MATERIALS AND METHODS

PLANT MATERIAL

The methanol extract from the bark of *G. speciosa* (GS extract) was obtained from Prof. Dr. Vichai Reutrakul, the Department of Chemistry, Faculty of Science, Mahidol University.

EXPERIMENTAL ANIMALS

Male Swiss albino mice weighing 20-40 g and male Sprague-Dawley rats weighing 40-60 g, 100-120 g and 180-200 g were obtained from the National Laboratory Animal Center, Nakorn Pathom. All animals were kept in the room maintained under environmentally controlled conditions of 24 $\pm$ 1^{o} C and 12 h light-12 h dark cycle. 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 of the animal except in the writhing response model, test drugs were administered intraperitoneally and in the ear edema model, a local application of test drug to outer and inner surfaces of the ear was performed. A 5% ethanol in 30% acacia was used as a vehicle except in ear edema model, where absolute ethanol was used. In the control group, animals received only vehicle in the same volume.

EXPERIMENTAL PROTOCOL

1. Anti-inflammatory activity study

1.1 Ethyl phenylpropiolate (EPP) and arachidonic acid (AA)-induced ear edema in rats

The methods of Brattsand *et al.* (1982) and Yong *et al.* (1984) were performed as follows:

Male rats weighing 40-60 g were used. AA and EPP were dissolved in acetone at the concentration of 50 mg/ml. Ear edema was induced by topical application of either AA or EPP to the inner and outer surfaces of both ears. Each rat received AA or EPP 1 mg/20 µl/ear. This dose of phlogistic was applied locally using an automatic microliter pipet to both the inner and outer surfaces of the ear.

Phenylbutazone and phenidone, used as reference drugs, as well as GS extract were dissolved in absolute ethanol and applied topically in a volume of 20 µl to the inner and outer surfaces of the ear by means of an automatic microliter pipet just before the irritants. The control group received absolute ethanol only. Before and at 15, 30, 60 and 120 min after edema induction, the thickness of each ear was measured with vernier calipers. The increase in ear thickness was compared with the vehicle-treated group and the percent inhibition was calculated as follows:

$$ED_x = ET_x - ET_o$$

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

where,

ED_x = edema thickness at time x

ET_x = ear thickness (μm) at time x

ET_o = ear thickness (μm) before application of EPP or AA

ED_c = edema thickness (μm) of control group at time x

ED_t = edema thickness (μm) of test group at time x

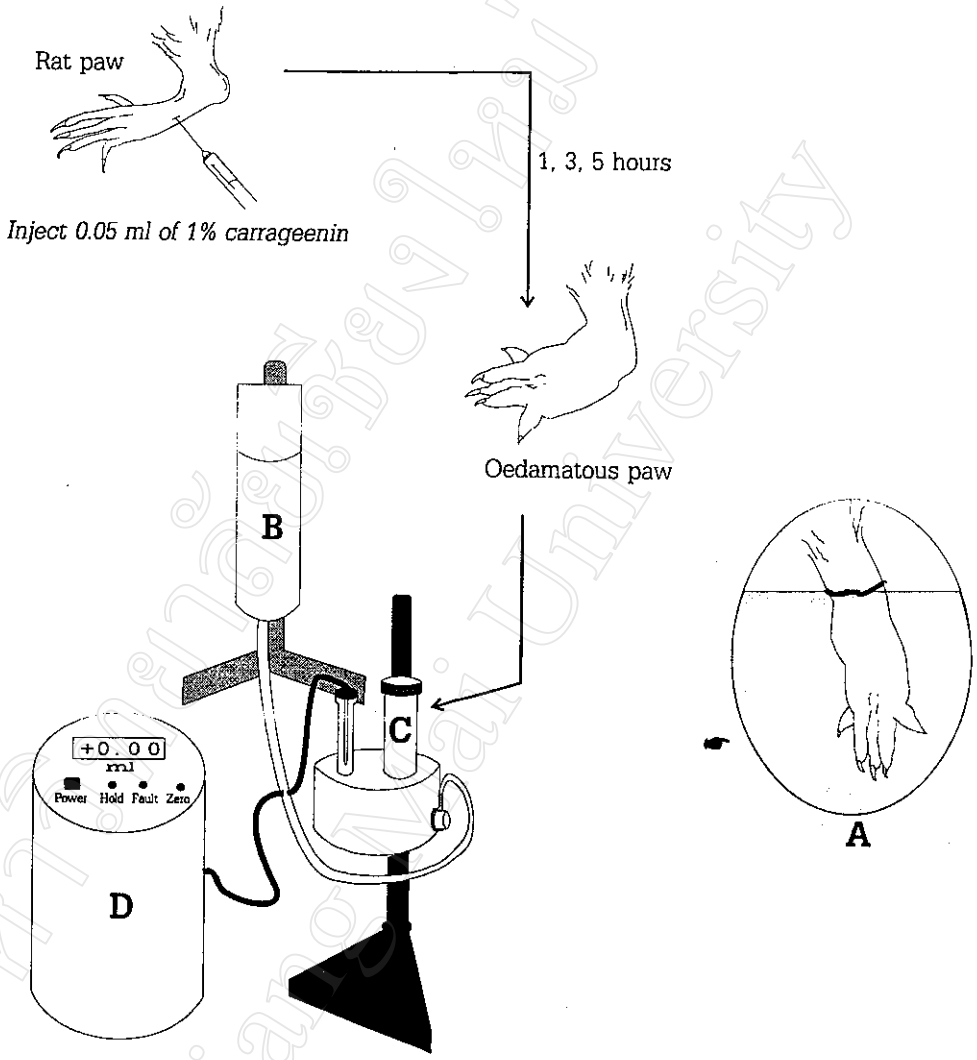
% EDI = percent edema inhibition of test compound at time x

1.2 Carrageenin-induced hind paw edema in rats

This method as described by Winter *et al.* (1962) was used for investigation of the inhibitory effect of anti-inflammatory drugs on the edema formation induced by carrageenin.

The scheme of experiment is shown in Figure 3. Male rats of 100-120 g body weight were divided into groups of 6 animals. Aspirin (acetylsalicylic acid), used as a reference drug, and GS extract were orally given 1 h prior to carrageenin injection. The control group received 5% ethanol in 30% acacia only. 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 hair line. Each paw volume was obtained from the average of 3 readings. In the group of carrageenin-induced paw edema, the paw volume was measured prior to and 1, 3 and 5 h after carrageenin injection



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.

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

1.3. Cotton pellet-induced granuloma formation in rats

This experiment was performed for investigation of the ability of agent to inhibit the proliferative component of the inflammatory process. The slight modification of the method described by Swingle and Shideman (1972) was performed as follows:

Absorbent cotton wool was cut into pieces weighing 20 $\pm$ 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^{o}C for 2 h.

Male rats of 180-200 g body weight were used. As shown in Figure 4 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.

The reference drugs, prednisolone and indomethacin as well as GS extract were administered orally in a once daily dosage regimen throughout

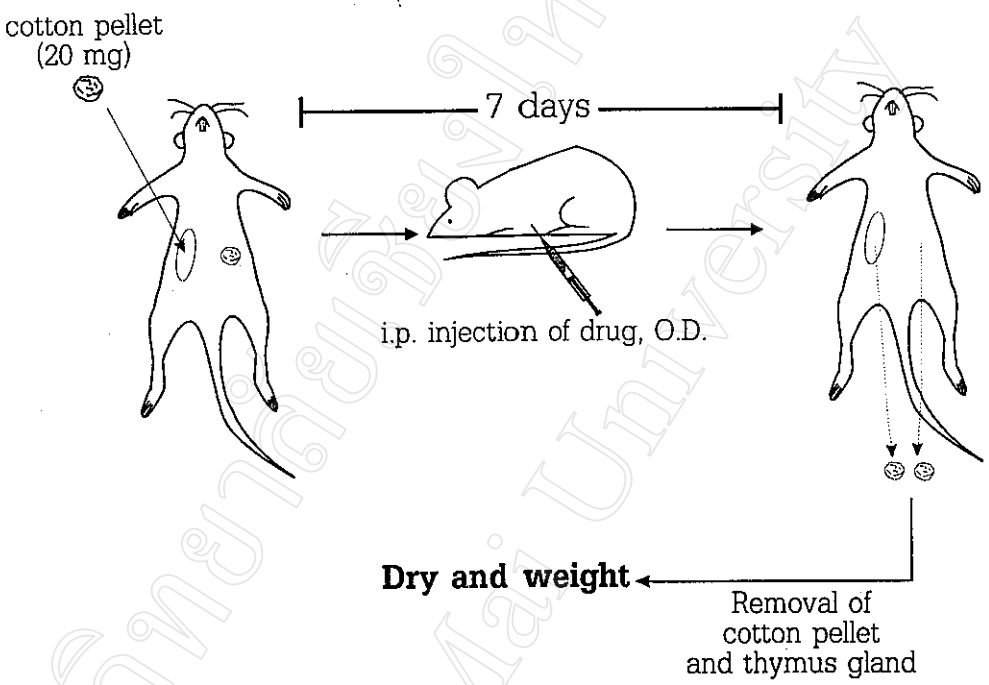


Figure 4. Diagram illustrating the method for cotton pellet-induced granuloma formation in rats.

the experimental period of 7 days whereas the control group received 5% ethanol in 30% acacia only. The animals were sacrificed on the eighth day after implantation. In order to obtain the data of many parameters, rats were first anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The trachea was cannulated with a polyethylene tube to facilitate spontaneous respiration and thereby increase the heart function. The cannula was inserted into the common carotid artery and blood was collected into a tube. The blood was centrifuged at 1000 r.p.m. for 5 min and the serum was separated.

After collection of the blood the rat was dead 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. The thymus was also dissected out. Both cotton pellets and thymuses were dried at 60° C for 18 h and their dry weight determined. The change in body weight from the first and the last day of experiment was also recorded.

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

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

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

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

where,

Wt_w = wet weight of grunuloma pellet (mg)

Wt_d = dry weight of grunuloma pellet (mg)

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

GW = granuloma weight (mg)

GI = granuloma inhibition

Samples of serum were sent to Central Laboratory, Faculty of Medical Technology, Chiang Mai University, for determination of the amount of alkaline phosphatase and total protein. The enzyme activity was expressed as units of enzyme/mg of serum protein.

The method for measurement of alkaline phosphatase activity and total protein in the serum, as described by Bessey *et al.* (1946) and Weichselbaum (1946), was briefly as follows: Alkaline phosphatase reagent was used to measure alkaline phosphatase activity by the kinetic rate method using a 2-amino-2-methyl-1-propanol (AMP) buffer. In the reaction, alkaline phosphatase catalyzed the hydrolysis of the colorless organic phosphate ester substrate, p-nitrophenylphosphate, to the yellow colored product, p-nitrophenol, and phosphate. This reaction occurred at an alkaline pH of 10.3. Protein reagent was used to measure the total protein concentration by a timed-endpoint biuret method. In this reaction, the peptide bonds in the protein sample bound to cupric ions in an alkaline medium to form colored peptide/copper complexes.

The SYNCHRON CX System automatically proportioned the appropriate serum of sample and reagent into the cuvette. A filled 0.5 ml sample cup was the optimum volume. The ratio used was one part sample

to 50 parts reagent. The system monitored the change in absorbance at 410 nm for measurement of alkaline phosphatase activity and at 560 nm for measurement of total protein. This change in absorbance was 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.

1.4. Acetic acid-induced writhing response in mice

This method was used for investigation the analgesic activity. The method was done essentially as described by Collier *et al.* (1968) and modified by Nakamura *et al.* (1986).

Male swiss albino mice weighing 30-40 g were used. A writhing response was produced by an injection of 0.75% acetic acid aqueous solution in a volume of 0.1 ml/10 g body weight into the peritoneal cavity and the animals were then placed into a transparent plastic box. The number of writhes, a response consisting of contraction of an abdominal wall, pelvic rotation followed by hind limb extention, was counted during continuous observation for 15 min beginning from 5 min after the acetic acid injection.

Aspirin, used as a reference drug, and GS extract were administered intraperitoneally 30 min before the acetic acid injection. The control group received 5% ethanol in 30% acacia only. Percentage of inhibition of writhing response was calculated.

2. Anti-ulcerogenic activity study

2.1 Preparation of rats for anti-ulcerogenic activity study

Sprague-Dawley rats weighing 180-200 g, were fasted 48 h, and water was given *ad libitum*. The water was withdrawn 1 h before starting the experiment; GS extract or the reference drug was given orally to the rats 1 h before gastric lesions were induced. The rats were divided into 5 groups of 5-6 animals.

Group 1 control group, received 5% ethanol in 30% acacia (used as a vehicle for all test drugs)

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

Group 3-5 test groups, received 75,150 and 300 mg/kg of GS extract, respectively.

Rats were given 5% ethanol in 30% acacia, reference drug, or GS extract 1 h before induction of gastric lesions.

2.2 Method used to induce gastric lesions

2.2.1 Indomethcin-induced gastric lesions

Indomethacin suspended in 0.5% carboxymethyl cellulose was injected intraperitoneally at a single dose of 30 mg/kg (Pal and Nagohandhury, 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 5).

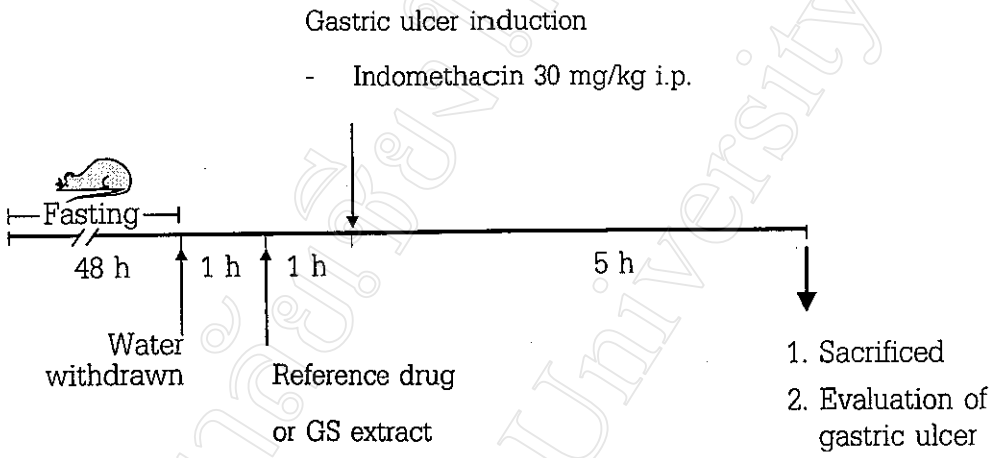


Figure 5. Diagram illustrating the procedure of indomethacin-induced gastric lesion in rat.

2.2.2 Pylorus ligation

Pylorus ligation was performed by following the method of Shay *et al.* (1945). 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 6). 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 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.

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

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)

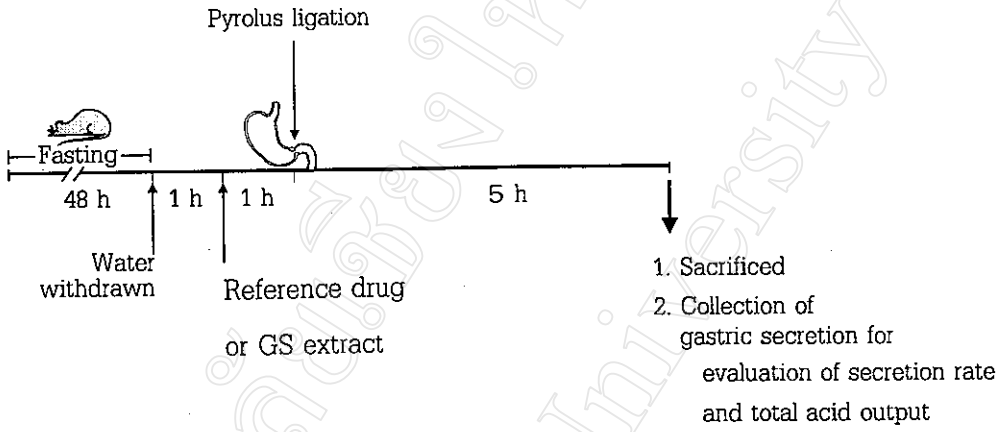


Figure 6. Diagram illustrating the procedure of pylorus ligation in rat.

2.3 Evaluation of gastric lesions

After the rats were sacrificed, the stomaches 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 under the microscope. The ulcer index was accessed using the severity score scale as follows (Minano *et al.*, 1987):

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 score in each group divided by the number of rats in that group was expressed as the ulcer index. 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

One-way ANOVA was applied to the results to evaluate the significance of differences. Regression analysis and test for correlation were also employed in some experiments.

DRUGS AND CHEMICALS

1. Drugs

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

1.2 Anesthetic ether B.P.1980 (The Government Pharmaceutical Organization, Bangkok, Thailand)

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

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

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

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

1.7 Phenidone (Riedel-de Haen AG, D-3016 Seelze 1, Germany)

1.8 Phenylbutazone (Sigma Chemical Company, St. Louis, U.S.A.)

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

2. Irritants

2.1 Absorbent cotton wool (Vidhyasom Co., Ltd., Thailand)

2.2 Arachidonic acid (Sigma Chemical Company, St. Louis, U.S.A.)

2.3 Carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

2.4 Glacial acetic acid B.P.C. 1973 (The Government Pharmaceutical Organization, Bangkok, Thailand)

2.5 Ethyl phenylpropiolate (EPP) (Fluka Chemicals Co., Ltd., Japan)

3. Vehicles

3.1 Acacia (Vidhyasom Co., Ltd., Bangkok, Thailand)

3.2 Acetone (Merck, Darmstadt, Germany)

3.3 Absolute ethanol (Merck, Darmstadt, Germany)