MATERIALS AND METHODS

Preparation of Methanolic Extract of *K. galanga*

The plant material was air-dried, ground to a moderately coarse powder and macerated with methanol at room temperature for 24 h and filtered. The maceration was repeated 3 times. The combined filtrate was concentrated *in vacou* at 55°C by means of a rotary evaporator (EYELA Tokyo Rikakikai Co., Ltd.) and the methanolic extract was then lyophylized. The yield of the methanolic extract was about 5% of dried material. The extract was kept in a refrigerator and suspended in 5% Tween 80 for using in the study.

Laboratory Animals

Adult male Sprague-Dawly rats, weighing between 170-300 g, were purchased from The National Laboratory Animal Center, Salaya, Mahidol University, Nakornpathom, Thailand. The animals were kept in an animal room where the temperature was maintained at 22 ± 2 °C under a 12 hour light-dark cycle. All animals were fed with standard diet (Pokphan Animal Feed Co., Ltd., Bangkok, Thailand) and water *ad libitum*. Guinea-pigs, weighing between 300-500 g were obtained from the Animal Center, Faculty of Medicine, Chiang Mai University.

Experiments

1. Preparation of rats for anti-ulcer activity study

Sprague-Dawly rats were fasted 48 h, and water was given *ad libitum*. The water was withdrawn 1 h before starting the experiment. The methanolic extract of *K. galanga* or the reference drug was given orally to the rats 1 h before gastric lesions were induced. The animals were divided into 5 groups of 5-6 rats.

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 3-5 test groups, received 3 doses (50, 100 and 150 mg/kg) of the methanolic extract of *K. galanga*.

Rats were given 5% Tween 80, or reference drug, or the methanolic extract of *K. galanga* 1 h before induction of gastric lesions. The procedures of anti-ulcer test are shown in Figure 3.

2. Methods used to induce gastric lesions

2.1. EtOH/HCl-induced gastric lesions

Each rat was administered 1 ml of EtOH/HCl (60 ml absolute ethanol + 1.7 ml HCl 36.5% + 38.3 ml water) per 200 g body weight, orally, according to the method modified from Yamahara *et al.* (1988). Cimetidine 100 mg/kg was used as a

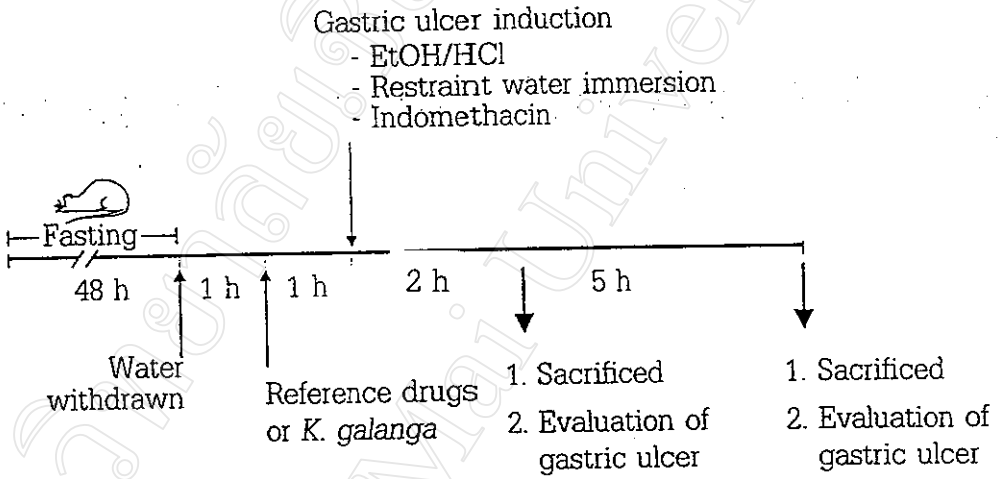

Figure 3 Diagram illustrated the procedure of anti-ulcer test; EtOH/HCl-, restraint water immersion stress-, and indomethacin-induced gastric ulcer in rats.

reference drug. Two hours later, the rats were sacrificed for determination of gastric ulcer.

2.2. Restraint water immersion stress-induced gastric lesions.

Rats were restrained in stainless steel cages and immersed up to their xiphoid in a water bath maintained at 22 ± 2 °C, according to the method of Takagi *et al.* (1963). Cimetidine 100 mg/kg was used as a reference drug. After 5 h of this exposure, the rats were sacrificed for determination of gastric ulcer.

2.3. Pylorus ligation

Pylorus ligation was performed by following the method of Shay *et al.* (1945). Rats were lightly anesthetized by ether. The abdomen was opened and the pylorus was ligated with linen thread (Figure 4). Suturing closed the abdomen. Five hours after ligation, the rats were sacrificed. The stomach was removed and gastric content was collected in graduated centrifuge tube. After centrifugation at 2,500 rpm for 5 min, the volume of gastric juice was measured and 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

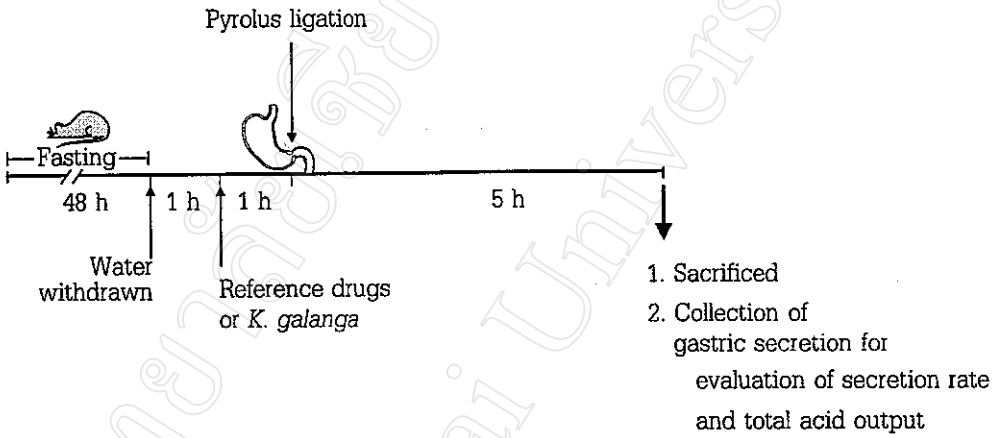

Figure 4 Diagram illustrated the procedure of pylorus ligation in rat.

Secretory rate and total acidity of gastric juice were expressed as ml and μEq per 100 g body weight of rat per hour, respectively.

Total acidity of gastric juice was calculated as follows:

$$N_1V_1 = N_2V_2$$

where N_1 = normality of of gastric juice

N_2 = normality of NaOH

V_1 = volume of gastric juice (ml)

V_2 = volume of NaOH (ml)

2.4. Indomethacin-induced gastric lesions

Indomethacin suspended in 5% Tween80 was injected intraperitoneal at a single dose of 30 mg/kg (Pal and Nagohandhury, 1991). Cimetidine (100 mg/kg) and misoprostol (100 μg/kg), per orally were used as reference drug. Five hours later, the rats were sacrificed for determination of gastric ulcer.

3. Evaluation of gastric lesions

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 examined for lesions. The ulcer index was accessed using of the following 2 methods.

3.1. Length of ulcer lesions. The length (mm) of each lesion was measured under a dissecting microscope (10x). Lesion size in mm was determined by measuring each lesion along its greatest diameter. The sum of the total lengths in each group divided by the number of rats in that group was expressed as the ulcer index. This method was used in evaluation of ulcer index in EtOH/HCl-induced, restraint water immersion stress-induced and pylorus ligation-induced gastric lesions models.

3.2. The severity score scale (Minano *et al.*, 1987) was assigned according to the following scale:

0 = no pathology

1 = mucosal edema and petechiae

2 = 1-5 small ulcer (1-2 mm)

3 = more than 5 small ulcers or one medium ulcer (3-4 mm)

4 = more than 2 medium ulcer or one large ulcer (> 4 mm)

5 = perforated ulcers

The sum of the total severity scores in each group divided by the number of rats in that group was expressed as the ulcer index. The gastric lesions of the indomethacin-

induced gastric lesions model were assessed by this method. 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

4. Gastric-wall mucus determination

Sprague-Dawly rats were fasted 48 h, and water was given *ad libitum*. The water withdrawal 1 h before starting the experiment. The animals were divided into four groups of 4-5 rats.

Group 1 normal group, received 5% Tween 80

Group 2 control group, received 5% Tween 80

Group 3 reference group, received cimetidine 100 mg/kg

Group 4 test group, received the methanolic extract of *K. galanga* 100 mg/kg

The rats of groups 2, 3, and 4 were administered 1 ml of EtOH/HCl orally, as previously described.

The rats were sacrificed 2 h later and stomachs were removed. Gastric wall mucus was determined by the method of Corne *et al.*, (1974). The stomachs were opened along the lesser

curvature, weighed and immersed in 0.1% w/v alcian blue solution for 2 h. The excessive dye was then removed by two successive rinses in 0.25M sucrose solution. Dye complexed with gastric wall mucus was extracted with 0.5M $MgCl_2$ for 2 h. The blue extract was then 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 spectophotometer (Milton Roy Company, U.S.A.). The quantity of alcian blue extract/g (net) of stomach was then calculated from standard curve of concentration and absorbance of alcian blue solution (Figure 5). Gastric wall mucus was calculated as follow:

$$\text{Gastric wall mucus} = \frac{\text{Conc. of alcian blue}}{\text{wt. of wet stomach}}$$

5. Isolated guinea-pig ileum experiment

The preparation was based on the method described in Pharmacological experiments on isolated preparations by staff of Department of Pharmacology, University of Edinburg (1970). The set up of isolated guinea-pig ileum is shown in Figure 6.

Guinea-pigs of both sexes weighing 300-400 g were fasted 48 h before the experiment. The animals were sacrificed, the midline incision of the abdomen was made and the ileum

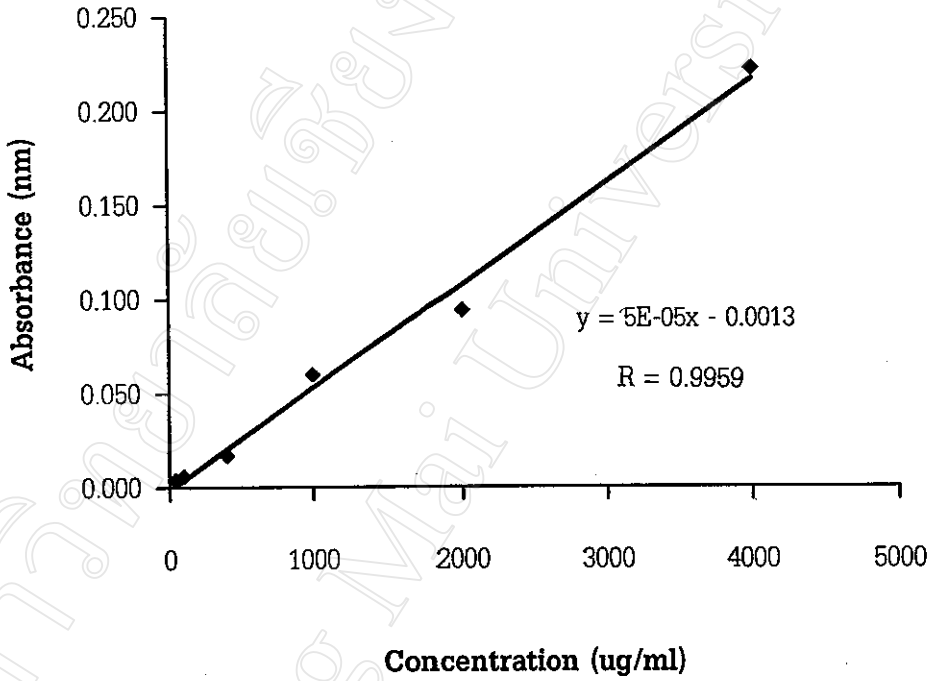

Figure 5 Standard curve of concentration absorbance of alcian blue solution.

was isolated. An ileum was suspended in 20 ml bath containing Tyrode solution (NaCl = 8.0, KCl = 0.2, $MgCl_2$ = 0.1, $CaCl_2$ = 0.2, NaH_2PO_4 = 0.05, $NaHCO_3$ = 1.0 and glucose = 1.0 g/l) with a controlled temperature of 37°C and aerated with 95% O_2 and 5% CO_2. Isometric contractions were recorded under a resting tension of 1.0 g via a force-displacement transducer (FTO3 Grass Instrument Co. Quincy, MA) and displayed on a polygraph (P7D, Grass Instrument Co.). After an equilibration period of 30 min, standard contractions produced by a reference spasmogen (acetylcholine or histamine) was recorded. The tissue was then washed out and the experiments were started. The methanolic extract of *K. galanga* was applied at various concentrations to determine dose response relationship. The effects of antagonists such as cholinergic antagonist (atropine), and histamine antagonist (chlorpheniramine) on the responses to the methanolic extract of *K. galanga* were also studied after their addition to the bath 5 min before the addition of the methanolic extract.

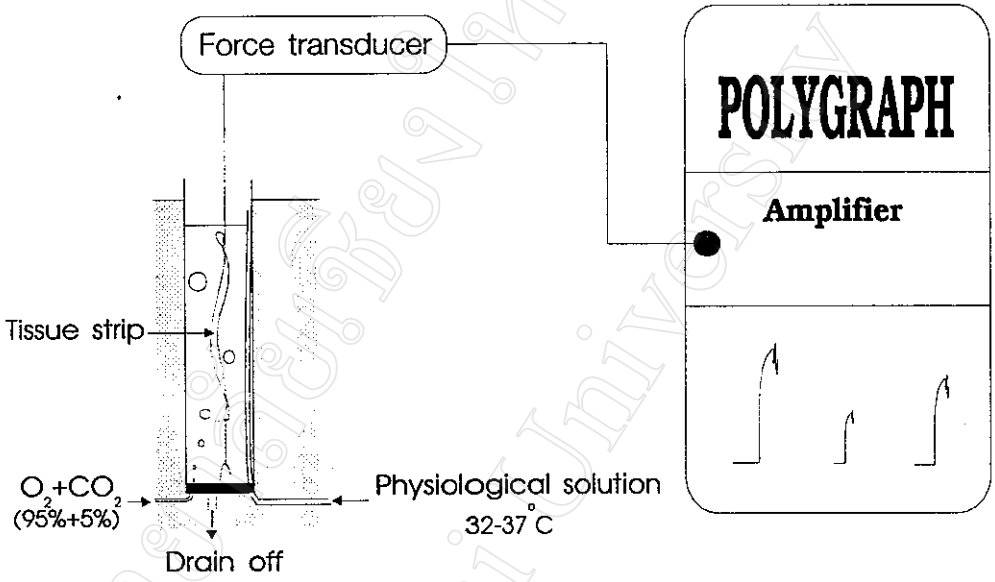

Figure 6 Diagram illustrated the set up of isolated guinea-pig ileum.

Drugs and Chemicals

Drugs

1. Acetylcholine (Calbiochem, Los Angelis, U.S.A.)
2. Atropine (The Government Pharmaceutical Organization, Bangkok, Thailand)
3. Chlorpheniramine (The Government Pharmaceutical Organization, Bangkok, Thailand)
4. Cimetidine (Tagamet^{R}, S.K.& F, U.S.A.)
5. Histamine Free Base, B grade (Calbiochem, Los Angelis, U.S.A.)
6. Indomethacin (BLH Trading Co., Ltd., Bangkok, Thailand)
7. Misoprostol (Diethelm & Co., Ltd., Bangkok, Thailand)
8. Sucralfate (Siam Pharmaceutical Co., Ltd., Bangkok, Thailad)

Chemicals

1. Alcian blue (Fluka Chemic AG)
2. Diethyl ether (BDH Laboratory Supplies Poole, England)
3. Ethanol (MERCK, Darmstadt, F.R. Germany)

4. Hydrochloric acid (BDH Laboratory Supplieds Poole, England)
5. Methanol (S.K. Tredding Co., Ltd., Chiang Mai, Thailand)
6. Phenolphthalein (MERCK, Darmstadt, F.R. Germany)
7. Tween 80 (S.K. Tredding Co., Ltd., Chiang Mai, Thailand)

Statistic Analysis

The data from the experiments were expressed as mean ± standard error of mean (S.E.M.). Statistical comparison between groups were analyzed by using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. Statistical comparisons between two groups were analyzed by Student's t-test. P values less than 0.05 were considered significant.