CHAPTER 2

MATERIALS AND METHODS

2.1 Plant extract

The methanol extract of *D. lomentaceum*, designated as DL extract, was kindly provided by Professor Dr. Vichai Reutrakul, Director of the Center for Innovation in Chemistry: The Program of Postgraduate Education and Research in Chemistry (PERCH-CIC). *D. lomentaceum* was collected from Ubon Ratchathani province in 2004. The dried ground stems of *D .lomentaceum* (4.7 kg) was sequentially extracted with hexanes, ethyl acetate (EtOAc) and MeOH as shown in Figure 3. After removal of the solvents under reduced pressure, the hexane (12.9 g, 0.27 % w/w), EtOAc (36.7 g, 0.78% w/w) and MeOH (292 g, 6.21% w/w) yielded methanol extract (292 g 6.21% w/w) extracts were obtained.

2.2 Experimental animals

Male Swiss albino mice weighing 30–40 g and male Sprague-Dawley rats weighing 100–120 g and 200–220 g obtained from the Nation Laboratory Animal Center, Nakorn Pathom, were used. All animals were be kept in a room maintained under environmental control conditions of 24±1 ºC, relative humidity 50±10%, and a 12 h light-dark cycle. All animals had free access to water and food. They were acclimatized for at least one week before starting the experiments. All animal experiments were approved by the Animal Ethics Committee, Facuty of Medicine Chiang Mai University.

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 all test drugs were intraperitoneal (IP) administered in an equivalent volume of 0.1 mL/10 g body weight of the mice.

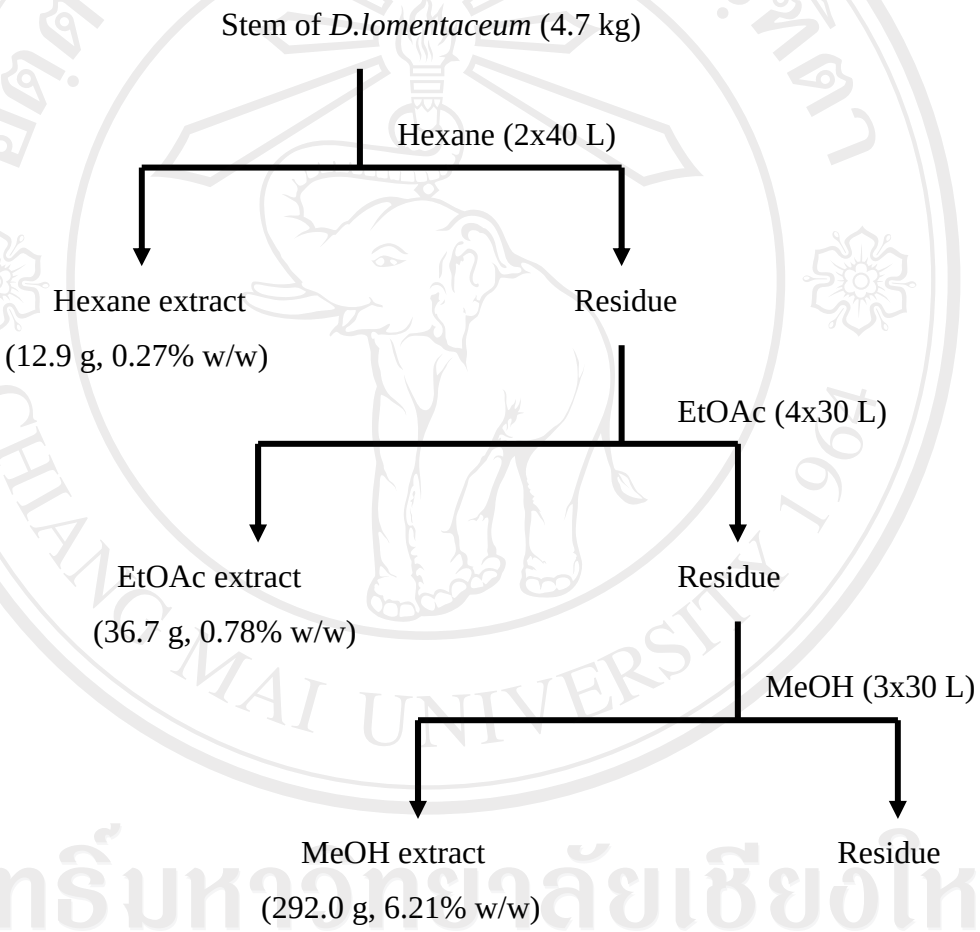


Figure 3 The extraction of stem of *D. lomentaceum*

2.5 Experimental protocols

2.5.1 Anti-inflammatory study

2.5.1.1 Carrageenin-induced hind paw edema in rats (81)

This experiment was performed to investigate the inhibitory effect of test agents on the hind paw edema formation induced by carrageenin. Male rats of 100-120 g body weight were divided into 5 groups of 6 animals per group.

Group 1: Control group, received 5% Tween 80

Group 2: Reference group, received 10 mg/kg of diclofenac

Group 3-5: Test groups, received 100, 200 and 400 mg/kg of the DL extract, respectively

Rats were pretreated with 5% Tween 80, diclofenac or various doses of the DL 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 4.) before and at 1, 3 and 5 h after carageenin injection. The diagram of experimental procedure is shown in Figure 5.

The edema volume of the paw and the percent edema inhibition by each test compound was 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) measure before carrageenin injection

$\%EI_x$ = percent edema inhibition of the DL extract at time x

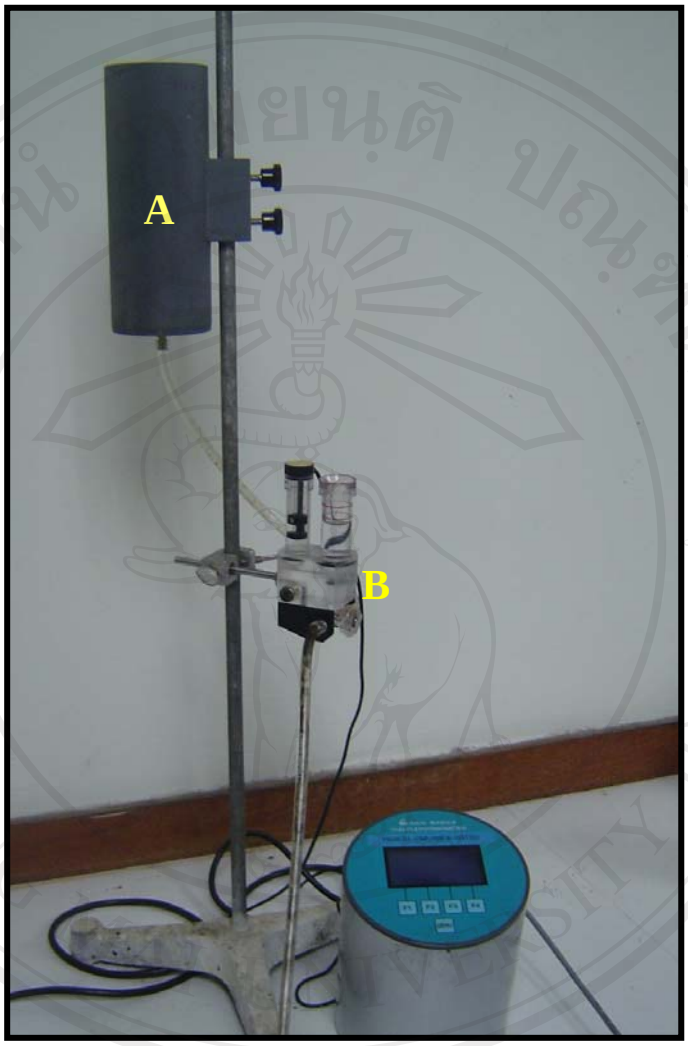


Figure 4. Plethysmometer

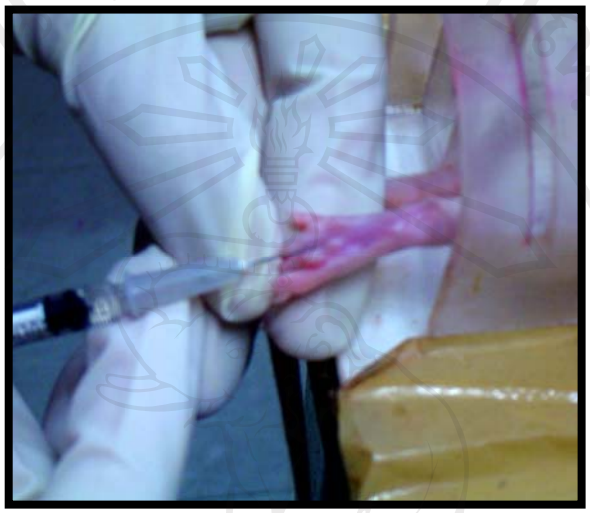
A: Water reservoir contained 0.05% NaCl in distilled water

B: Measuring chamber

Measuring initial paw volume

Oral drug administration of test compound

1 h



Subplantar injection of carrageenin

Measuring paw edema volume at 1, 3 and 5 h after carrageenin injection.

Figure 5. Diagram illustrating subplantar injection of carrageenin and the water displacement method for measuring rat paw volume

2.5.1.2 AA-induced hind paw edema in rats (87)

This experiment was performed to investigate the inhibitory effect of test agents on the edema formation induced by AA. Male rats of 100-120 g body weight were used and divided into 6 groups of 6 animals per group.

Group 1 Control group, received 5% Tween 80
Group 2 Reference group, received 10 mg/kg of diclofenac
Group 3 Reference group, received 5 mg/kg of prednisolone
Group 4-6 Test groups, received 100, 200 and 400 mg/kg of the DL extract, respectively

Tween 80 (5%), diclofenac, prednisolone and various doses of the DL 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.

Paw volume of rats was measured prior to and at 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 (88)

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±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 ^{o}C for 2 h. Male rats of 200-220 g body weight were used and divided into 4 groups of 6 animals per group.

Group 1 Control group, received 5% Tween 80
Group 2 Reference group, received 5 mg/kg/day of diclofenac
Group 3 Reference group, received 5 mg/kg/day of prednisolone
Group 4 Test group, received 400 mg/kg/day of the DL extract

A. Granuloma formation and transudation

The abdominal skin was shaved and disinfected with 70% alcohol. 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 6. The DL extract and prednisolone as well as diclofenac were administered orally in once daily dosage regimen throughout the experimental period of 7 days whereas the control group received 5% Tween 80 only. On the eight day after cotton pellet implantation, rats were anesthetized with pentobarbital sodium (50 mg/kg, ip), blood was collected for alkaline phosphatase activity measurement as detailed in B. subsequently animals were sacrificed and experiments A, C and D were performed. 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°C for 18 h and their dry weight was determined. The changes in granuloma weight and transudative weight of test group were compared with those of the control and reference groups. 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 before implantation (mg)

GW = granuloma weight (mg)

% GI = percent granuloma inhibition

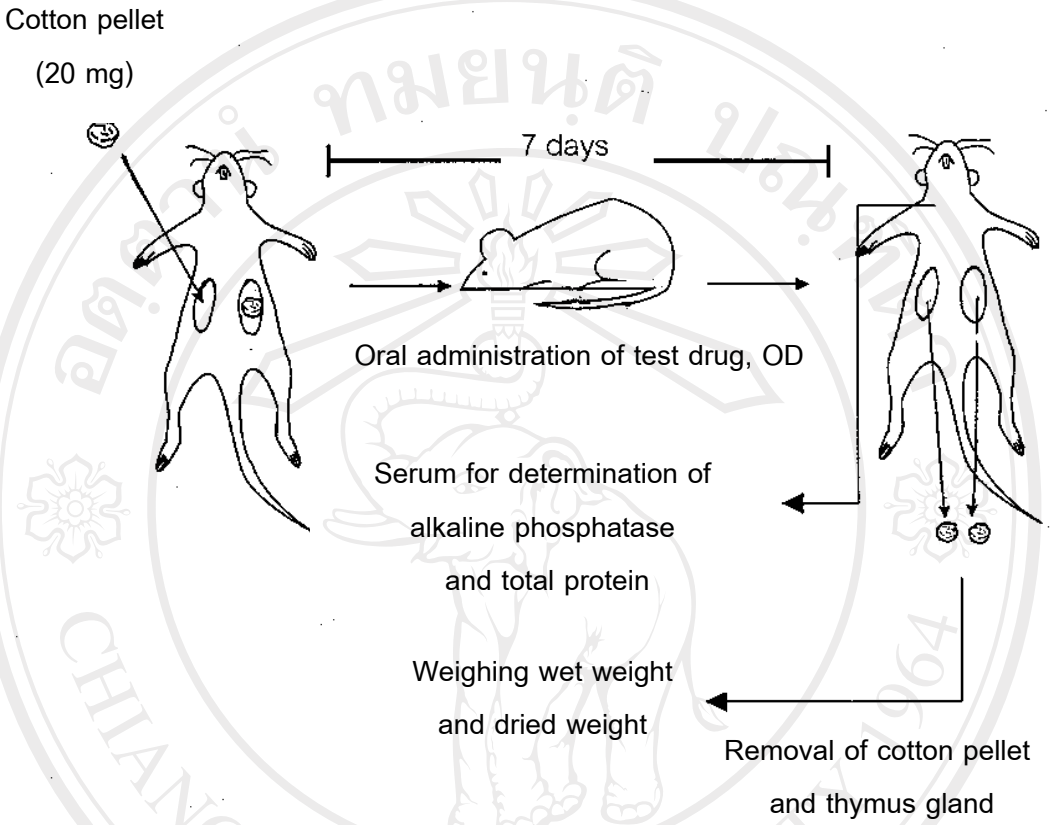


Figure 6. Diagram illustrating the method for cotton pellet-induced granuloma formation in rat

B. Alkaline phosphatase activity

The same animals in the cotton pellet-induced granuloma formation model were used for determination of alkaline phosphatase activity as described by Bessey *et al.* (105). On the eight day after cotton pellets implantation, blood of the anesthetized rats was collected into a glass tube by cardiac puncture technique. Samples of serum were sent to the Associated Medical Sciences Clinical Services Center, Faculty of Associated Medical Sciences, Chiang Mai University for determination of alkaline phosphatase and total protein.

C. The body weight gain and the thymus weight

The same animals in the cotton pellet-induced granuloma formation model were used. The body weight gain and the thymus dry weight of the DL extract treated rats were compared with those of the control and the reference groups as described by Swingle and Shideman (88). The neck skin 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 the experiment was also recorded.

D. Evaluation of ulcerogenic effect (106)

The stomach of the same animals from the cotton pellet-induced granuloma formation model 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 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

2.5.2.1 Formalin test in mice

The analgesic activity of the extract was tested using the formalin test and compared with reference drugs (94). The formalin test comprises the early phase and the late phase assessment of the analgesic effect. Male Swiss-albino mice weighing 30-40 g were used and divided into two sets of 8 groups (6 animals per group).

Group 1 Control group, received 5% Tween 80

Group 2 Reference group, received 5 mg/kg of diclofenac

Group 3 Reference group, received 10 mg/kg of morphine

Group 4-8 Test groups, received 5, 25, 50, 100 and 200 mg/kg of the DL extract, respectively

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 7).

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 8).

The percent inhibition of the licking response to represent analgesic activity of test compounds 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

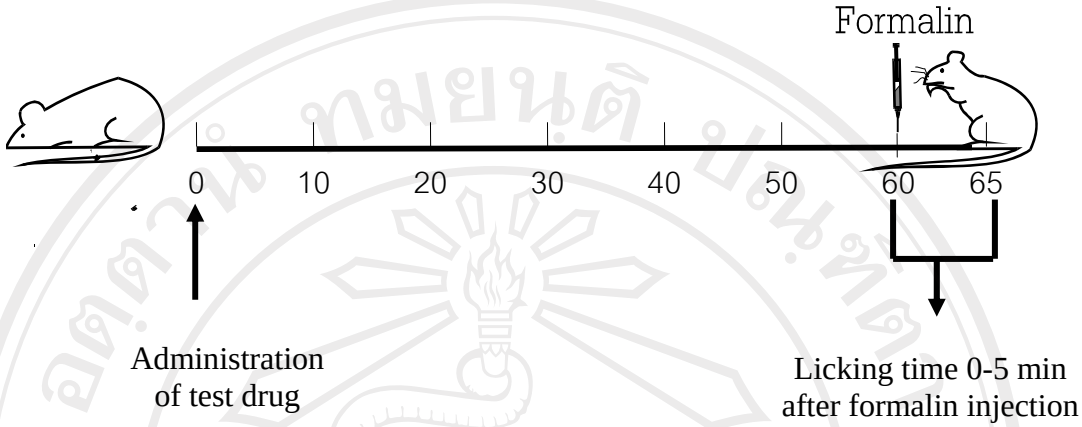


Figure 7. Diagram illustrating the method for formalin test (early phase) in mice.

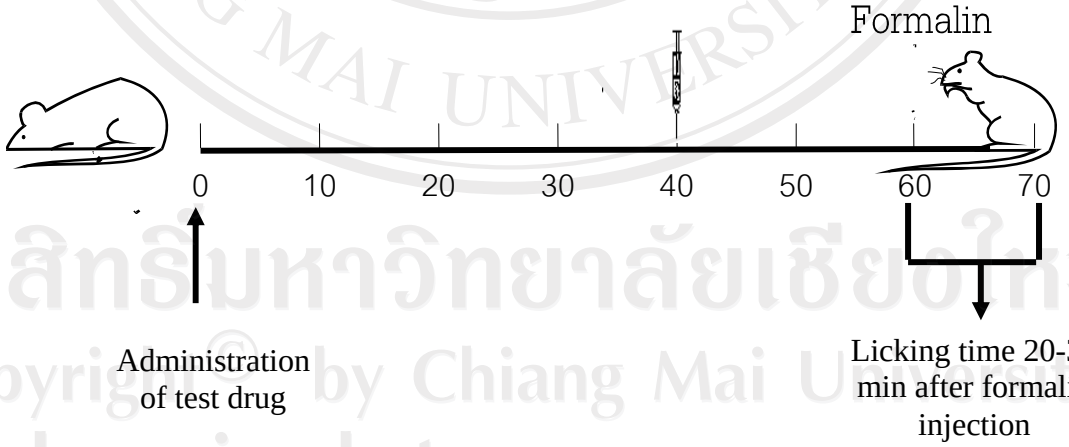


Figure 8. Diagram illustrating the method for the formalin test (late phase) in mice.

2.5.3 Antipyretic study

2.5.3.1 Yeast-induced hyperthermia in rats

The antipyretic activity of the DL extract was tested and compared with diclofenac by using yeast-induced hyperthermia in rats following the slightly modified method described by Teotino *et al*. (107). Male rats weighing 200-220 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 of diclofenac

Group 3 Test group, received 400 mg/kg of the DL extract, respectively

Before pyrexia was induced, rats were restrained in plastic cages and 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 9). 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 hours 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 DL extract, diclofenac 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 rats is shown in Figure 10.

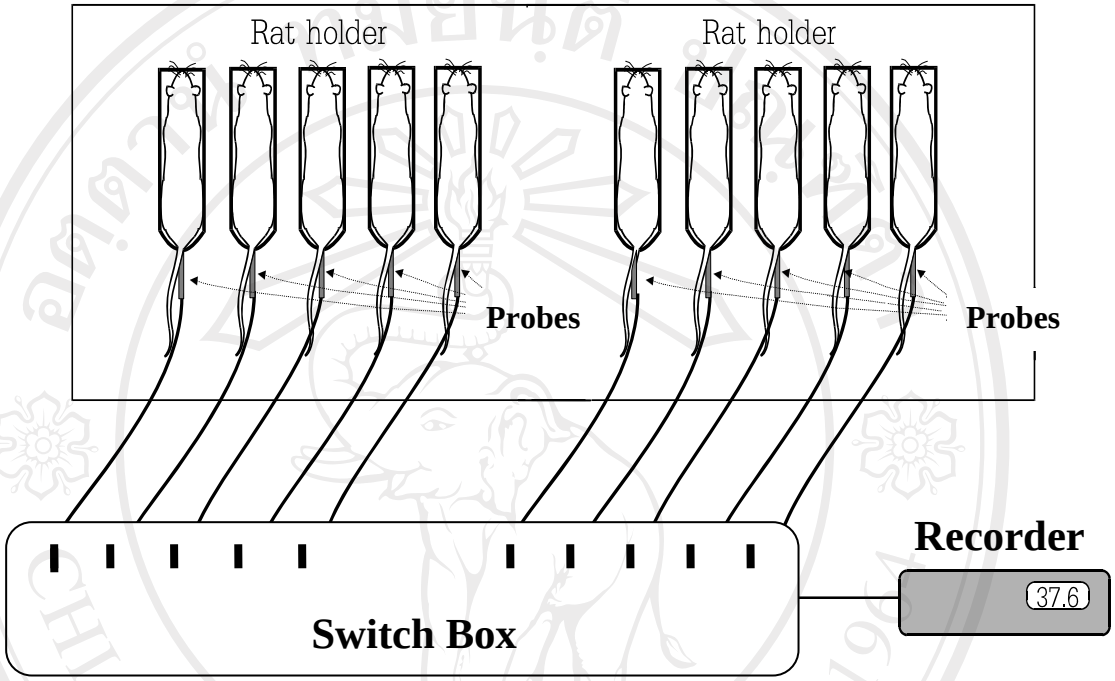


Figure 9. Diagram illustrating an instrument for recording rectal temperature (ten channel electric thermometer with rectal probes and a recorder).

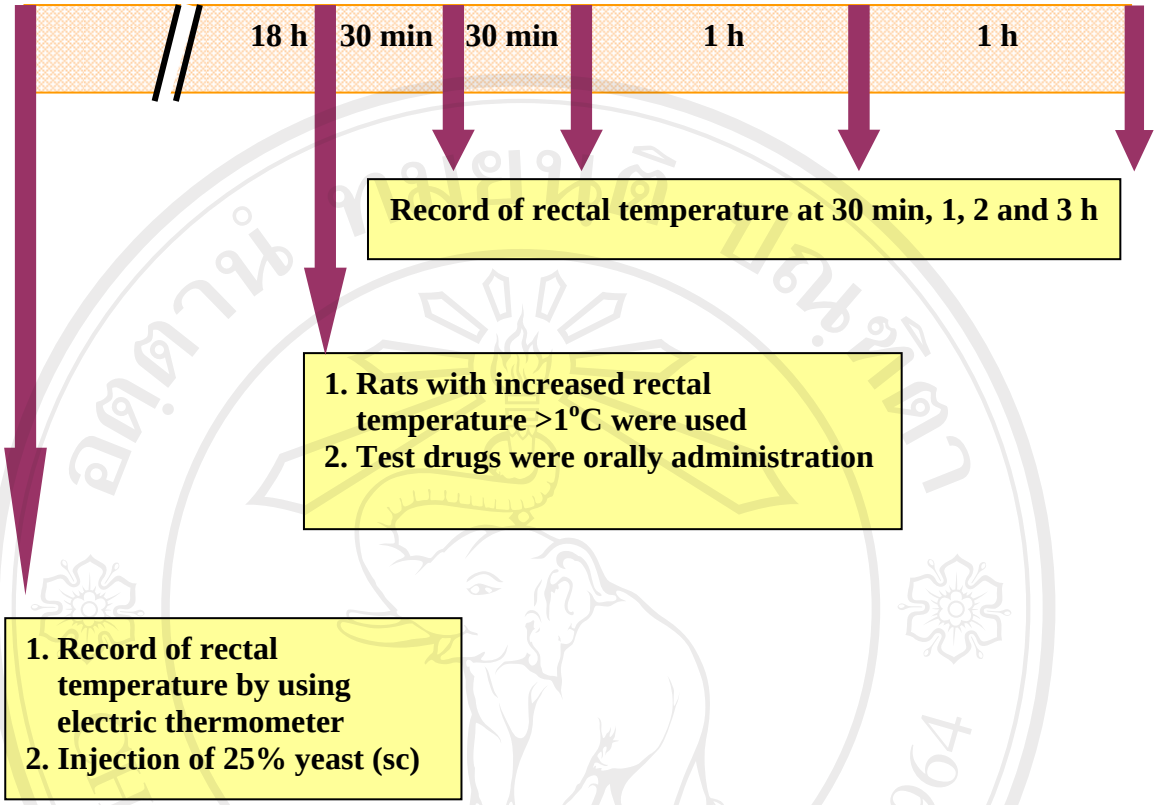


Figure 10. Diagram illustrating the procedure for testing antipyrexia of test drugs in yeast-induced hyperthermia in rat.

2.6 Statistical analysis

Data from all experiments were expressed as mean±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 Diclofenac (Sigma Chemical Company, St. Louis, U.S.A.)

2.7.1.2 Prednisolone (Scherisone®, Schering Bangkok Ltd., Nontaburi, Thailand).

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

2.7.1.4 Morphine (The Government Pharmaceutical Organization Bangkok, Thailand)

2.7.2 Irritants

2.7.2.1 Adsorbent cotton wool (Vidhyasom Co., Ltd., Bangkok, 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)