CHAPTER 2

MATERIAL AND METHODS

2.1 PLANT EXTRACT

Murdannia loriformis was collected from Petchaburi province during November. The plant material was authentic and identical to the voucher specimen QBG.No.25135 which is kept at the herbarium section of Queen Sirikit Botanical Garden. Dry powder of *M. loriformis* weighing 700 g was extracted for 24 h with 4 L of 80% ethanol using stirrer at room temperature (repeated 4 times). The extract was filtered through filter paper by suction. The filtrate was evaporated to dryness by using a rotating evaporator at 55 ^{o}C and lyophilized. The residue obtained (96.7 g) is designated as ML extract.

2.2 EXPERIMENTAL ANIMALS

Male and female Sprague-Dawley rats weighing 100-110 g and 190-210 g as well as male Swiss albino mice weighing 30-35 g obtained from Nation Animal Laboratory Center, Nakornpathom, were used. All animals were kept in a room maintain under environmentally control conditions of 24 $\pm$ 1 ^{o}C and 12 h light-12 h dark cycle. All animals had free access to water and standard diet (Pokphan Animal Feed Co., Ltd., Bangkok, Thailand). They were acclimatized for at least one week before starting the experiments.

2.3 PREPARATION OF TEST DRUGS

All test drugs were suspended in 5% polysorbate 80 U.S.P. (Tween 80)

2.4 DRUG ADMINISTRATION

All test drugs were orally administered in an equivalent volume of 0.5 ml/100 g body weight of the rats and 0.1 ml/10 g body weight of the mice.

2.5 EXPERIMENTAL PROTOCOL

2.5.1 ANTI-INFLAMMATORY STUDY

2.5.1.1 Carrageenin-induced hind paw edema in rats (66)

This experiment was performed to investigate the inhibitory effect of test agents on the hind paw edema formation induced by carrageenin. Male rats of 100-110 g body weight were divided into 5 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 10 mg/kg indomethacin

Group 3-5 test groups, received 100, 200 and 400 mg/kg of the ML extract

The rats were pretreated with 5% Tween 80, indomethacin or the various doses of the ML extract 1 h prior to carrageenin injection. Acute inflammation was produced by subplantar administration of 0.05 ml of 1% carrageenin in sterile normal saline solution (NSS) into the right hind paw of the rats. Paw volume was measured using a plethysmometer (model 7150, Ugo Basile, Italy, Figure 3.) before injection and immediately at 1, 3 and 5 h after carageenin injection. The scheme of experiment is shown in Figure 4.

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

$$EV_X = PV_X - PV_0$$

$$\%EI_X = \frac{EV_X \text{ of control group} - EV_X \text{ of test group}}{EV_X \text{ of control group}} \times 100$$

where,

EV_X = edema volume (ml) at time x

PV_X = paw volume (ml) at time x

PV_0 = paw volume (ml) measure before carrageenin injection

$\%EI_X$ = percent edema inhibition of the ML extract at time x

A: water reservoir contained 0.05% NaCl in distilled water

B: measuring chamber

Figure 3. Plethysmometer

Measuring initial paw volume

Oral drug administration

1 h

Measuring paw edema volume

at 1, 3 and 5 h after carrageenin injection.

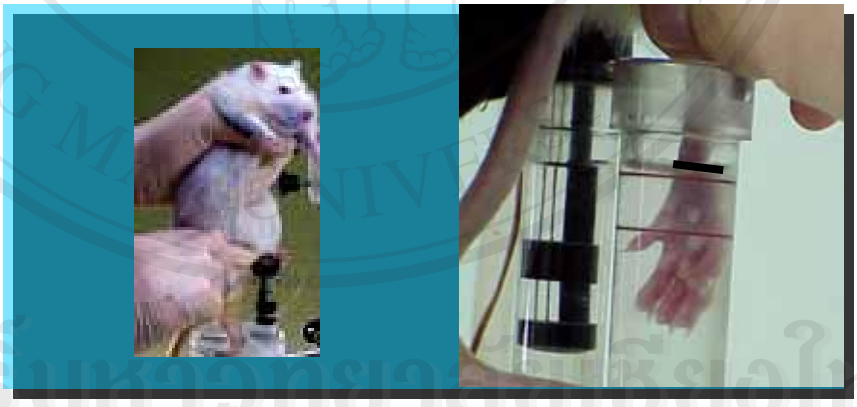

Figure 4. Diagram illustrating the water displacement method for measuring rat paw volume.

2.5.1.2 Arachidonic acid-induced hind paw edema in rats (72)

This experiment was performed to investigate the inhibitory effect of test agents on the edema formation induced by AA. Male rats of 100-110 g body weight were used and divided into 6 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 10 mg/kg indomethacin

Group 3 reference group, received 40 mg/kg phenidone

Group 4-6 test groups, received 100, 200 and 400 mg/kg ML extract

5% Tween 80, indomethacin, phenidone and various doses of the ML extract were administered 2 h prior to AA injection. A volume of 0.1 ml of 0.5% AA in 0.2 M carbonate buffer (pH 8.4) was injected intradermally into the plantar of the right hind paw.

The paw volume was measured 1 h after AA injection. Paw volume of rats, edema volume of paw and the percent edema inhibition of each compound were measured by the same method as described in carrageenin-induced hind paw edema.

2.5.1.3 Cotton pellet-induced granuloma formation in rats (73)

This experiment was performed for investigation of the ability of an agent to inhibit the proliferative components of the subchronic and chronic inflammatory processes. The method was slightly modified as follows:

Adsorbent cotton wool was cut into pieces weighing 20 $\pm$ 1 mg and made into pellets. The pellets were sterilized in a hot air oven (model 25, Arthur H. Thomas CO., U.S.A.) at 120 ^{0}C for 2 h. Male rats of 190-210 g body weight were used and divided into 4 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 5 mg/kg indomethacin

Group 3 reference group, received 5 mg/kg prednisolone

Group 4 test group, received 100, 200 and 400 mg/kg ML extract

A. Granuloma formation

The abdominal skin was shaped and disinfected with 70% alcohol. After that, 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 scheme of experiment is shown in Figure 5. The ML extract and prednisolone as well as indomethacin were administered orally in a once daily dosage regimen throughout the experimental period of 7 days whereas the control group received 5% Tween 80 only. The animals were sacrificed on the eighth day after cotton pellet implantation, the abdominal skin was then opened. The implanted pellets were dissect out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. Cotton pellets were dried at 60 ^{0}C for 18 h and their dry weight determined. The changes in granuloma weight and transudative weight of test group was compared with the control and reference group. The percent granuloma inhibition of the extract was calculated according to the following formulae:

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

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

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

where,

Wt_w = wet weight of granuloma pellet (mg)

Wt_d = dry weight of granuloma pellet (mg)

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

GW = granuloma weight (mg)

%GI = percent granuloma inhibition

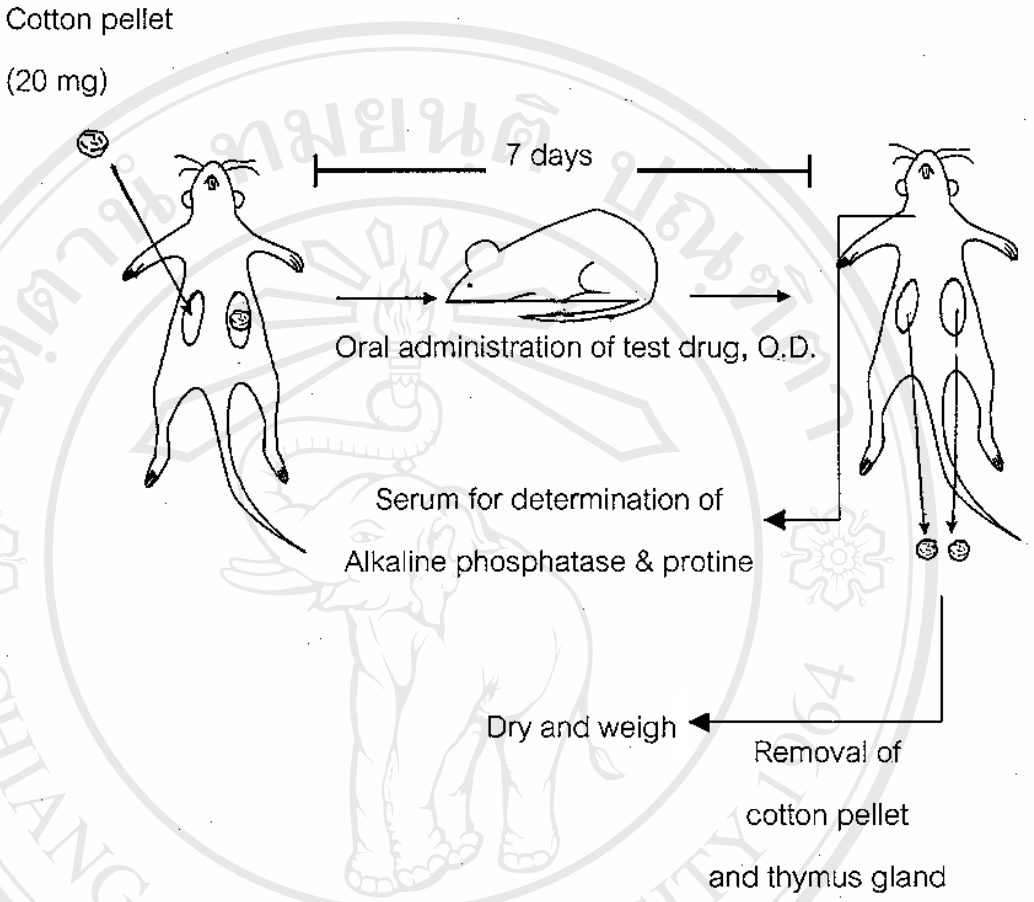

Figure 5. Diagram illustrating the method for cotton pellet-induced granuloma formation in rats

B. Measurement of the body weight gain and the thymus weight

The body weight gain and thymus dry weight of ML extract rats were compared with those of control group and reference groups. This method was described by Swingle and Shideman (73). The animals in the cotton pellet-induced granuloma formation model were used. After collection of the blood, the chest of the rat was opened and the thymus was dissected out. The thymuses were dried at 60 ^{o}C for 18 h and their dry weights were determined. The change in body weight from the first and the last day of experiment was also recorded.

C. Measurement of the alkaline phosphatase activity in serum

This method was described by Bessey *et al*. (118). The animals in the cotton pellet-induced granuloma formation model were used. On the eight day after cotton pellets implantation, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). Blood was collected into a glass tube by cardiac puncture technique. Samples of serum were sent to Suandok Medical Lab (1/42 Chiangmai-Lampang Rd., Hangdong, Chiangmai 50230) for determination of the alkaline phosphatase and total protein.

D. Evaluation of ulcerogenic effect (119)

The animals from the cotton pellet-induced granuloma formation model were used. After the rat was sacrificed, the stomach was 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 a dissecting microscope (10X). Lesion size in mm was determined by measuring each lesion along its greatest diameter, and the grade of lesion was scored according to the following scale:

0 = no pathology

1 = mucosal edema and petechiae

2 = one to five small ulcers (1 to 2 mm)

3 = more of five small ulcers or one medium ulcer (3 to 4 mm)

4 = two or more medium ulcers or large ulcers (>4 mm)

5 = perforated ulcers

2.5.2 ANALGESIC STUDY

The analgesic activity of the extract was tested and compared with reference drugs using the formalin test (79). The formalin test comprises the early phase and the late phase assessment of the analgesic effect. Male Swiss-albino mice weighing 35-40 g were used and divided into two sets of 6 groups (6 animals per group)

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 10 mg/kg indomethacin

Group 3 reference group, received 50 mg/kg codeine

Group 4-6 test groups, received 10, 20 and 40 mg/kg ML extract

In the early phase assessment, 20 μl of 1% formalin in NSS was injected subcutaneously into the right dorsal hind paw of the mice 60 min after test drug administration. Then, between 0-5 min after formalin injection, the time in seconds the mice spent for intensive licking the right dorsal hind paw was determined (Figure 6).

In the late phase assessment, another set of mice as above was used. The formalin was injected 40 min after test drug administration and the licking time was determined between 20-30 min after formalin injection (Figure 7).

The licking time and the percent pain inhibition of each test compound was obtained by the following calculation

$$\%\text{Inhibition} = \frac{L_c - L_t}{L_c} \times 100$$

where,

L_c = Licking time (sec) of control group

L_t = Licking time (sec) of test group

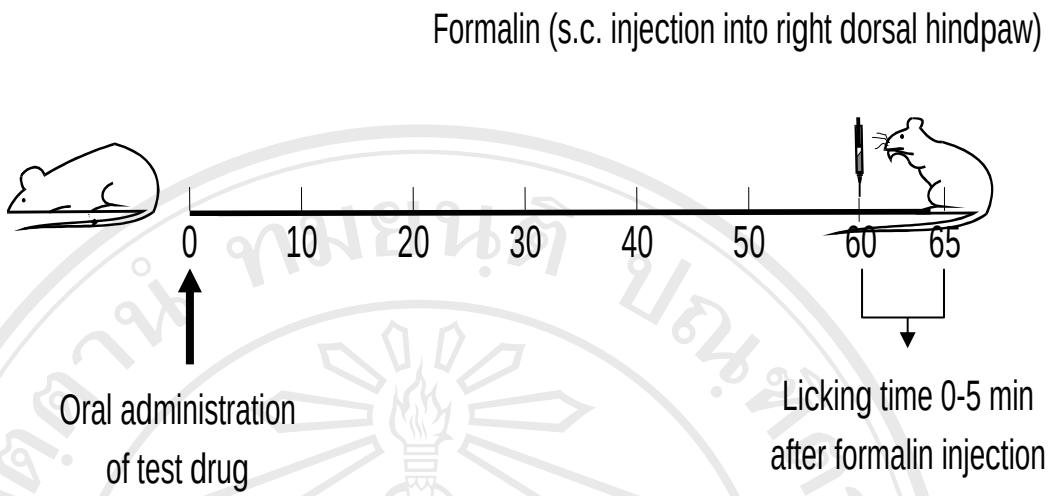

Figure 6. Diagram illustrating the method for formalin test (early phase) in mice

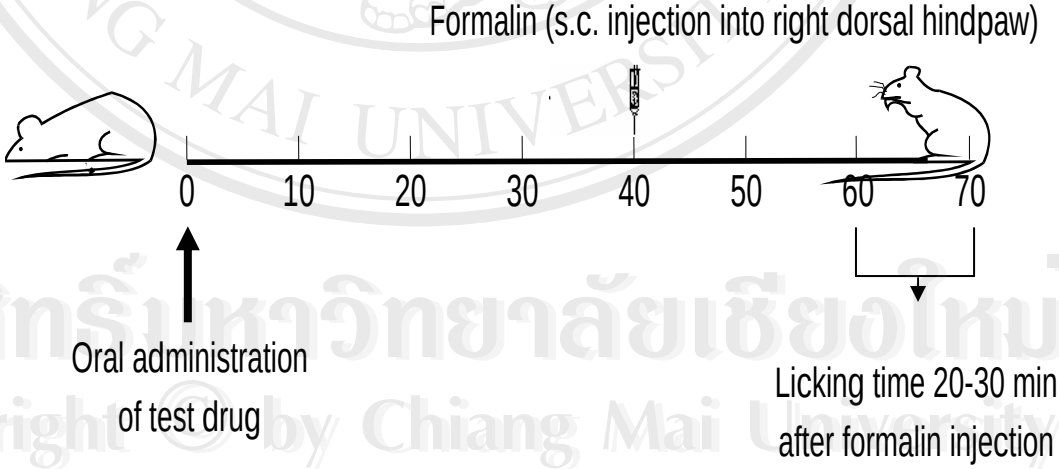

Figure 7. Diagram illustrating the method for formalin test (late phase) in mice.

2.5.3 ANTIPYRETIC STUDY

The antipyretic activity of the ML extract was tested and compared with indomethacin by using yeast-induced hyperthermia in rats following the method described by Teotino *et al*. with slightly modification (120). Male rats weighing 190-210 g were used and divided into 3 groups of 6 animals.

Group 1 control group, received 5% Tween 80

Group 2 reference group, received 10 mg/kg indomethacin

Group 3 test group, received 400 mg/kg ML extract

They were housed and maintained under uniform environmental conditions. Disturbances likely to affect them were avoided. Before pyrexia was induced, the rats 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 depth (Figure 8). In order to adapt the rats to the handling procedure for probe insertion, the 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 25% yeast in NSS. Eighteen hour after yeast injection, the rectal temperatures were again recorded. Those animals which showed a rise in rectal temperature of more than 1 ^{o}C were used. The ML extract, indomethacin and 5% Tween 80 were then administered orally and the rectal temperatures of animals were recorded at 30 min, 1 h, 2 h and 3 h following drug treatment. Diagram illustrating the procedure of the yeast-induced hyperthermia in rat is shown in Figure 9.

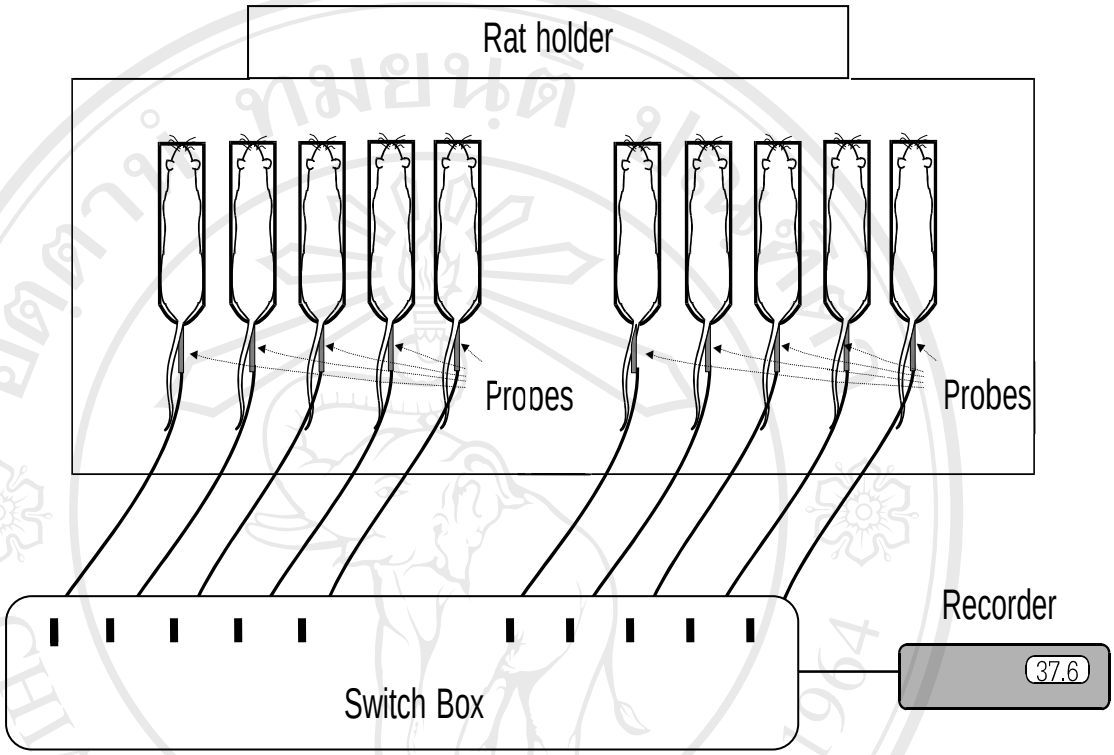

Figure 8. Diagram illustrating an instrument for recording rectal temperature.
(ten channel electric thermometer with rectal probes and a recorder)

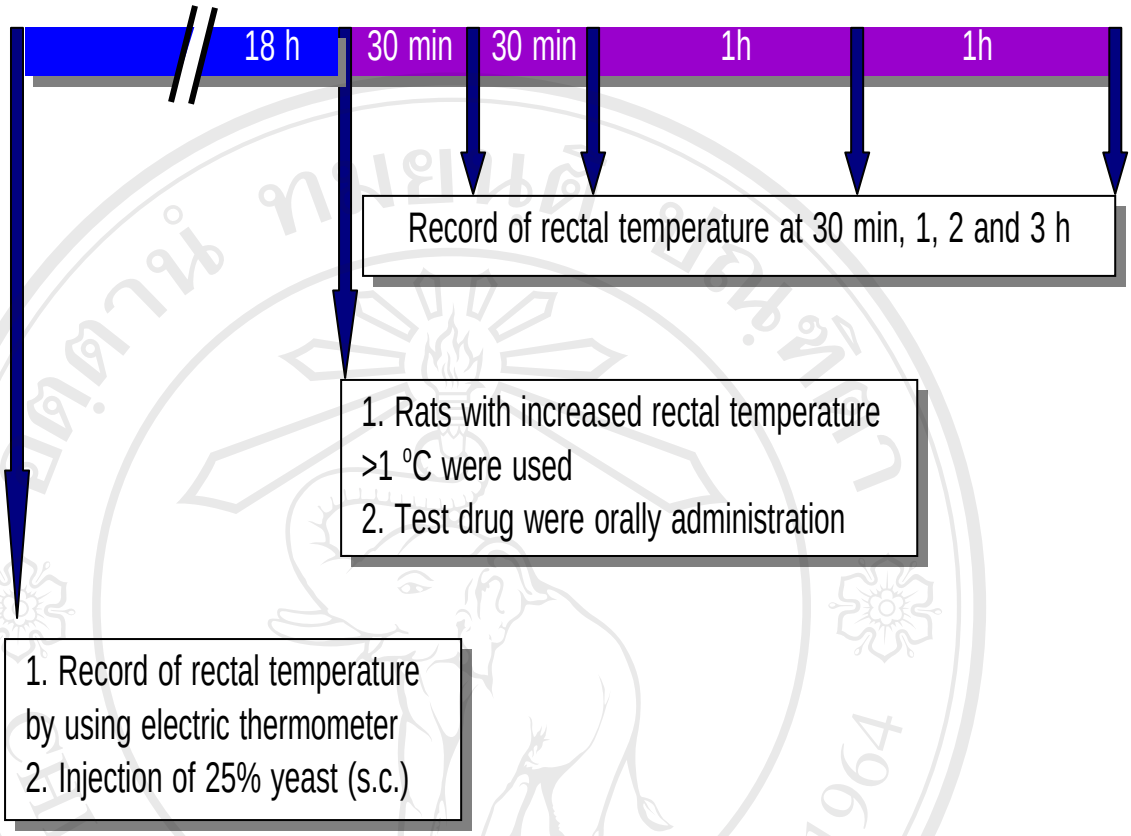

Figure 9. Diagram illustration the procedure of the yeast-induced hyperthermia in rat.

2.5.4 ACUTE TOXICITY

the procedure was conducted according to the Organization of Economic Cooperation and Development (OECD) guidelines for testing of chemicals (84, 85). Rats of both sexes with 190-210 g body weight were used and divided into 2 groups of 5 males and 5 females/ group.

Group 1 control group, received 5% Tween 80

Group 2 test groups, received 5000 mg/kg ML extract

Rats were deprived of food but not water 16-18 h prior to administration of test substances. The test substances were given in a single oral dose by gavage using a stomach tube. Signs and symptoms observed after the administration of test sample were recorded at 1, 2, 4 and 6 h after test substance administration and then once daily for 7 days. The visual observations included changes in the skin and fur, eyes and mucous membrane, and also respiratory, circulatory, autonomic and central nervous system, as well as somatomotor activity and behavioral pattern.

The rats which died during the experimental period were autopsied and gross pathological changes of the internal organs (heart, lungs, livers, spleen, kidneys, adrenal glands, sex organs, thymus, brain, eyes, stomach, intestine, etc.) were recorded. The surviving rats were sacrificed on the 8 th day to examine any gross pathological changes of the internal organs. Any changes of internal organs compared with those of the control group were recorded.

2.6 STATISTICAL ANALYSIS

The data from the experiments were expressed as mean $\pm$ standard error of mean (S.E.M.). Statistical comparison between groups was analyzed by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test and *P* values less than 0.05 were considered significant.

2.7 DRUGS AND CHEMICALS

2.7.1 Drugs

2.7.1.1 Indomehacin (Sigma Chemical Company, St. Louis, U.S.A.)

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

2.7.1.3 Prednisolone (Scherisone®, Schering <Bangkok> Ltd., Nontaburi, Thailand.)

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

2.7.1.5 Codeine (The Government Pharmaceutical Organization (GPO), Thailand)

2.7.2 Irritants

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

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

2.7.2.3 λ-Carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

2.7.2.4 Formalin (Chiang Mai Winner R.O.P. Co., Ltd., Thailand)

2.7.2.5 Brewer's yeast (Sigma Chemical Company., St Louis, U.S.A.)

2.7.3 Vehicles

2.7.3.1 Polysorbate 80 (Tween 80, Sigma Chemical Company, St Louis, U.S.A.)

2.7.3.2 Carbonate buffer (pH 8.4)