MATERIALS AND METHODS

PLANT MATERIAL

The hexane extract from the stem of *Diospyros variegata* was obtained from Prof. Dr. Vichai Reutrakul, Department of Chemistry, Faculty of Science, Mahidol University, and designated as *D. variegata* hexane extract or DVHE.

EXPERIMENTAL ANIMALS

Male Sprague-Dawley rats weighing 100-120 g and 200-250 g were obtained from the National Laboratory Animal Center, Nakorn Pathom. All animals were housed individually in a light- and temperature-controlled room on a 12 : 12 h light-dark cycle, with the temperature of 24 $\pm$ 1°C and relative humidity of 50 $\pm$ 10%. The animals had free access to water and food and were acclimatized at least one week before starting the experiments.

DRUG ADMINISTRATION

All test drugs were orally administered in an equivalent volume of 0.5 ml/100 g body weight. A 5% Tween 80 was used as a vehicle. In the control group, animals received only vehicle in the same volume.

EXPERIMENTAL PROTOCOL

1. Anti-inflammatory activity study

1.1 Carrageenin-induced hind paw edema in rats

The method as described by Winter *et al.* (1962) was used for evaluation of the inhibitory effect of DVHE on the edema formation induced by carrageenin in comparison with aspirin, the standard nonsteroidal anti-inflammatory drug.

Male rats of 100-120 g body weight were divided into 5 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 300 mg/kg of aspirin

Group 3-5 test groups, received 75, 150 and 300 mg/kg of DVHE

DVHE, aspirin and 5% Tween 80 were orally given 1 h prior to carrageenin injection. Lambda carrageenin was made up as a 1% suspension in sterile normal saline solution (NSS). A volume of 0.05 ml of 1% carrageenin was injected intradermally into the plantar side of the right hind paw of an unanesthetized rat which was restrained in a plastic cage.

Foot volume of animal was determined by means of a volume displacement technique using a plethysmometer (model 7150, 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 hairline. Each paw volume was obtained from the average of 3 readings. The paw volume was measured prior to and 1, 3 and 5 h after carrageenin injection. The scheme of experiment is shown in Figure 3.

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

$$EV_x = PV_x - PV_o$$

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

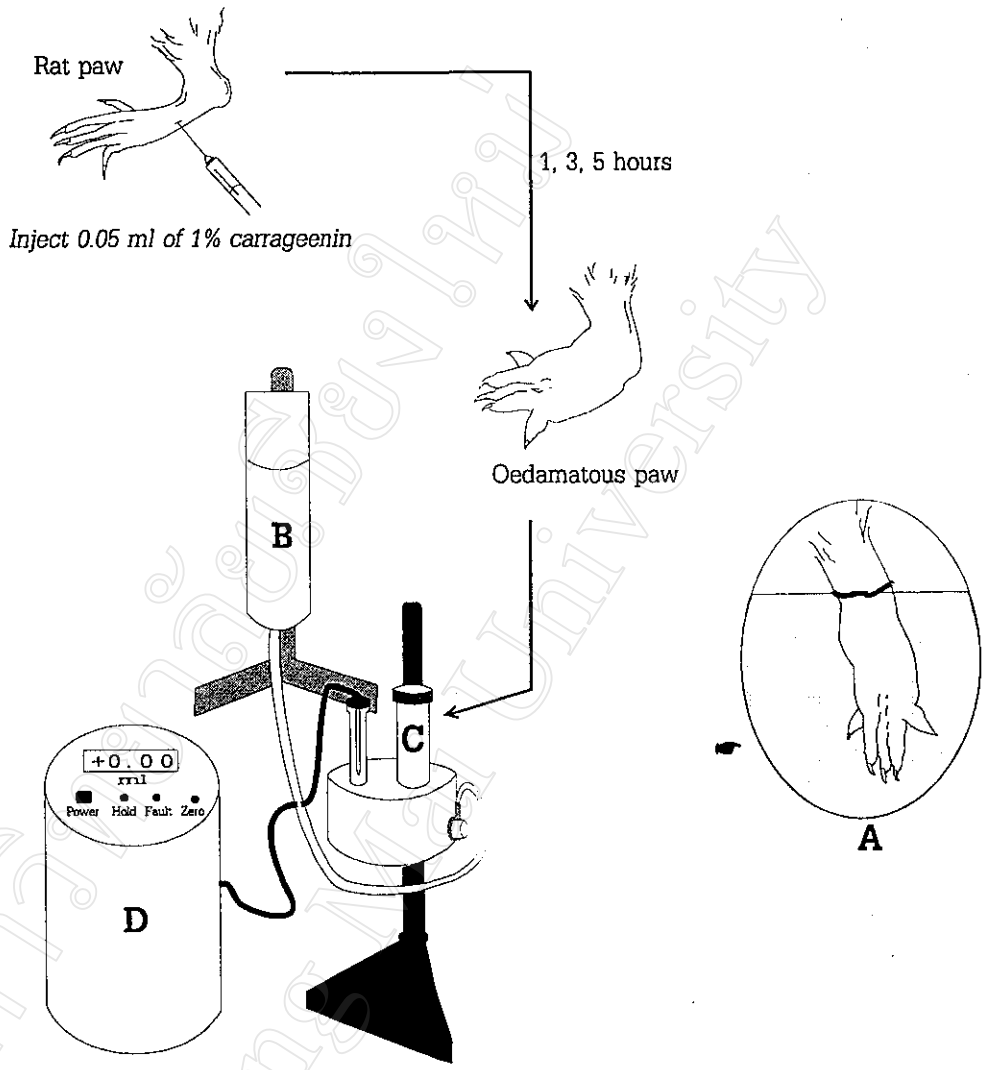
where

EV_x = edema volume (ml) at time x

PV_x = paw volume (ml) at time x

PV_o = paw volume (ml) measured before carrageenin injection

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



A : measured paw with line indicated anatomical hair line marked

B : water reservoir contained 0.05% NaCl in distilled water

C : measuring chamber

D : plethysmometer

Figure 3. Diagram illustrating the water displacement method for measuring rat paw volume

1.2 Carrageenin-induced pleurisy in rats

Male rats of 200-250 g body weight were divided into 8 groups of 6 animals.

Group 1-2 control groups, received 5% Tween 80

Group 3-4 reference groups, received 300 mg/kg of aspirin

Group 5-6 reference groups, received 5 mg/kg of prednisolone

Group 7-8 test groups, received 300 mg/kg of DVHE

Lambda carrageenin was made up as a 1% suspension in sterile normal saline solution (NSS). A volume of 0.15 ml of 1% carrageenin was injected into the right pleural space of animals under light ether anesthetization. Each intrapleural injection was given between the third and the fifth rib on the right side of the mediastinum after sterilization with 0.5% tincture hibitane. DVHE, aspirin and 5% Tween 80 were orally given 30 min prior to carrageenin injection. The scheme of experiment is shown in Figure 4 (Gagon *et al.*, 1966; Velo *et al.*, 1973; Vinegar *et al.*, 1973).

To determine the anti-inflammatory activity of DVHE, the following experiments were performed.

1.2.1 Exudate collection and volume determination

Each animal was killed by a high dose of pentobarbital at 3 h (animal groups 1,3,5,7) or 6 h (animal groups 2,4,6,8) after carrageenin injection and the skin and pectoral muscles retracted leaving the ribcage exposed. A longitudinal incision was made between the third and fifth ribs on each side of the mediastinum. A tuberculin syringe with soft canula was used to aspirate the fluid from the right and left aspects of the thoracic cavity. The exudate volume was determined as ml. Any exudate showing evidence of blood contamination was rejected. The percentages of inhibition of exudate formation were calculated for each group and at each measurement, comparing with the control group, using the following ratio:

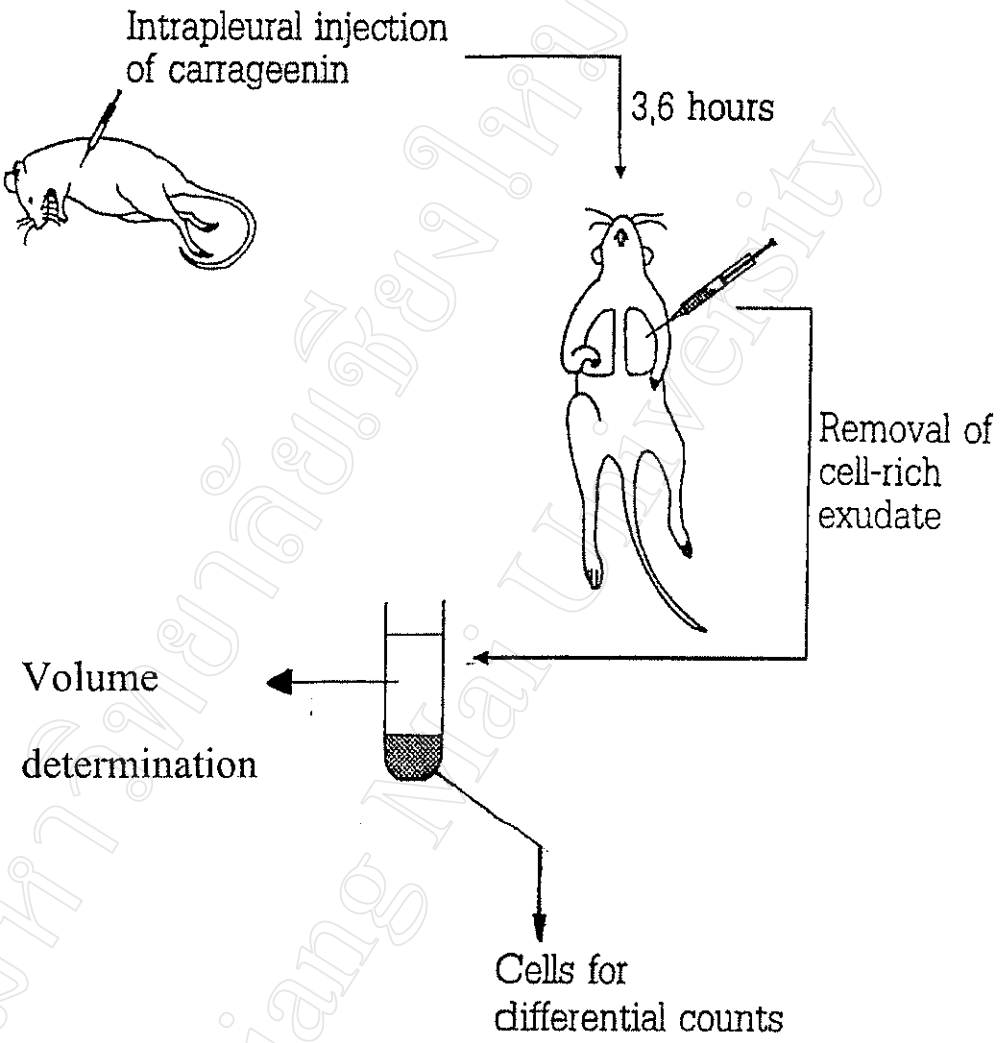


Figure 4. Diagram illustrating the method for carrageenin-induced pleurisy in rats

$$\% \text{ ExVI} = \frac{\text{ExV of control group - ExV of test group}}{\text{ExV of control group}} \times 100$$

where

ExV = exudate volume (ml)

% ExVI = percent exudate volume inhibition of test compound

1.2.2 Leukocyte determination

The exudate was drawn up by using the white cell pipet (Corning®, No. 7049A, U.S.A.) and then diluted with 3% acetic acid by slowly aspirating into the pipet. After shaking well, the first few drops were discarded and the rest was then filled into a hemocytometer (Boeco®, Western Germany). The total leukocyte count was determined by using a microscope and assessed as cells/ml. The percentages of inhibition of leukocyte accumulation in the exudate were calculated for each group and at each measurement, comparing with the control group, using the following ratio:

$$\% \text{ TLNI} = \frac{\text{TLN of control group - TLN of test group}}{\text{TLN of control group}} \times 100$$

where

TLN = total leukocyte number

% TLNI = percent total leukocyte number inhibition of test compound

1.3 Cotton pellet-induced granuloma formation in rats

The slight modification of the method described by Swingle and Shideman (1972) was performed as follows:

Male rats with an average weight of 200 g were divided into 4 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 300 mg/kg of aspirin

Group 3 reference group, received 5 mg/kg of prednisolone

Group 4 test group, received 300 mg/kg of DVHE

Adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made up to a pellet. The pellets were then sterilized in a hot air oven (model 25, Arthur H. Thomas CO., U.S.A.) at 120°C for 2 h. As shown in Figure 5, the abdominal skin was shaved and disinfected with 0.25% tincture hibitane and 70% alcohol. Thereafter two pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether anesthetization and sterile technique. The suture was then made and the animal was allowed to recover.

DVHE, the reference drugs (prednisolone and aspirin) as well as 5% Tween 80 were administered orally in a once daily dosage regimen throughout the experimental period of 7 days. The animals were sacrificed on the eighth day after implantation 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. Both cotton pellets were dried at 60°C for 18 h and their dry weight determined.

The granuloma formation, transudative weight and the percent granuloma inhibition of the test compound were calculated according to the following formulae:

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

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

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

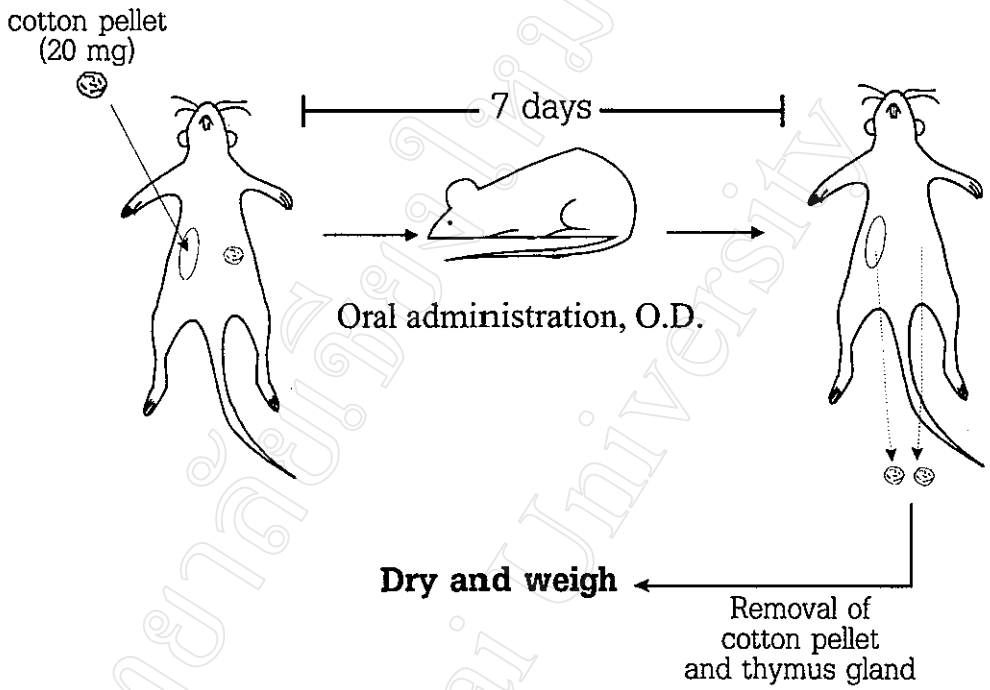


Figure 5. Diagram illustrating the method for cotton pellet-induced granuloma formation in rats

where

Wt_w = wet weight of granuloma (mg)

Wt_d = dry weight of granuloma (mg)

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

GF = granuloma formation (mg/mg cotton)

GI = granuloma inhibition

1.4 Measurement of the alkaline phosphatase activity in serum

The animals used in this method were those in the experiment of cotton pellet-induced granuloma formation in rats (Ismail *et al.*, 1997). In additional, the normal group of rats (the rats which were not implanted with cotton pellet) was included.

On the eighth day after implantation just before the dissection of cotton pellets and the thymus, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The trachea was canulated with a polyethylene tube to facilitate spontaneous respiration and thereby increase the heart function. The canula was inserted into the common carotid artery and blood was collected into a tube. The serum was separated by centrifugation of the blood sample at 1,000 rpm for 5 min.

Samples of serum were sent to the Clinical Service Center, Faculty of Associated Medical Science, Chiang Mai University, for determination of the amount of alkaline phosphatase and total protein immediately after collection. For determining alkaline phosphatase in the serum, calorimetric procedure described by Bessey *et al.* (1946) was followed using p-nitrophenyl phosphate as substrate and expressed as units/l. The level of total protein in the serum was estimated calorimetrically using biuret method referred by Weichselbaum (1946) and expressed as g/dl. The alkaline phosphatase activity was expressed as units of enzyme/mg of serum protein X 10^{-4}.

The method for measurement of alkaline phosphatase activity and total protein in serum, was performed as previously described by Bessey *et al.* (1946) and Weichselbaum (1946). In brief, a 2-amino-2-methyl-1-propanol (AMP) buffer was used to measure alkaline phosphatase activity by the kinetic rate method. In the reaction, alkaline phosphatase hydrolyzed the substrate p-nitrophenyl phosphate to yield phosphoric acid and p-nitrophenol. With an excess of substrate and defined conditions as to pH, temperature, and buffer molarity, the rate of reaction was constant and was proportion to the concentration of the enzyme. The rate of reaction could be determined by measuring the change in absorbance at 405 nm which was the absorbance maximum for the reaction product p-nitrophenol.

Biuret reagent and standard protein was used to measure the total protein concentration by a timed-endpoint biuret method. In the reaction, protein reacted with cupric ions in an alkaline sodium potassium tartrate solution to form a complex colored compound. Any compound that possessed its molecular structure pairs of carbamyl groups linked through nitrogen or carbon (or peptide linkages) would show a positive biuret reaction, resulting in a violet-colored solution. Proteins contained these linkages and gave the characteristic color which was directly proportional to the amount of protein present.

The SYNCHRON CX System automatically proportioned the appropriate serum of sample and reagent into the cuvette. The system monitored the change in absorbance at 405 nm for measurement of alkaline phosphatase activity and at 540 nm for measurement of total protein. These changes in absorbance were directly proportional to the activity of alkaline phosphatase or total protein in the sample and were used by the SYNCHRON CX System to calculate and express alkaline phosphatase activity or total protein concentration.

1.5 Measurement of the thymus weight and the body weight gain

The animals in the experiment of cotton pellet-induced granuloma formation were used. After collecting the blood sample and the dissection of cotton pellet, the chest of the animal was opened. The thymus was dissected out and then dried at 60°C for 18 h. Their dry weight was determined and expressed as mg/100 g body weight.

The change in body weight from the first and the last day of experiment was also recorded (Lisciani *et al.*, 1984).

2. Ulcerogenic activity study

Sprague-Dawley rats weighing 200-250 g were divided into 3 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 300 mg/kg of aspirin

Group 3 test group, received 300 mg/kg of DVHE

DVHE, aspirin as well as 5% Tween 80 were administered orally in a once daily dosage regimen throughout the experimental period of 7 days. The animals were sacrificed on the eighth day. The abdomen was opened, the stomach was then removed and opened along the greater curvature, rinsed with isotonic saline solution and pinned out on a wax plate. The glandular portion of the stomach was examined for lesions under the microscope (10X) (Murakami *et al.*,1982; Saxena *et al.*,1987). The ulcer index was assessed using the sum of the length (mm) of ulcer lesions. Lesion size in mm was determined by measuring each lesion along its greatest diameter. The sum of the lengths in each group divided by the number of rats in that group. If the test group did not show any ulcerogenic effect (ulcer index = 0), the study for anti-ulcerogenic activity was then performed.

3. Anti-ulcerogenic activity study

3.1 Preparation of rats for anti-ulcerogenic activity study

Sprague-Dawley rats weighing 200-250 g, were fasted 48 h, and water was given *ad libitum*. The water was withdrawn 1 h before starting the experiment. The rats were divided into 3 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received a reference drug (cimetidine 100 mg/kg or misoprostol 100 µg/kg)

Group 3 test group, received 300 mg/kg of DVHE

Rats were orally given DVHE, reference drug or 5% Tween 80 1 h before induction of gastric lesions.

3.2 Method used to induce gastric lesions

3.2.1 Indomethacin-induced gastric lesions

Indomethacin suspended in 5% Tween 80 was injected intraperitoneally at a single dose of 30 mg/kg (Pal and Nag Chaudhuri, 1991). Misoprostol 100 µg/kg per oral, was used as reference drug. Five hours later, the rats were sacrificed for determination of gastric ulcer (Figure 6).

3.2.2 Pylorus ligation

Pylorus ligation was performed by following the method of Shay *et al.* (1945). Cimetidine 100 mg/kg per oral, was used as reference drug. Rats were slightly anesthetized by ether. The abdomen was opened and the pylorus was ligated with linen thread. Suturing closed the abdomen. Five hours after ligation, the rats were sacrificed (Figure 7). The abdomen was opened and a ligature was placed around the esophagus close to the diaphragm. The stomach was removed and gastric content was collected in a graduated centrifuge tube. After centrifugation at 2,500 rpm for 5 min, the volume of gastric juice was measured and expressed as ml. Gastric secretory rate was expressed as ml per

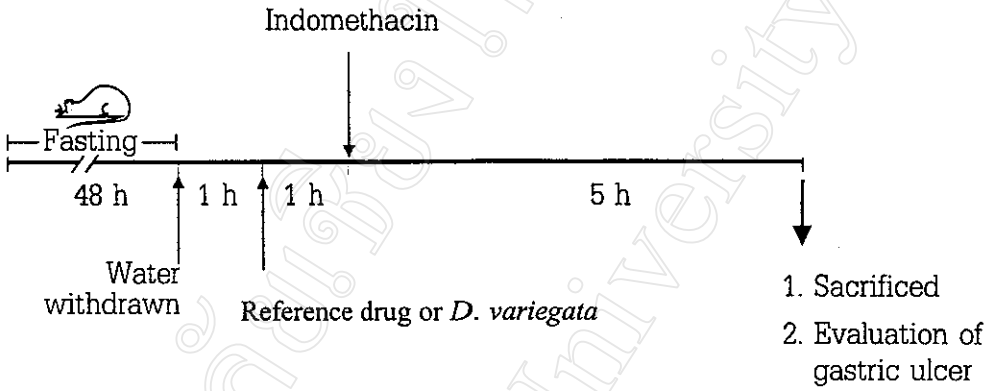


Figure 6. Diagram illustrating the procedure of indomethacin-induced gastric lesion in rats

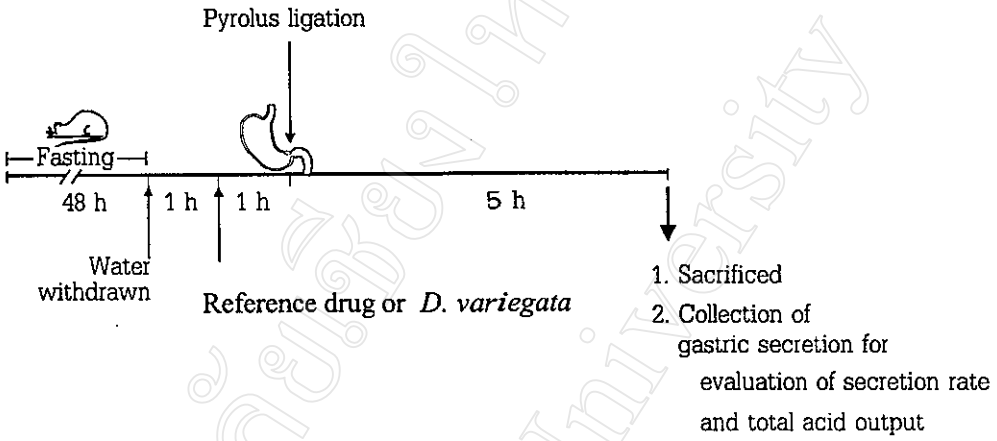


Figure 7. Diagram illustrating the procedure of pylorus ligation in rats

100 g body weight of rat per hour. The total acidity of the supernatant was determined by titration with 0.1 N NaOH to end point of pH 7.4 using phenolphthalein as an indicator and was expressed as mEq/l.

Total acidity of gastric juice was calculated as follows:

$$N_1V_1 = N_2V_2$$

Where

N_1 = normality of gastric juice (Eq)

N_2 = normality of NaOH (Eq)

V_1 = volume of gastric juice (ml)

V_2 = volume of NaOH (ml)

3.3 Evaluation of gastric lesions and calculation the percent inhibition of gastric ulcers

In the model indomethacin-induced gastric lesions, the ulcer index was assessed using the sum of the length of ulcer lesions in the same way as in the study of ulcerogenic activity.

The percent inhibition of gastric ulcers is calculated as follows:

$$\% \text{ inhibition} = \frac{UI_c - UI_t}{UI_c} \times 100$$

Where

UI = Ulcer index

UI_c = Ulcer index of control group

UI_t = Ulcer index of test group

STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Turkey test. P values less than 0.05 were considered significant.

DRUGS AND CHEMICALS

1. Drugs

1.1 Aspirin (acetylsalicylic acid) (Vidhyasom Co., Ltd., Bangkok, Thailand)

1.2 Cimetidine (Tagamet®, Smith & Kline Beecham (Thailand), Bangkok, Thailand)

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

1.4 Misoprostol (Cytotec®, Searle (Thailand) Ltd., Bangkok, Thailand)

1.5 Pentobarbital sodium injection U.S.P. (Nembutal®, Abbott Laboratories, North Chicago, U.S.A.)

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

2. Chemicals

2.1 Lambda carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

2.2 Diethyl ether (Merck, Darmstadt, F.R. Germany)

2.3 Glacial acetic acid (J.T. Baker, Phillipsburg, U.S.A.)

2.4 Phenolphthalein (Merck, Darmstadt, F.R. Germany)

2.5 Polyoxyethylene-sorbitan monooleate (Tween 80, Sigma Chemical Company, St. Louis, U.S.A.)

2.6 Sodium hydroxide (Merck, Darmstadt, F.R. Germany)