MATERIALS AND METHODS

Compound : Labda-7,12(*E*),14-triene-17-oic acid (DS4)

The compound was isolated from *C. oblongifolious* and kindly provided by Associate Professor Dr. Amorn Petsom (the Department of Chemistry, Faculty of Science, Chulalongkorn University).

DS4 was dissolved in absolute ethanol or suspended in 0.5% sodium carboxymethylcellulose (CMC), and stored in a refrigerator.

Laboratory Animals

Adult male Sprague-Dawley rats, weighing between 180-200 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 h light dark cycle. All animals were fed with standard diet (Pokphan Animal Feed Co., Ltd., Bangkok, Thailand) and water *ad libitum.*

Assessment of anti-gastric ulcer activity

Preparation of rats for anti-gastric ulcer activity study (Figure 5)

Sprague-Dawley rats were fasted 48 h, and water was given *ad libitum.* The water was withdrawn 1 h before starting the experiment. DS4 or the reference drug was administrated intraperitoneally to the rats 1 h before induction of gastric lesions.

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

Group 1	control group, received ethanol (1 ml/kg)
Group 2	reference group, received a reference drug (cimetidine 50 mg/kg)
Group 3-5	test groups, received 3 doses (25,50 and 100 mg/kg) of DS4 (dissolved in absolute ethanol)

In the study in which DS4 or reference drug was administered orally to the rats 1 h before induction of gastric lesions the rats were divided into 4 groups of 6 rats as follows :

Group 1	control group, received 0.5% CMC (5ml/kg)
Group 2	reference group, received a reference drug (cimetidine 100 mg/kg)
Group 3-4	test groups, received 2 doses (100 and 400 mg/kg) of DS4 (suspended in 0.5% CMC)

Methods used to induce gastric lesions

1. EtOH/HCl-induced gastric lesions

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

2. Restraint water immersion stress-induced gastric lesions

The method described by Takagi *et al.*, (1963) was used. The rats were placed individually in each compartment of stainless steel cages and immersed vertically up to the level of the xiphoid in a water bath (22±2 °C) to induce stress ulcers for 5 hours. After this time, the rats were sacrificed for determination of gastric lesions.

3. Indomethacin-induced gastric lesions

The method of Morimoto *et al.* (1991) was followed. Indomethacin suspended in 0.5% CMC was injected intraperitoneally at a single dose of 30 mg/kg. Five hours later, the rats were sacrificed for determination of gastric lesions.

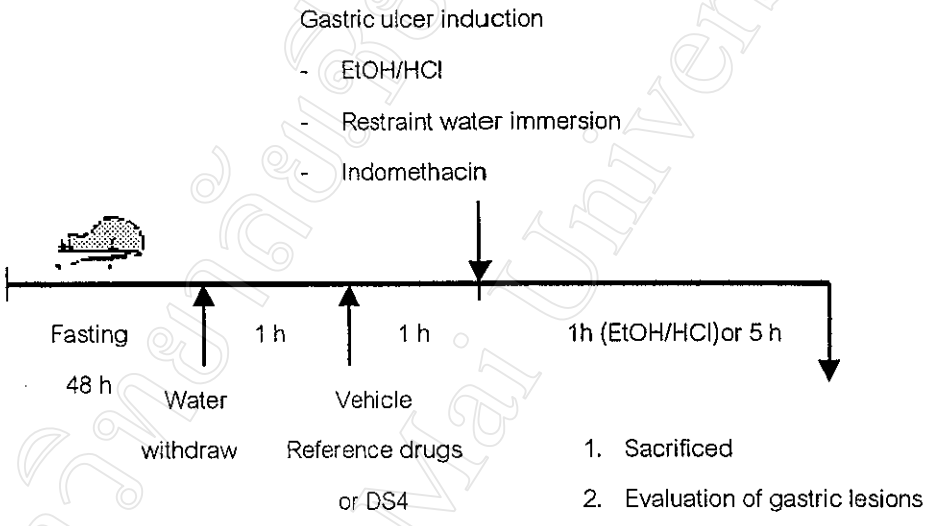

Figure 5 Diagram illustrating the procedure of anti-gastric ulcer test in rats

Evaluation of gastric lesions

After the rats were sacrificed by an overdose of ether, the stomachs were removed and opened along the greater curvatures, 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). Lesion size in mm was determined by measuring each lesion along its greatest diameter. 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:

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

where UI = Ulcer index

UI_c = Ulcer index of control group

UI_t = Ulcer index of test group

Pylorus ligation Experiment (Figure 6)

The pylorus was ligated according to the method of Shay *et al.* (1945). 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 stomachs was excised and the gastric juice was collected, centrifuged at 2,500 rpm for 5 min and its volume was measured and 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 acid output was calculated and expressed as μEq/100g body weight of rat/hour. Each stomach was also determined for gastric ulcer.

Total acidity of gastric juice was calculated as follow:

$$N_1V_1 = N_2V_2$$

where N_1 = normality of gastric juice (μEq)

N_2 = normality of NaOH (μEq)

V_1 = volume of gastric juice (ml)

V_2 = volume of NaOH (ml)

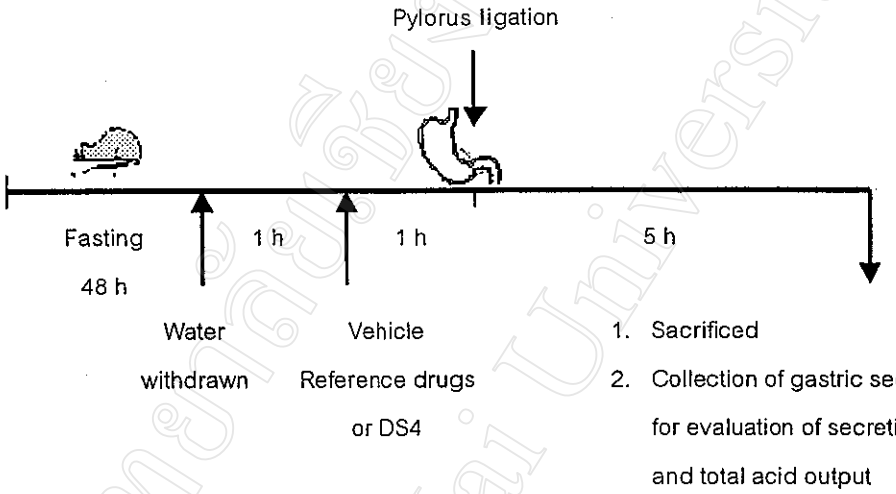

Figure 6 Diagram illustrating the procedure of pylorus ligation in rat experiment

Gastric-wall mucus determination (Figure 7)

Sprague-Dawley rats were fasted 48 h, and water was given *ad libitum*. The water was withdrawn 1 h before starting the experiment.

The experimental animals were divided into 7 groups of 6 rats.

Group 1	normal group, received ethanol (1 ml/kg)
Group 2	control group, received ethanol (1 ml/kg)
Group 3	reference group, received cimetidine 50 mg/kg
Group 4	reference group, received misoprostol 50 µg/kg
Group 5-7	test groups, received 3 doses (25,50 and 100 mg/kg) of DS4 dissoved in absolute ethanol

The rats were pre-treated with DS4 or the reference drugs intraperitoneally. One hour later, the rats of groups 2-7 were administered 1 ml of EtOH/HCl orally, (as previously described) and 1 h later sacrificed and the stomach was removed. Gastric wall mucus was determined by the method of Corne *et al.* (1994). 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 mucous 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 spectrophotometer (Milton Roy Company. U.S.A.). The quantity of alcian blue extract/g (wet) of stomach was then calculated from a standard curve of concentration and absorbance of alcian blue (Figure 8).

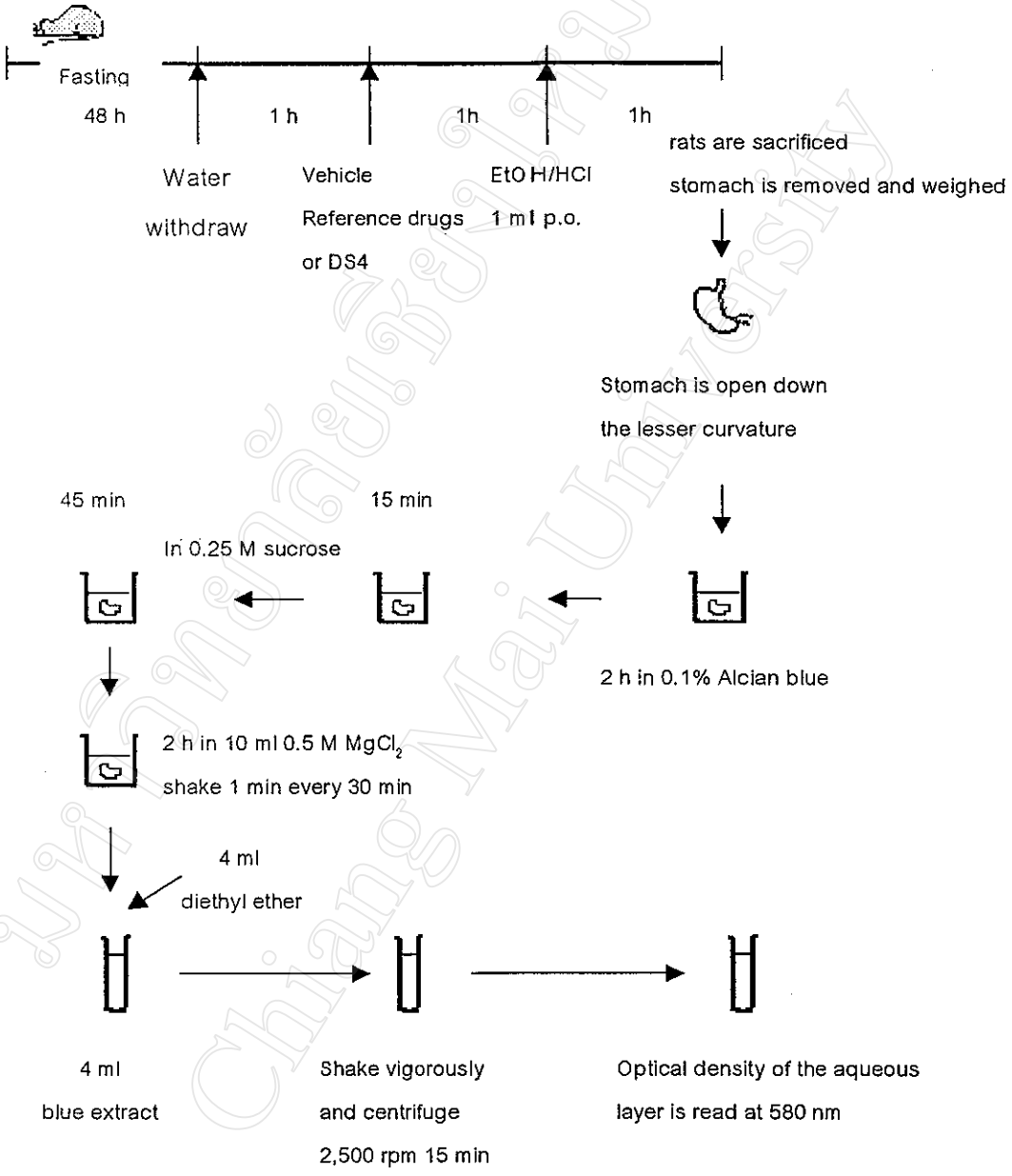

Figure 7 Diagram illustrating the gastric-wall mucus determination

Gastric wall mucus was calculated as follow:

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

Hippocratic screening test

The study was performed according to the method of Malone and Robichaud (1962). Non-fasted male Sprague-Dawly rats weighing between 180-200 g were used. Various doses of DS4 were administered orally to groups of 3 rats. Signs and symptoms observed at 5,10,15,30,60,120 and 240 min after DS4 administration was graded and recorded in the standard working sheet (Figure 9).

The treated animals which were alive after 7 days were sacrificed and a necropsy was performed to examine the internal organs (heart, lung, liver, kidney, intestine, etc.) for any unusual signs.

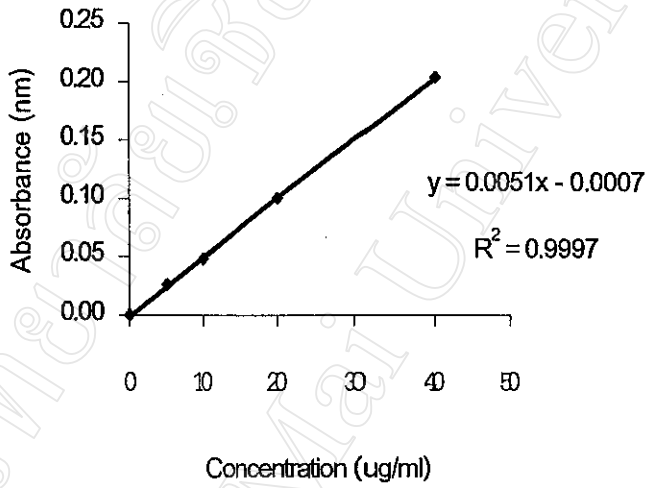

Figure 8 Standard curve of concentration absorbance of alcian blue solution

The Standard Working Sheet for Hippocratic Screening

Date	Qualitative and Semi-Quantitative Screening and Toxicity Report of												
Vehicle for sample :				Test Animal :					Fast ?				
Conc :				Sex :		Mark :			Color mark				
MI. Inj				Weight (G)									
Route Inj	Time inj					Evaluted by :							
Time : (min) post dosage	0	5	15	30	60	120	Time : (min) post dosage	0	5	15	30	60	120
PARAMETER	Response						PARAMETER	Response					
CNS							EARS, ORAL MUCOSA						
Motor Activity							Blanching						
Loss. Righting Reflex							Hyperemia						
Analgesia							Cyanosis						
Resp. Rate							GENERAL						
Resp. Depth							Salivation						
Loss. Corneal Reflex							Tail Erection						
Paraltsis : legs							Pilomotor Erection						
Screen grip : H.L. loss							Micturation						
Screen grip : F.L. loss							Diarrhoea						
Fine Body Tremors							Robichaud Test						
Coarse Body Tremors							Circling Motions						
Fasciculations							Tail Lashing						
Clonic Convulsions							Abdominal Griping						
Tonic Convulsions							Rectal Temp. C						
Mixed Type Convulsions							Startle Reaction						
EYES													
Enopthalmos							Head Tap : Aggressive						
Exophthalmos							Head Tap : Passive						
Palpebral Ptosis							Head Tap : Fearful						
Pupil size, mm.							BodyBody Touch : Passive						
Nystagmus							Body Touch : Fearful						
Lacrimation							Statue Positions						
Bloody Tear							Excess Curiosity						

DEATH AND AUTOPSY NOTES

Figure 9 The standard working sheet for hippocratic screening

Drugs and Chemicals

Drugs

1. Cimetidine
   - tablets (Tagamet®, S.K.& F, U.S.A.)
   - injection (Siam Pharmaceutical Co., Ltd., Bangkok, Thailand)
2. Indomethacin (BLH Trading Co., Ltd., Bangkok, Thailand)
3. Prostaglandin (Cytotec®, G.D. Searle (Thailand) Ltd.)

Chemicals

1. Alcian blue (Fluka Chemical AG., Switzerland)
2. Diethyl ether (BDH Laboratory Supplies Poole, England)
3. Ethanol (MERCK, Darmstadt, F.R. Germany)
4. Hydrochloric acid (MERCK, Darmstadt, F.R. Germany)
5. Magnesium chloride (MERCK, Darmstadt, F.R. Germany)
6. Phenolphthalein (MERCK, Darmstadt, F.R. Germany)
7. Sodium acetate (MERCK, Darmstadt, F.R. Germany)
8. Sodium carboxymethylcellulose (Srichand United Dispensary Ltd.)
9. Sodium hydroxide (May & Baker Ltd., Dagenham, England)
10. Sucrose (MERCK, Darmstadt, F.R. Germany)

Statistic Analysis

The data from the experiments were expressed as mean $\pm$ standard error of 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. Statistical comparisons between two groups were analyzed by Student's t-test. P values less than 0.05 were considered significant.