MATERIALS AND METHODS

PLANT MATERIAL

Clerodendrum petasites S. Moor was collected by Professor Dr. Tawatchai Santisuk, Department of Forestry Herbarium, Bangkok. The plant was identified and found to be identical with the voucher specimen (QBG 4469) deposited at the Herbarium of the Queen Sirikit Botanical Garden, Chiang Mai, Thailand.

PLANT EXTRACT

The methanol extract from *C. petasites*, designated as CP extract, was kindly prepared by Professor Dr. Vichai Reutrakul, the Department of Chemistry, Faculty of Science, Mahidol University.

EXPERIMENTAL ANIMALS

Male Swiss albino mice weighing 30-40 g and male Sprague-Dawley rats weighing 40-60 g, 100-120 g and 180-220 g were purchased from the National Laboratory Animal Center, Nakorn Pathom. All animals were kept in the room maintained under environmentally controlled conditions of 24 $\pm$ 1°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 ear edema model, a local application of test drug to outer and inner surfaces of the ear was performed. A 5% polysorbate 80 U.S.P. (Tween 80) was used as a vehicle except in ear edema model, where absolute ethanol and acetone were used. In the control group, animals received only vehicle in the same volume and the same route.

EXPERIMENTAL PROTOCOL

1. Anti-inflammatory activity study

1.1 Ethyl phenylpropiolate (EPP)-induced ear edema in rats

This method was used for screening and evaluation of inhibitory activity on the edema formation induced by EPP. The method described by Brattsand *et al.* (1982) was performed as follows:

Male rats of 40-60 g body weight were divided into 8 groups of 5 animals.

Group 1 control group, received acetone (a vehicle for reference drug)

Group 2 control group, received absolute ethanol (a vehicle for test drug)

Group 3-5 reference groups, received phenylbutazone 0.25, 0.5 and 1 mg/20 µl/ear, respectively

Group 6-8 test groups, received CP extract 1, 2 and 4 mg/20 µl/ear, respectively

EPP (used as an irritant for edema induction) was dissolved in acetone at the concentration of 50 mg/ml. Ear edema was induced by topical application of EPP to the inner and outer surfaces of both ears using an automatic microliter pipet. Each rat received EPP 1 mg/20 µl/ear.

Phenylbutazone was dissolved in acetone, whereas CP extract was dissolved in absolute ethanol. Each of the test drugs was 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 irritant. The control groups received vehicle only in the same volume. The thickness of each ear was measured with vernier calipers before and at 15, 30, 60 and 120 min after edema induction.

The increase in ear thickness caused by test drugs was compared with their vehicle-control 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

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 test drugs on the edema formation induced by carrageenin.

Male rats of 100-120 g body weight were divided into 7 groups of 6 animals.

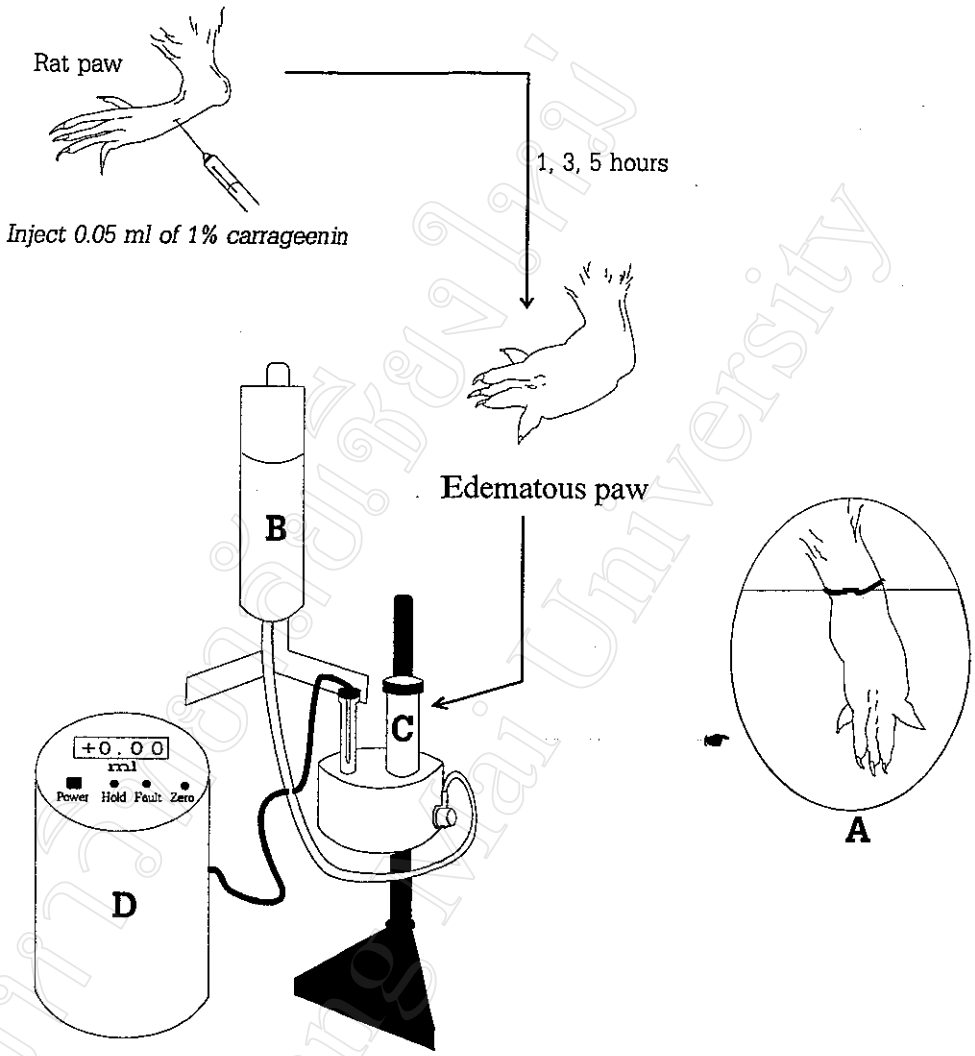
Group 1 control group, received vehicle

Group 2-4 reference groups, received aspirin 75, 150 and 300 mg/kg, respectively

Group 5-7 test groups, received CP extract 300, 600 and 1200 mg/kg, respectively

Aspirin (acetylsalicylic acid), CP extract and vehicle 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 unanaesthetized 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



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.

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

1.3 Arachidonic acid (AA)- induced hind paw edema in rats

This method as described by DiMartino *et al.* (1987), was used for investigation of the inhibitory effect of anti-inflammatory drugs on the edema formation induced by AA.

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

Group 1 control group, received vehicle

Group 2 reference group, received aspirin 300 mg/kg

Group 3 reference group, received phenidone 40 mg/kg

Group 4-6 test groups, received CP extract 300, 600 and 1200 mg/kg, respectively

Phenidone, aspirin, CP extract as well as vehicle were given 2 h prior to AA injection. AA was dissolved in 0.2 M carbonate buffer (pH 8.4) in a concentration of 0.5% and a volume of 0.1 ml was injected into the plantar side of the right hind paw.

Foot volume of animal was determined similarly to the method performed in the model of carrageenin-induced paw edema. The paw volume was measured prior to and 1 h after AA injection.

The edema volume of the paw and the percent edema inhibition of each test compound were obtained by the calculating formula described in the model using carrageenin as edema inducer.

1.4 Cotton pellet-induced granuloma formation in rats

This experiment was used 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:

Male rats of 180-220 g body weight were divided into 6 groups of 6 animals.

Group 1 control group, received vehicle

Group 2 reference group, received aspirin 300 mg/kg

Group 3 reference group, received prednisolone 5 mg/kg

Group 4 test groups, received CP extract 1200 mg/kg

Adsorbent 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.

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.

Prednisolone, aspirin and CP extract as well as vehicle 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. In order to obtain the data of many parameters, rats were first anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. The cotton pellets then were dried at 60^{o} C for 18 h and their dry weight was determined.

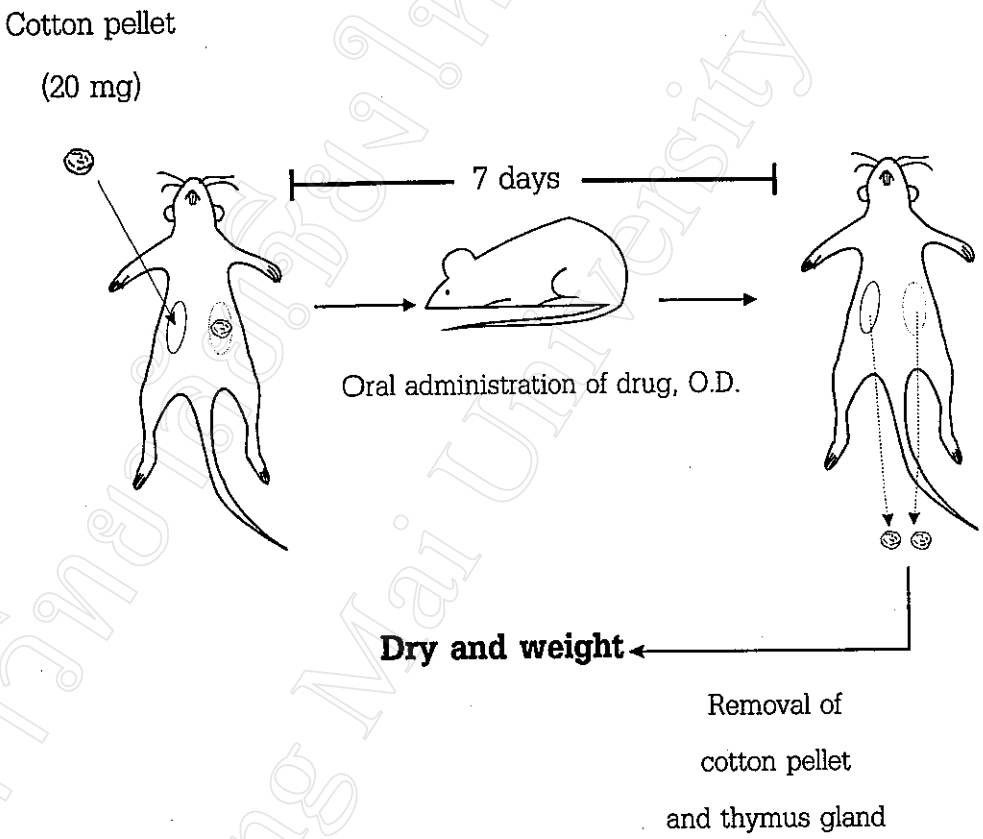


Figure 4. Diagram illustrating the method for cotton pellet-induced granuloma formation in rats.

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

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

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

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

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

1.5 Measurement of alkaline phosphatase activity in the serum (Bessy *et al.*, 1946)

The animals in the cotton pellet-induced granuloma formation model were used in this method.

On the eighth day after implantation, rats were anaesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). Cardiac puncture was performed and the blood was collected into a tube. The blood was centrifuged at 1000 r.p.m. for 5 min and the serum was separated.

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), was briefly described 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.

1.6 Measurement of thymus weight and body weight gain (Swingle and Shideman, 1972)

The animals in the cotton pellet-induced granuloma formation model were used in this experiment. On the eighth day, the rat was dead after collection of the blood. The chest was then opened and the thymus was dissected out. The thymuses were dried at 60° C for 18 h and their dry weight was determined. The change in body weight from the first and the last day of experiment was also recorded.

2. Ulcerogenic activity study

Evaluation of gastric lesions

The animals in the cotton pellet-induced granuloma formation model were also used in this experiment. 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 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 of lesions in each group divided by the number of rats in that group was expressed as the gastric lesions.

3. Analgesic test

Acetic acid-induced writhing response in mice

This method was used for investigation the analgesic activity. The procedure 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 and divided into 5 groups of 6 animals.

Group 1 control group, received vehicle

Group 2 reference group, received aspirin 300 mg/kg

Group 3-5 test groups, received CP extract 300, 600 and 1200 mg/kg, respectively

A typical "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 extension, was counted during continuous observation for 15 min beginning from 5 min after the acetic acid injection.

Aspirin, CP extract and vehicle were administered orally 1 h before the acetic acid injection. Percentage of inhibition of writhing response was calculated.

4. Antipyretic test

Yeast-induced hyperthermia in rats

The antipyretic activity of test fractions was tested and compared with aspirin, using the method described by Teotino *et al.*, (1963) as follows:

Male rats weighing 180-220 g were divided into 3 groups of 8 animals.

Group 1 control group, received vehicle

Group 2 reference group, received aspirin 300 mg/kg

Group 3 test group, received CP extract 300 mg/kg

The animals were housed and maintained under uniform environmental conditions. Disturbances likely to excite them were avoided. Before pyrexia was induced, the animals were restrained in plastic cages and the initial rectal temperatures were recorded using a ten channel electric thermometer (EXACON), model MC 8940, EXACON Scientific Instruments Aps, Denmark) connected with the probes (model H-RRA, EXACON Instruments Aps, Denmark) which were inserted into the rat rectums to about 5 cm dept (Figure 5). In order to adapt the rats to handling procedure for probe insertion, basal rectal temperatures were taken 1 h after probe insertion. Thereafter hyperthermia was induced in rats by subcutaneous injection of 1 ml/100 g body weight of 20% yeast in NSS. When the temperature was at a peak, 18 h after yeast injection, the rectal temperatures were again recorded. Those animals that showed a rise in rectal temperature of more than 1 ^{o}C were used. Aspirin, CP extract and vehicle were then administered orally and the rectal temperatures of animals were recorded at 30 minutes interval for two hours following drug treatment.

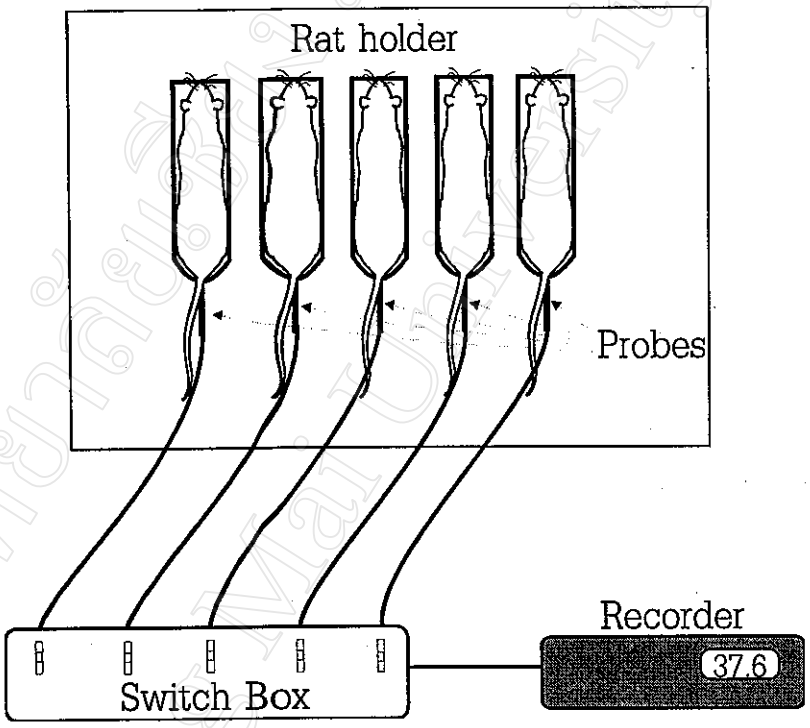


Figure 5. Diagram illustrating the method for yeast-induced hyperthermia.

5. Hippocratic screening test

The effect of high doses of CP extract on the general behavior of conscious animals was the object of this experiment. The method as described by Malone and Robichaud (1962) was performed.

Nonfasted rats of both sexes, weighing between 180-220 g, were used. CP extract and vehicle were administered orally. Five animals were used for complete evaluation in each dosage level. The behavioral changes, which could be observed in the animal after receiving treatment, were recorded on the standardized work sheet (Figure 6) prior to and 5,15, 30 minutes, 1, 2, 4, 6 and 24 hours after the drug treatment, then once daily thereafter for 7 days. Autopsy was performed either immediately after death of the animals or after killing on the eighth day of the experiment. Any changes in vital organs compared with those of control animals were recorded.

According to OECD guidelines for testing of chemicals, if an oral dose of 5000 mg/kg of test compound causes no death and no change in general behavior of the animals, it is considered to be non toxic, therefore it is not necessary to test other doses.

The standard working sheet for Hippocratic screening test

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

Figure 6. Standardized work sheet for Hippocratic screening.

STATISTICAL ANALYSIS

One-way ANOVA, student's t-test and Mann Whitney U test were 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 Pentobarbital sodium injection U.S.P. (Nembutal®, Abbott Laboratories, North Chicago, U.S.A.)
   - 1.4 Phenidone (Riedel-de Haen AG, D-3016 Seelze 1, Germany)
   - 1.5 Phenylbutazone (Sigma Chemical Company, St. Louis, U.S.A.
   - 1.6 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)
   - 2.6 Brewers yeast (Sigma Chemicals Co., U.S.A.)
3. Vehicles
   - 3.1 Acetone (Merck, Darmstadt, Germany)
   - 3.2 Absolute ethanol (Merck, Darmstadt, Germany)
   - 3.3 Polysorbate 80 (Tween 80, Sigma Chemical Company, St. Louis, U.S.A.
   - 3.4 Carbonate buffer (pH 8.4)