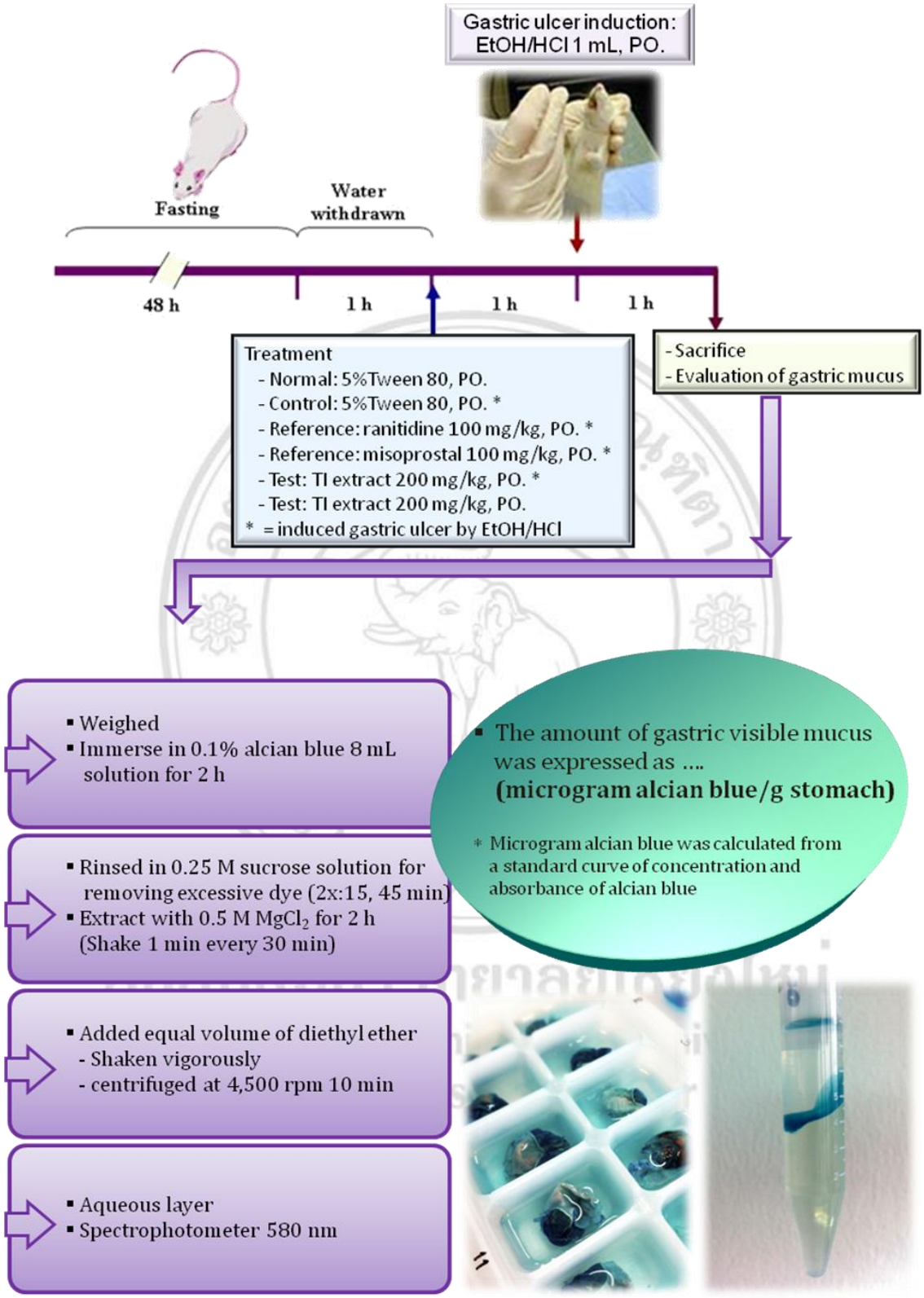

Figure 2.11 Diagram illustrating the procedure of gastric visible mucus secretion.

2.6.4 Acute toxicity study [75]

Adult (7 weeks old) female Sprague Dawley rats weighing 180-200 g were randomly divided into 2 groups of 5 rats each. Rats were deprived of food but not water for 16-18 h before administration of test substances. Five percent tween80 was orally given to the rats in vehicle control group. In test group, TI extract was administered by oral gavage at a dose of 2,000 mg/kg body weight. Visual observation of signs and symptoms such as changes in the skin, fur, eyes, and mucus membrane were made and recorded at 1, 2, 4, 6 h and then once daily for 14 days after the administration of test substances. Survival rats were sacrificed on the 15th day to examine any gross pathological changes of the internal organs. Any changes of the intestinal organs compared with those of the control group were recorded.

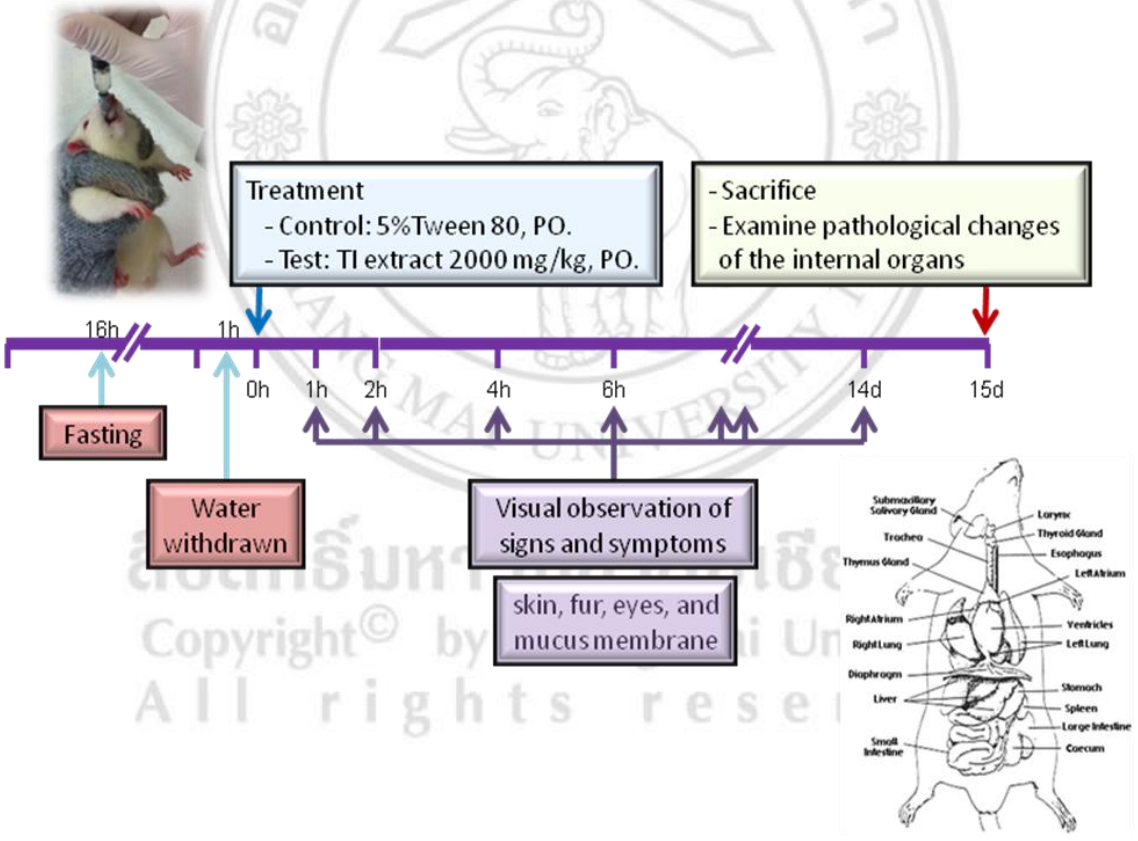

Figure 2.12 Diagram illustrating the procedure of acute toxicity study.

2.7 Statistical analysis

The results from the experiment were expressed as mean±standard error of the mean (S.E.M.). Statistical comparisons between groups were analyzed by using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. *P* values less than 0.05 were considered significant.