CHAPTER 2

MATERIALS AND METHODS

Preparation of an aqueous extract of *Ulva reticulata* Forsskal

A green marine alga: *U. reticulata* was collected from Paklok bay, Phuket province, Thailand, in March 2006 and authenticated by Associate Professor Dr. Yuwadee Peerapornpisal (Department of Biology, Faculty of Science, Chiang Mai University). Figure 4 is a diagram of the preparation of an aqueous extract of *U. reticulata*. The alga was cleaned by removing the contamination and washed with tap water, air dried in shade, and dried in a 50-60^{o}C air oven for 24-48 h. The dried sample was boiled at 50^{o}C in distilled water for 24 h and filtered through 4 layers of gauzes. The filtrate was evaporated by means of a rotary evaporator and lyophilized to obtain a dry aqueous extract of *U. reticulata*. The yield of the aqueous extract of *U. reticulata* was 48 % of dried material. The extract was dissolved in distilled water before using in the study.

Laboratory Animals

Male Sprague-Dawley rats and male guinea-pigs weighing between 200-250 g and 300-500 g, respectively, were purchased from the National Laboratory Animal Center, Salaya, Mahidol University, Nakornpathom, Thailand. All animals were kept in an animal room, conditions controlled at temperature of 22 $\pm$ 2^{o}C and under a 12 h light dark cycle. They had free access to water, and food from the Perfect Companion Co., Ltd., Bangkok, Thailand. The animals were acclimatized for at least one week before starting the experiments.

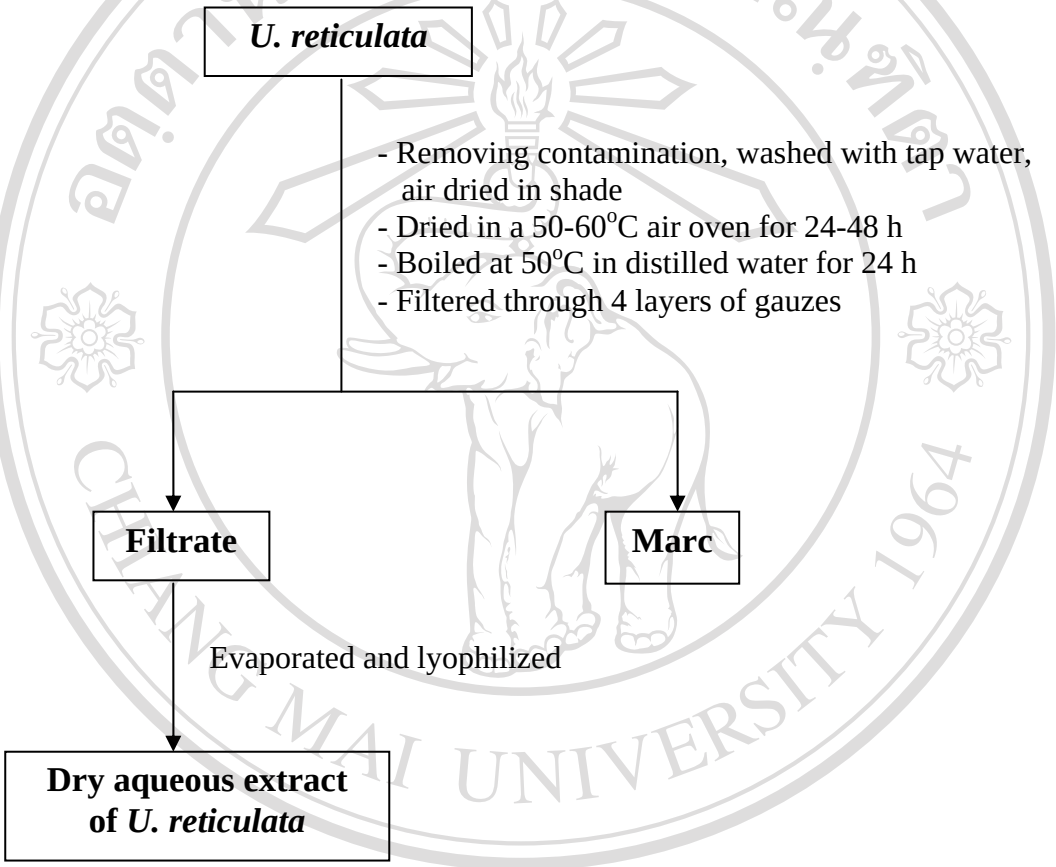

Figure 4 Diagram illustrated the preparation of an aqueous extract of *U. reticulata*

Experimental models

Experiments used for investigation of anti-gastric ulcers activity and involved mechanism(s)

1. Preparation of rats for experimental models

The rats were fasted 48 h and water *ad libitum.* The water was withdrawn 1 h before starting the experiment. The aqueous extract of *U. reticulata*, the reference drug (cimetidine) or vehicle (distilled water) was given orally 1 h before induction of gastric ulcers.

The rats were divided into 5 groups of 6-8 rats as follows:

Group 1 control group, received distilled water

Group 2 reference group, received cimetidine 100 mg/kg

Group 3-5 test groups, received 3 doses (100, 200 and 500 mg/kg) of the aqueous extract of *U. reticulata*

2. Induction of gastric ulcers

The gastric ulcers were induced in rats by 4 methods as follows:

2.1 Restraint water immersion stress-induced gastric ulcers in rats

The method described by Takagi *et al*.(1963) was used (61). The rats were placed individually in each compartment of stainless steel cages and immersed up to the level of the xiphoid in a water bath at 22 ± 2^{o}C for 5 h to induce stress ulcers. After that the rats were sacrificed for determination of gastric ulcers (Figure 5).

2.2 HCl/EtOH-induced gastric ulcers in rats

Gastric ulcers were induced according to the method modified from that of Mizui and Doteuchi (1983) (62). Each rat was administered 1 ml of HCl/EtOH (60 ml absolute ethanol + 1.7 ml HCl + 38.3 ml water) orally. One hour later, the rats were sacrificed for determination of gastric ulcers (Figure 6).

2.3 Indomethacin-induced gastric ulcers in rats

The method used was described by Djahanguiri *et al*. (1969) (63). The rats were given indomethacin suspended in 0.5% carboxymethylcellulose by an intraperitoneal injection at a dose of 30 mg/kg. After 5 h the rats were sacrificed for determination of gastric ulcers (Figure 7).

2.4 Histamine-induced gastric ulcers in rats

Histamine given by an intraperitoneal injection at the dose of 10 mg/kg was used for induction of gastric ulcer in rats according to the method of Rajeshkumar *et al*. (2001) (64). The rats were sacrificed 4 h after the administration of the ulcer agent and stomach was determined of gastric ulcers (Figure 8).

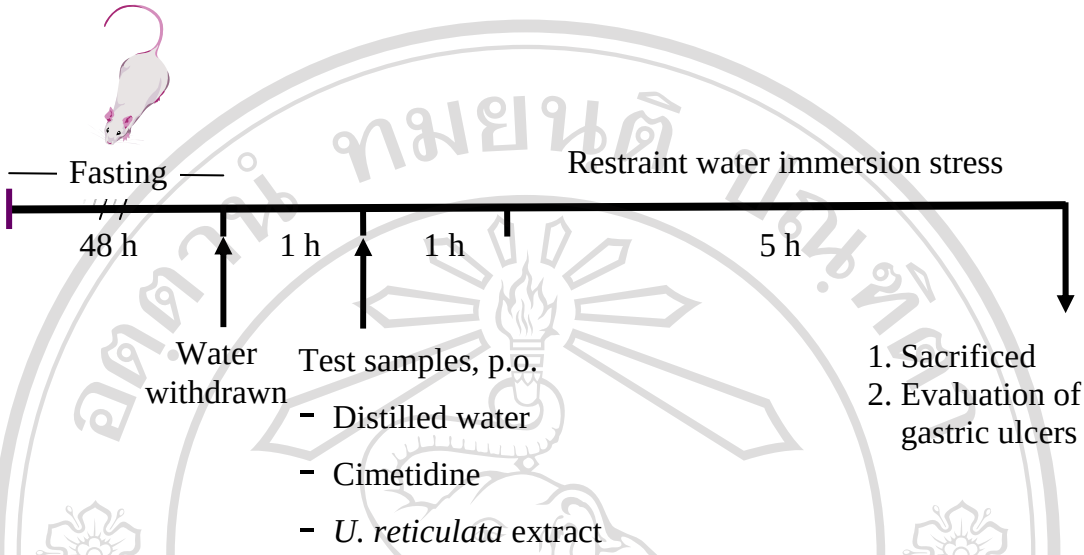

Figure 5 Diagram illustrated the protocol of anti-gastric ulcer test using the restraint water immersion stress-induced gastric ulcer in rat experiment

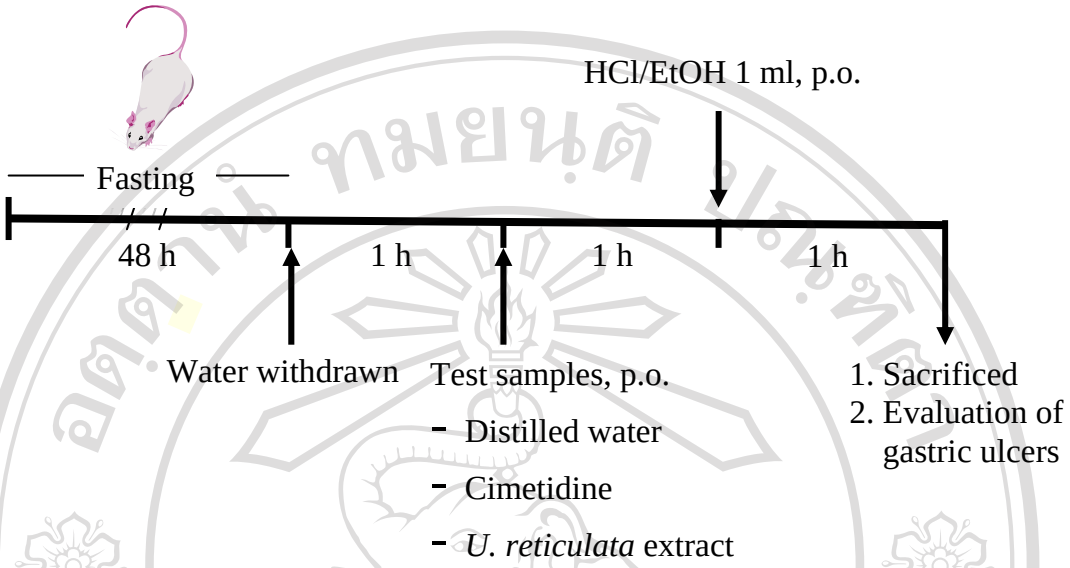

Figure 6 Diagram illustrated the protocol of anti-gastric ulcer test using HCl/EtOH-induced gastric ulcer in rat experiment

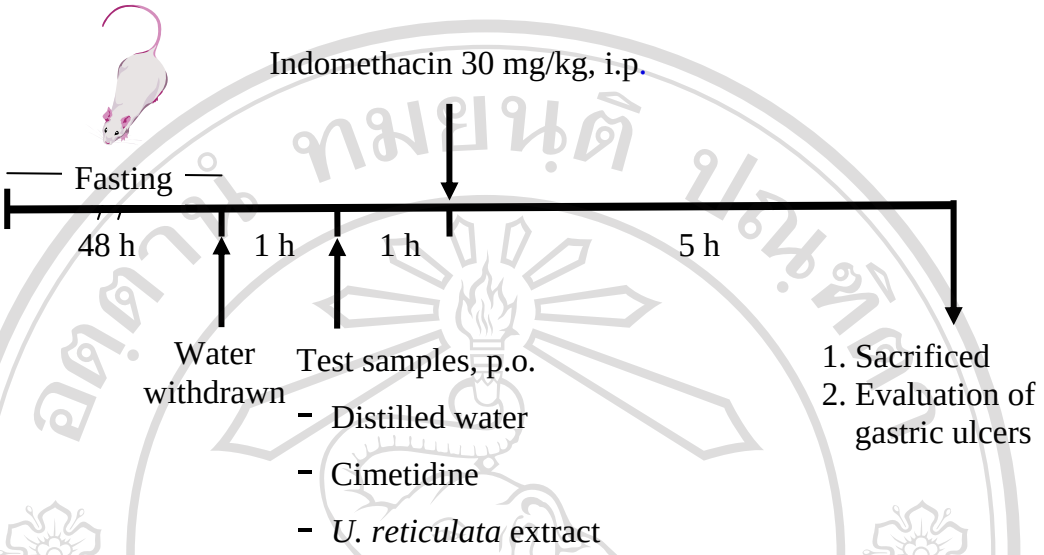

Figure 7 Diagram illustrated the protocol of anti-gastric ulcer test using indomethacin-induced gastric ulcer in rat experiment

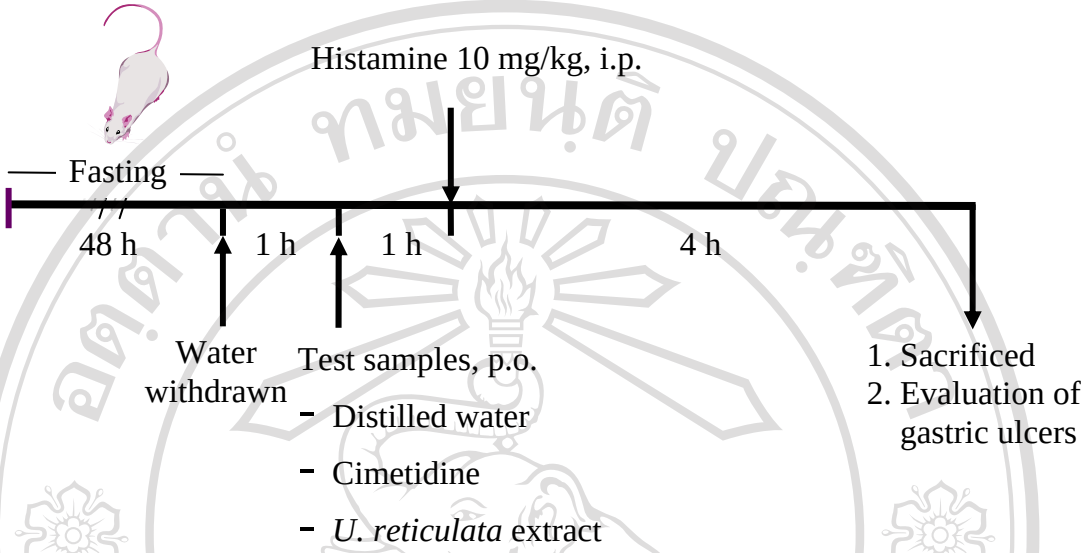

Figure 8 Diagram illustrated the protocol of anti-gastric ulcer test using histamine-induced gastric ulcer in rat experiment

Evaluation of gastric ulcers

The rat was sacrificed, the stomach was opened along greater curvature, rinsed with isotonic saline and pinned out on a wax plate. The glandular portion of the stomach was examined for gastric ulcer.

The length in mm of each ulcer was measured under a dissecting microscope (10x). The sum of the total length in each group divided by the number of rats in that group was expressed as an ulcer index.

$$\text{Ulcer index (UI)} = \frac{\text{Sum of the total length of lesions in each group}}{\text{Number of rats in that group}}$$

The percent inhibition of gastric ulcers was calculated as follows:

$$\% \text{ Inhibition} = \frac{Ul_c - Ul_t}{Ul_c} \times 100$$

where Ul = Ulcer index

Ul_c = Ulcer index of control group

Ul_t = Ulcer index of test group

3. Pylorus ligation experiment

The pylorus ligation experiment described by Shay *et al*. (1945) was followed (65). The rats were pyloric ligated under light ether anesthesia. The stomach was carefully replaced and the abdominal wall was sutured. Five hours after the ligation, the rats were sacrificed by an overdose of ether. The stomach was excised and the gastric juice was collected, centrifuged and its volume and pH was measured. The total acidity of the supernatant was determined by titration with 0.1 N NaOH to an end point of pH 7.4 using phenolphthalein as an indicator. Total acidity of gastric juice was expressed as ml and uEq per 100 g body weight of rat per hour, respectively (Figure 9).

Total acidity of gastric juice was calculated as follows:

$$N_1V_1 = N_2V_2$$

where N_1 = normality of gastric juice (uEq)

N_2 = normality of NaOH (uEq)

V_1 = volume of gastric juice (ml)

V_2 = volume of NaOH (ml)

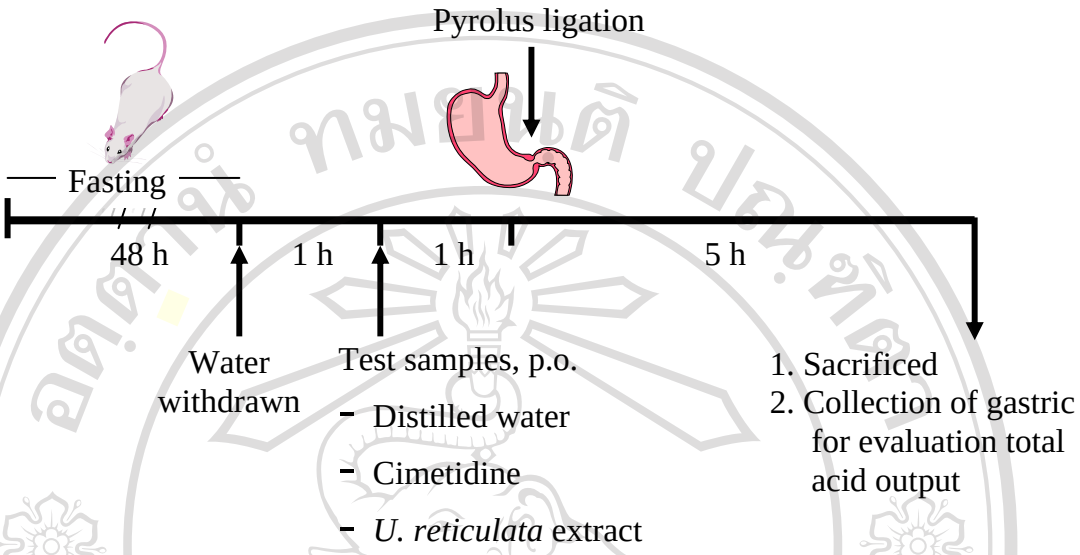

Figure 9 Diagram illustrated the protocol of pylorus ligation in rat experiment

4. Gastric-wall mucus determination experiment

Gastric wall mucus was determined by the method of Corne *et al.* (1974) (66). The rats were fasted 48 h, and water *ad libitum*. The water was withdrawn 1 h before starting the experiment. The rats were divided into 6 groups of 6 rats.

Group 1 normal group, received distilled water
Group 2 control group, received distilled water
Group 3 reference group, received cimetidine 100 mg/kg
Group 4-6 test groups, received 3 doses (100, 200 and 500 mg/kg) of the aqueous extract of *U. reticulata*

The rats of control group (group 2), reference group (group 3), and test group (group 4-6) were administered 1 ml of HCl/EtOH 1 ml orally to induce gastric ulcer and 1 h later sacrificed and the stomach was removed. The stomach was opened along the lesser curvature, weighed and immersed in 0.1% w/v alcian blue solution for 2 h. The excessive dye was removed by two successive rinses in 0.25 M sucrose solution. Dye complexed with gastric wall mucus was extracted with 0.5 M $MgCl_2$ for 2 h. The blue extract was shaken vigorously with an equal volume of diethyl ether and the resulting emulsion was centrifuged at 2,500 rpm for 15 min. The optical density of alcian blue in the aqueous layer was read against a buffer blank at 580 nm using a spectrophotometer (Milton Roy Company, U.S.A.) (Figure 10). The quantity of alcian blue extract/g wet stomach was calculated from a standard curve of concentration and absorbance of alcian blue (Figure 11).

Gastric wall mucus was calculated as follow:

$$\text{Gastric wall mucus} = \frac{\text{Concentration of alcian blue}}{\text{Weight of wet stomach}}$$

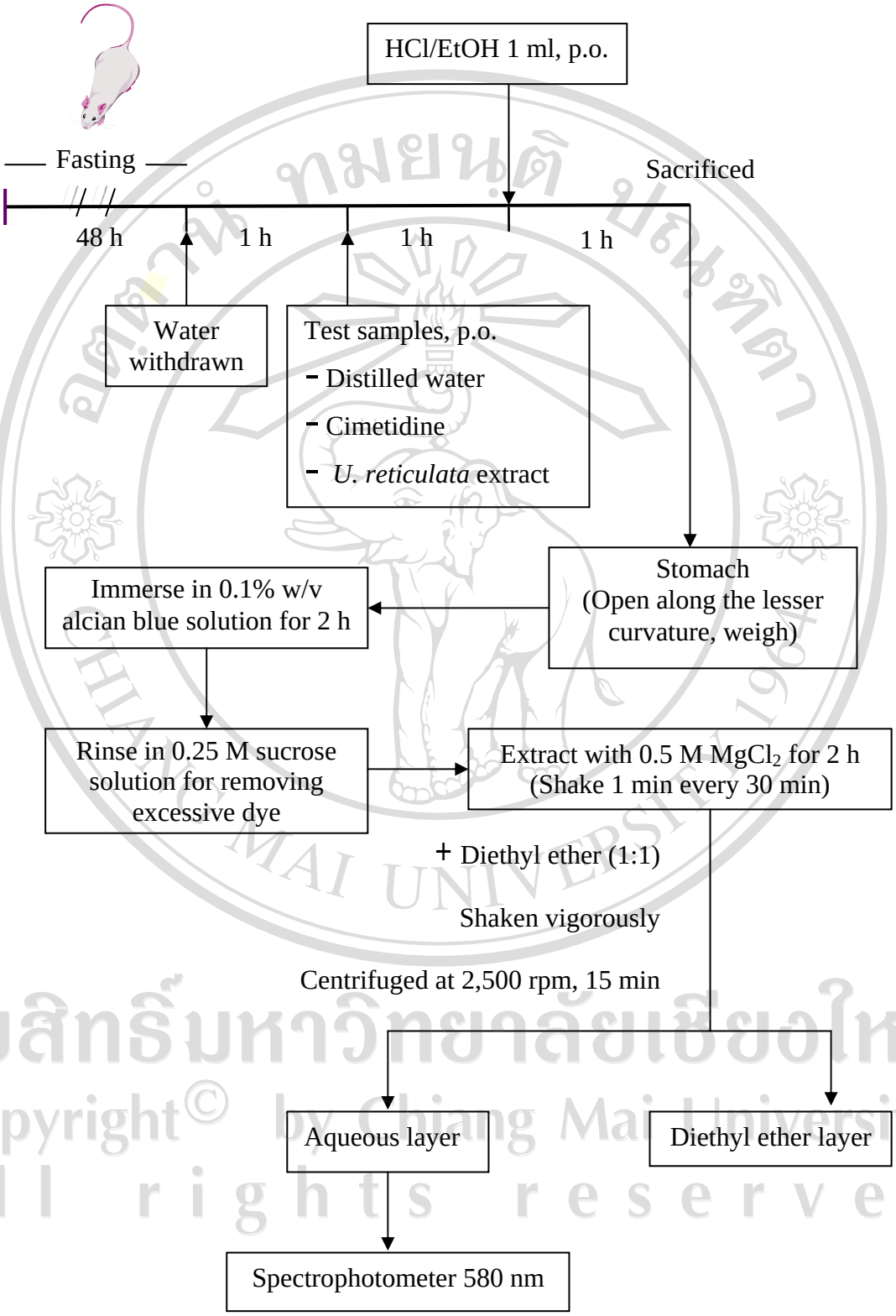

Figure 10 Diagram illustrated the protocol of gastric wall mucus determination

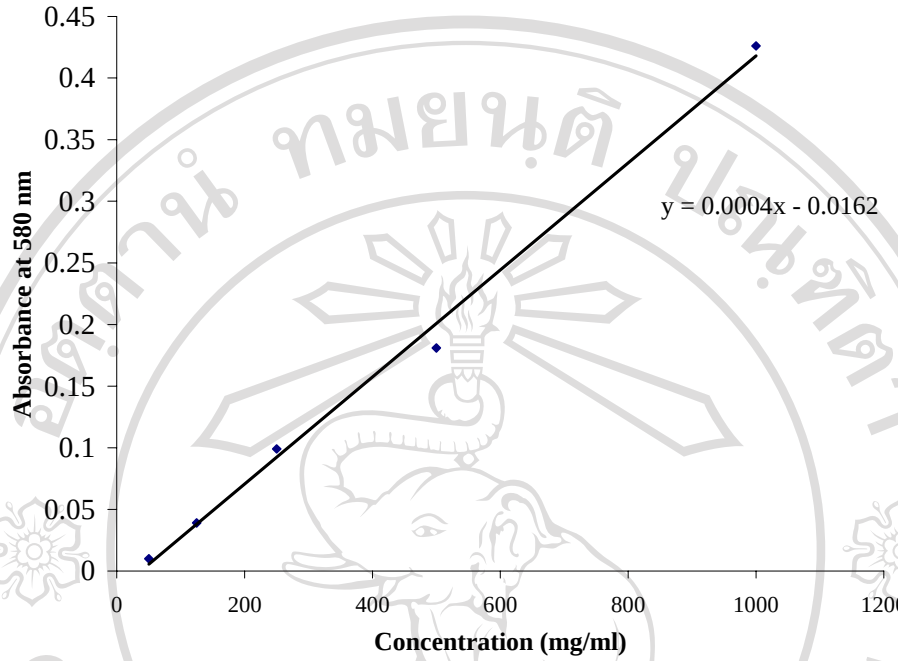

Figure 11 Standard curve of concentration-absorbance of alcian blue

5. Isolated guinea-pig right atria experiment

The preparation of isolated guinea-pig atria was performed in the book of "Pharmacological Experiments on Isolated Preparations" (67). Male guinea-pig was sacrificed by a blow on the head. The chest was opened and the right atrium was dissected and then suspended in a 20 ml organ bath containing Feigen's solution (NaCl = 9.0, $NaHCO_3$ = 0.6, KCl = 0.42, $CaCl_2$= 0.62 and glucose = 1.0 g/l) with a control temperature of 37^{o}C and aerated continuously with 95% O_2 and 5% CO_2 gas mixture. The force and rate of atrium contraction were recorded via a force displacement transducer (FT03 Grass Instrument Co., U.S.A.) under a resting tension of 1 g and displayed on a polygraph recorder (Model 7D, Grass Instrument Co., U.S.A.). The set up of experiment is shown in Figure 12. A 30 min equilibration period was allowed before starting the experiment. Histamine 10^{-5} M was used to induce an increased heart rate (68). The inhibitory effect of various concentrations of the aqueous extract of *U. reticulata* on histamine induced increased heart rate was determined.

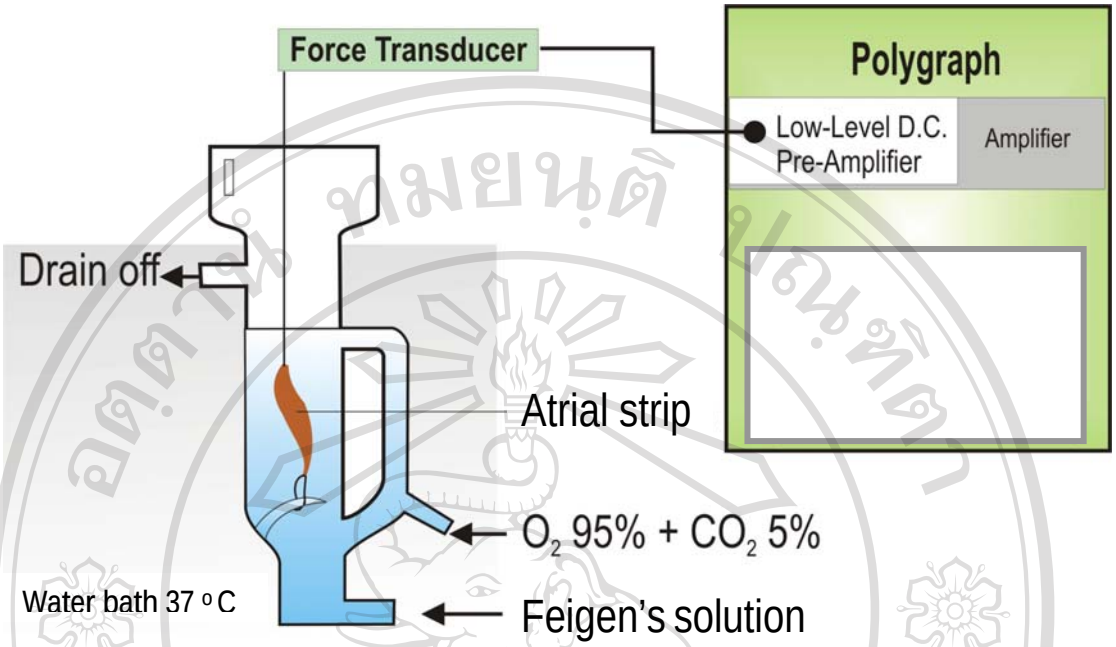

Figure 12 Diagram illustrated the set up of isolated guinea-pig right atria experiment

Drugs and chemicals

Drugs

1. Cimetidine (Siam Pharmaceutical Co., Ltd., Thailand)
2. Histamine (Sigma Chemical Company, St. Louis, U.S.A.)
3. Indomethacin (Sigma Chemical Company, St. Louis, U.S.A.)

Chemicals

1. Alcian blue (Fluka Chemicals Co., Ltd., Japan)
2. Calcium chloride (MERCK, Darmstadt, F.R. Germany)
3. Diethyl ether (BDA Laboratory Supplies, Poole, England)
4. Ethanol (MERCK, Darmstadt, F.R. Germany)
5. Glucose (May & Baker Ltd., Dagenham, England)
6. Hydrochoric acid (BDA Laboratory Supplies, Poole, England)
7. Magnesium chloride (MERCK, Darmstadt, F.R. Germany)
8. Potassium chloride (May & Baker Ltd., Dagenham, England)
9. Phenolphthalein (MERCK, Darmstadt, F.R. Germany)
10. Sodium chloride (MERCK, Darmstadt, F.R. Germany)
11. Sodium hydrogen carbonate (MERCK, Darmstadt, F.R. Germany)
12. Sodium hydroxide (MERCK, Darmstadt, F.R. Germany)

Statistical analysis

The data from the experiments were expressed as mean $\pm$ 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.