CHAPTER 2
MATERIALS AND METHODS

2.1 Preparation of the extract

The rhizomes of *S. involucratus* were collected from Chiang Rai province in April 2009. The voucher specimen (number 144) was deposited at the School of Health Science, Mae Fah Luang University, Thailand. The air dried powdered rhizome was macerated with 95% ethanol for two days and filtered. The marc was remacerated again for two times with 95% ethanol each time for two days and filtered. The combined filtrate was concentrated in vacuo at 55 °C and lyophilized to obtain a dry ethanolic extract (6% w/w yield). The extract was subsequently reconstituted in 5% Tween80 to required concentrations for the experiments.

Gas chromatography/Mass spectrometry (GC/MS) analysis of the extract was carried out on a gas chromatograph (GC 7890 Agilent Technologies) fitted with a DB-5MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness). The GC oven temperature was programmed from 50 °C held for 5 min, raised to 200 °C at 10 °C/min, then to 250 °C at 5 °C/min and held for 10 min. The injection temperature was 250 °C; and the flow rate of carrier gas, helium, was at 1.5 ml/min; 1:25 split ratio. The GC was coupled to a mass selective detector (Agilent HP 5973). The MS operating parameters were as follows: ionization voltage, 70 eV; ion source temperature, 230 °C. Identification of the trichloroethylene (TCE) components were performed by comparison of their relative retention times and mass spectra with those in the NIST05a.L Database (Agilent Technologies Inc.).

2.2 Test substance administration

SI extract and reference drugs, including diclofenac were suspended in 5% Tween80 for using in all experiments except in the ethyl phenylpropiolate (EPP)-

induced ear edema model which SI extract was suspended in acetone and diclofenac was suspended in 5% dimethylsulfoxide (DMSO) in acetone.

The animals received the test substances orally at the volume of 0.2 mL/100 g body weight of rats and 0.1 mL/10 g body weight of mice except in the EPP-induced ear edema model which they received the test substances topically (20 µL/ear).

2.3 Experimental animals

Male Swiss albino mice 30-40 g body weight; male Sprague-Dawley rats 40-60 g, 100-120 g, 160-180 g, and 180-200 g body weight; female Sprague-Dawley rats 180-200 g body weight were purchased from the National Laboratory Animal Center, Salaya, Mahidol University, Nakornpathom, Thailand. All animals were kept in a room under environmental control conditions of 24 ± 1 °C, relative humidity 50 ± 10% and 12 h light-dark cycle. They received free water and standard diet, and were acclimatized for at least one week before starting the experiments. All experiments were approved by The Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Protocol number 8/2553).

2.4 Experimental models

2.4.1 Anti-inflammatory activity

A. EPP-induced ear edema [48]

Male rats weighing 40-60 g were divided into 4 groups of 3 animals.

Group 1: control group of reference drug (received 5% DMSO in acetone)

Group 2: control group of SI extract (received acetone)

Group 3: reference group (received 5 mg/ear of diclofenac)

Group 4: test group (received 5 mg/ear of SI extract)

The ear edema was induced by topical application of 1 mg EPP (20 µL/ear) after application of each test substance by the automatic microliter pipette to outer and inner surfaces of the both ears. The ear thickness was determined by the digital vernier calipers before and at 15, 30, 60, and 120 min after EPP application. The scheme of this method is shown in Figure 4. The increases of ear thickness in each

test group were compared with those of its control group and the percentage of inhibition was calculated by using the following formulae:

$$ED_x = ET_x - ET_0$$

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

where:

ED_x = edema thickness at time x (µm)

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

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

ET_0 = ear thickness before application of EPP (µm)

ET_x = ear thickness at time x (µm)

$\% EDI_x$ = percent edema inhibition of test substance at time x

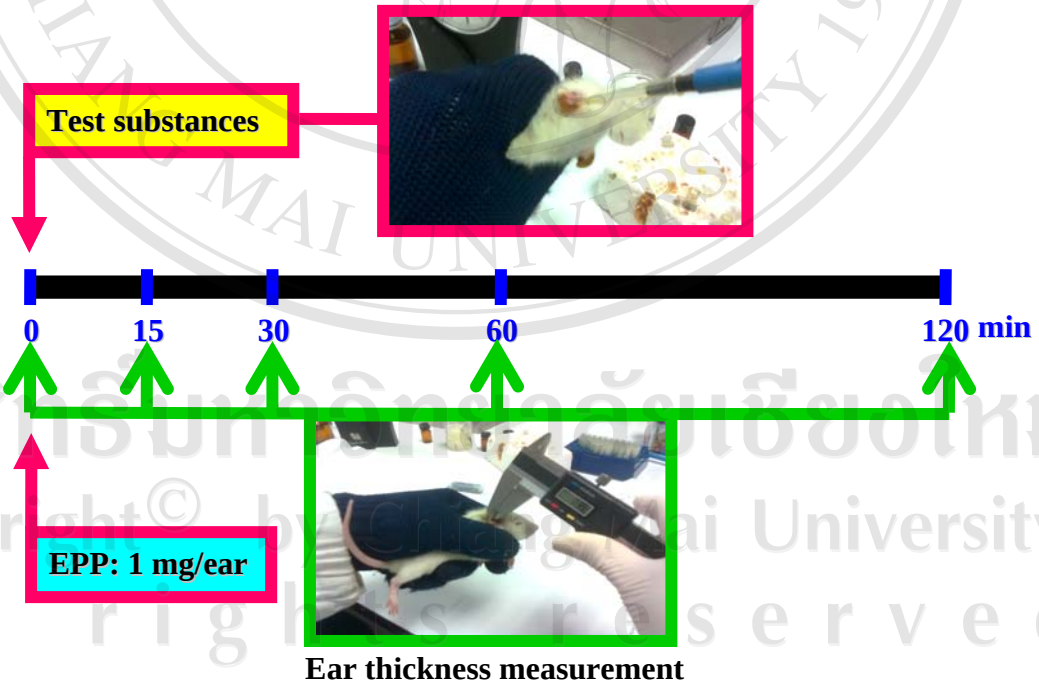

Figure 4 Method of EPP-induced ear edema in rats.

B. Carrageenin-induced hind paw edema [49]

Male rats weighing 100-120 g were divided into 5 groups of 6 animals.

Group 1: control group (received 5% Tween80)

Group 2: reference group (received 10 mg/kg of diclofenac)

Group 3-5: test groups (received 75, 150, and 300 mg/kg of SI extract, respectively)

The paw edema was induced by injecting 0.05 mL of 1% carrageenin in sterile normal saline solution (NSS) intradermally into the plantar surface of the right hind paw after administration of each test substance for 1 h. The paw volume was determined by means of a volume displacement technique using the plethysmometer (model 7150, Ugo Basile, Italy) before and at 1, 3, and 5 h after carrageenin injection. The scheme of this method is shown in Figure 5. The increases of paw volume in each test group were compared with those of the control group and the percentage of inhibition was calculated by using the following formulae:

$$EV_x = PV_x - PV_0$$

$$\% EI_x = \frac{EV_c - EV_t}{EV_c} \times 100$$

where:

EV_x = edema volume at time x (mL)

EV_c = edema volume of control group at time x (mL)

EV_t = edema volume of test group at time x (mL)

PV_0 = paw volume before injection of carrageenin (mL)

PV_x = paw volume at time x (mL)

$\% EI_x$ = percent edema inhibition of test substance at time x

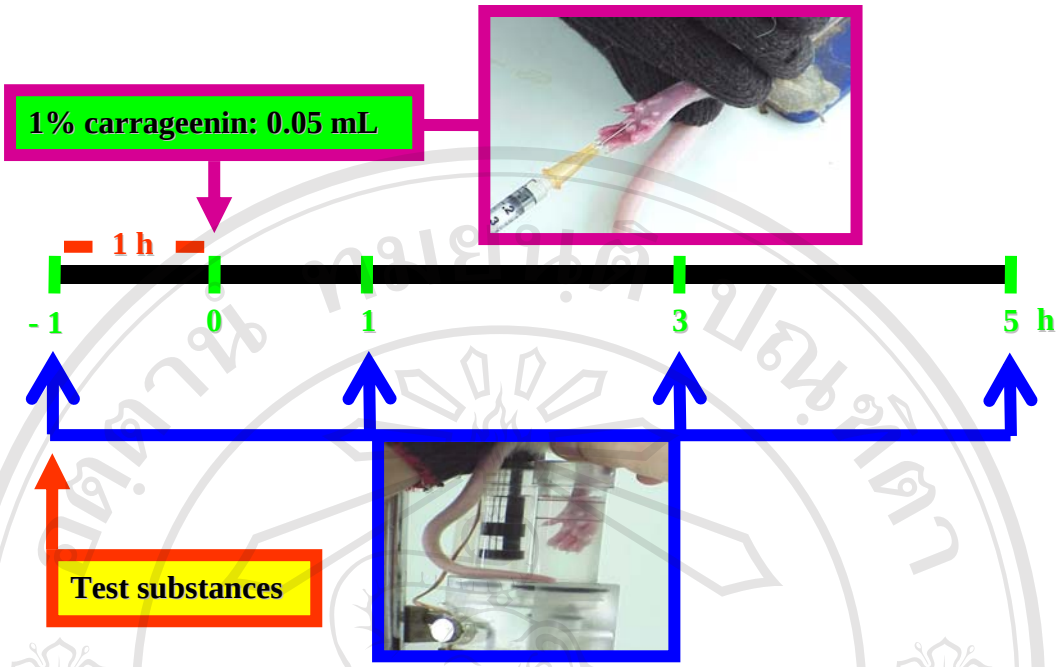

Figure 5 Method of carrageenin-induced hind paw edema in rats.

C. AA-induced hind paw edema [50]

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

Group 1: control group (received 5% Tween80)

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 75, 150, and 300 mg/kg of SI extract, respectively)

The paw edema was induced by injecting 0.1 mL of 0.5% AA in 0.2 M carbonate buffer (pH 8.4) intradermally into the plantar surface of the right hind paw after administration of each test substance for 2 h. The paw volume was determined by means of a volume displacement technique using the plethysmometer (model 7150, Ugo Basile, Italy) before and at 1 h after AA injection. The scheme of this method is shown in Figure 6. The increases of paw volume in each test group were compared with those of the control group and the percentage of inhibition was calculated as same as in carrageenin-induced hind paw edema model.

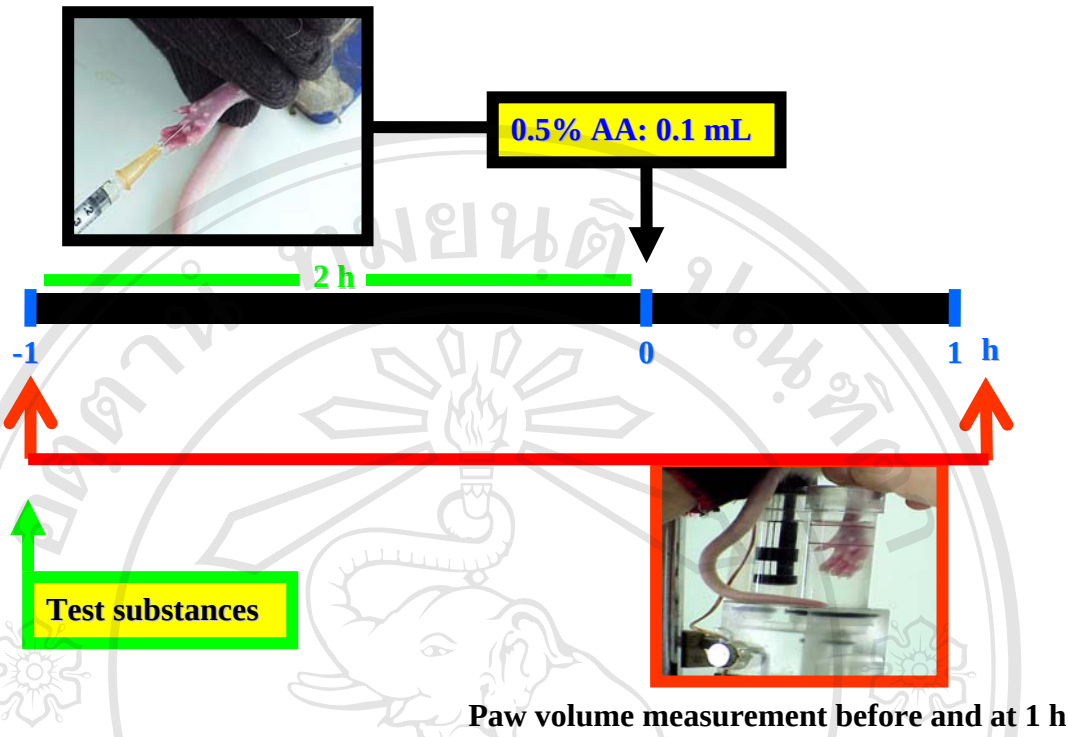

Figure 6 Method of AA-induced hind paw edema in rats.

D. Cotton pellet-induced granuloma formation [51]

This model was used for investigation of anti-inflammatory activity of the extract by its inhibitory effect on the transudative and proliferative components of chronic inflammation induced by cotton pellet. The model was slightly modified as follow:

The 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 °C for 2 h. Male rats weighing 180-200 g were divided into 5 groups of 6 animals.

Group 1: normal group (received 5% Tween80)

Group 2: control group (received 5% Tween80)

Group 3: reference group (received 5 mg/kg/d of diclofenac)

Group 4: reference group (received 5 mg/kg/d of prednisolone)

Group 5: test group (received 300 mg/kg/day of SI extract)

The abdominal skin was shaved and disinfected with 70% alcohol. Then, two pellets were implanted subcutaneously, one on each side of an abdomen in all groups except in the normal group, under light ether anesthesia and sterile technique. The suture was made after that and the animal was allowed to recover.

Each test substance was administered three times daily for 7 days. Diclofenac and prednisolone were given at doses of 1, 2, and 2 mg/kg in the morning, afternoon, and evening, respectively, whereas SI extract was given at a fixed dose of 100 mg/kg in the same manner.

Measurement of granuloma weight and transudative weight

The implanted cotton pellets were removed and weighed for the wet weight, then dried at 60 °C for 18 h and weighed for the dry weight. The granuloma weight, transudative weight, and percentage of granuloma inhibition were calculated by using the following formulae:

$$TW = Wt_w - Wt_d$$

$$GW = \frac{Wt_d - Wt_i}{Wt_i}$$

$$\%\ GI = \frac{GW_c - GW_t}{GW_c} \times 100$$

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/mg cotton)

GW_c = granuloma weight of control group (mg/mg cotton)

GW_t = granuloma weight of test group (mg/mg cotton)

TW = transudative weight (mg)

% GI = percent granuloma inhibition

Measurement of body weight

The body weight of all rats at the end of the study was recorded.

Measurement of thymus weight

After blood collection, the chest was opened and the thymus was cut out and weighed immediately for the wet weight. Then it was dried at 60 °C for 18 h and weighed again for its dry weight.

Measurement of alkaline phosphatase (ALP) activity in serum [52]

On the 8th day after cotton pellet implantation, all rats were anesthetized by intraperitoneal injection of thiopental sodium (50 mg/kg). Blood was collected by cardiac puncture technique and sent to Veterinary Diagnostic Laboratory of Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University for determination of the ALP and total protein. Measurement of the ALP activity (U of enzyme/mg of serum protein x 10^{-4}) in serum was calculated by using the following formula:

$$\text{ALP activity} = \frac{\text{ALP (U/L)}}{\text{Total protein (g/dL)}}$$

Measurement of ulcerogenic effect [53]

After the animal was sacrificed, the stomach was cut out and opened along the greater curvature. It was cleaned with distilled water and pinned out on the wax plate before evaluating for lesions under the dissecting microscope (10X).

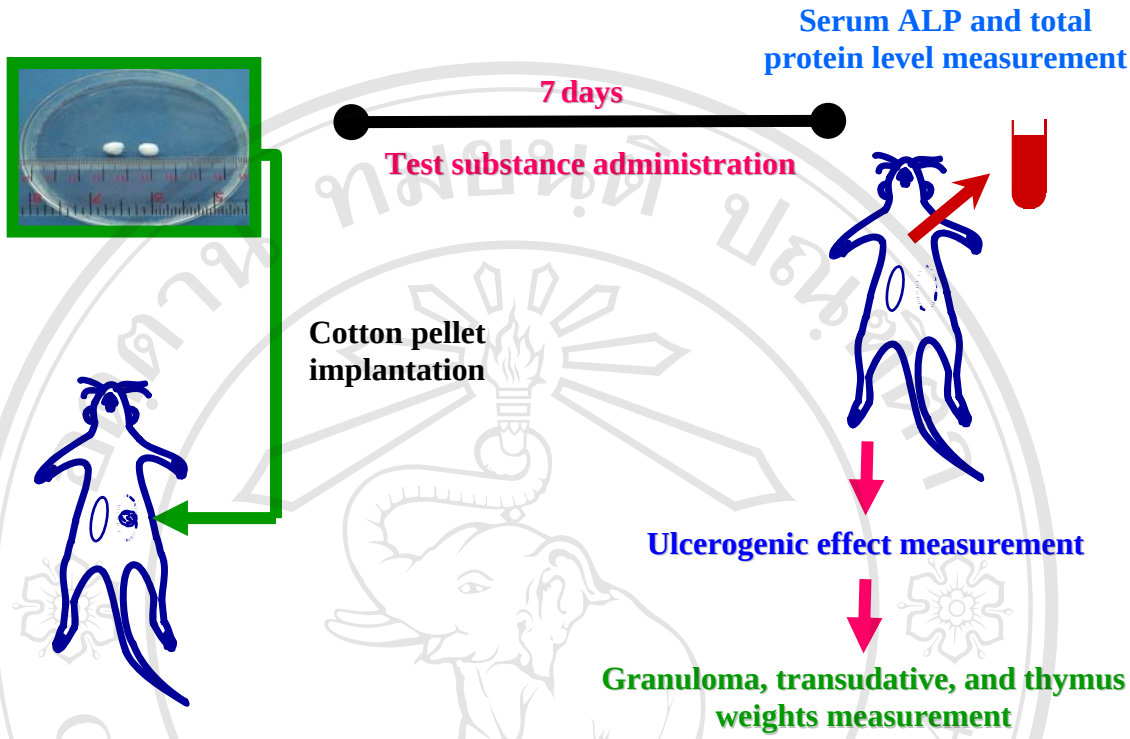

Figure 7 Method of cotton pellet-induced granuloma formation in rats.

2.4.2 Analgesic activity

A. Acetic acid-induced writhing response [54, 55]

Male mice weighing 30-40 g were divided into 5 groups of 6 animals.

Group 1: control group (received 5% Tween80)

Group 2: reference group (received 10 mg/kg of diclofenac)

Group 3-5: test groups (received 18.75, 37.5, and 75 mg/kg of SI extract, respectively)

The writhing response was induced by injecting of 0.75% acetic acid aqueous solution in a volume of 0.1 mL/10 g body weight into the peritoneal cavity after administration of each test substance for 1 h. The number of writhes, the 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 acetic acid injection. The scheme of this method is shown in Figure 8. The

number of writhes in each test group was compared with those of the control group and the percentage of inhibition was calculated by using the following formula:

$$\% \text{ WI} = \frac{NW_c - NW_t}{NW_c} \times 100$$

where:

NW_c = number of writhes of control group (time)

NW_t = number of writhes of test group (time)

% WI = percent writhing response inhibition of test substance

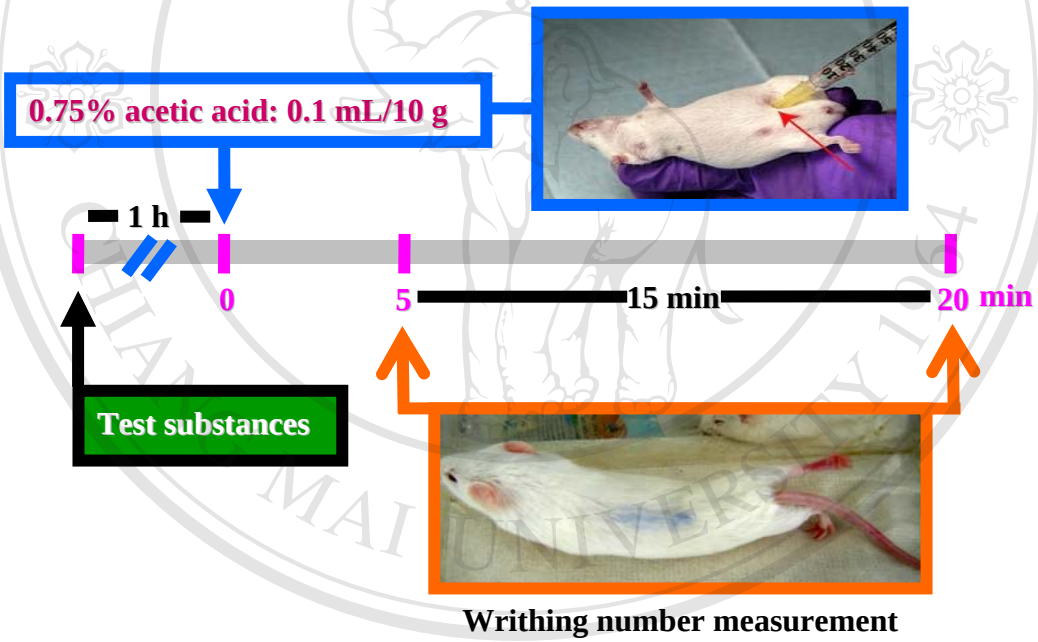

Figure 8 Method of acetic acid-induced writhing response in mice.

B. Tail-flick test [56, 57]

Male rats weighing 160-180 g were divided into 6 groups of 6 animals.

Group 1: control group (received 5% Tween80)

Group 2: reference group (received 10 mg/kg of diclofenac)

Group 3: reference group (received 200 mg/kg of codeine)

Group 4-6: test groups (received 18.75, 37.5, and 75 mg/kg of SI extract, respectively)

The spinal reflex was induced by placing the tail of rat (3 cm from tip) on a flush mounted photocell window of the tail-flick apparatus (model 7360, Ugo Basile, Italy). Heat was applied by the infrared lamp (50 W bulb) mounted in a reflector. A pedal switch was depressed, the infrared lamp turned on and a timer started. When the rat felt pain and moved (flicked) its tail away from the heat, this automatically stopped the timer and switched off the lamp. The reaction time was presented on a digital display. The voltage was adjusted to give a normal reaction time of 2-4 sec. The cut-off time of 10 sec was a maximum time for the rat that did not move its tail away from the heat to avoid tissue damage. The reaction time was determined before and at 1, 2, and 3 h after each test substance administration. The scheme of this method is shown in Figure 9. The reaction time of each group was compared with its baseline and the percentage of maximum possible response was calculated by using the following formula:

$$\% \text{ maximum possible response} = \frac{T_t - T_b}{10 - T_b} \times 100$$

where:

T_b = reaction time of baseline (sec)

T_t = reaction time of test (sec)

10 = cut-off time (sec)

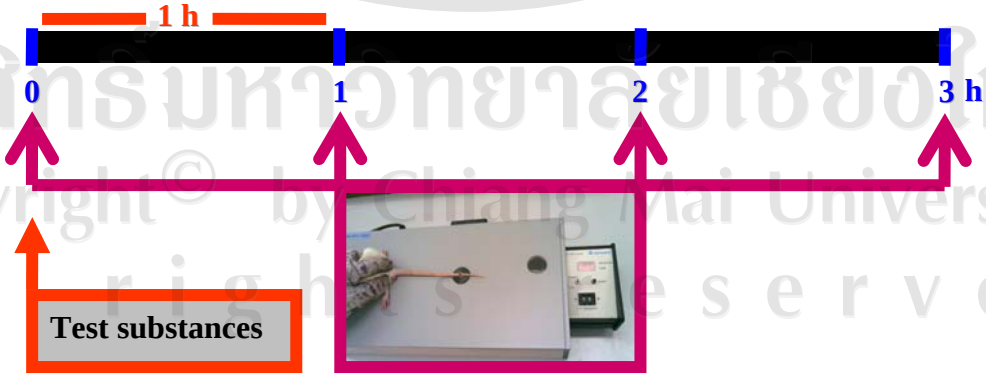

Figure 9 Method of tail-flick test in rats.

2.4.3 Antipyretic activity

A. Yeast-induced hyperthermia [58]

Male rats weighing 180-200 g were divided into 5 groups of 6 animals.

Group 1: control group (received 5% Tween80)

Group 2: reference group (received 10 mg/kg of diclofenac)

Group 3-5: test groups (received 75, 150, and 300 mg/kg of SI extract, respectively)

The hyperthermia was induced by subcutaneous injection of 25% yeast in sterile NSS in a volume of 1 mL/100 g body weight. The rectal temperature (°C) was measured by using the twelve-channel electrical thermometer (LETICA, model TMP 812 RS, Panlab S.L., Spain) connected with the probes which were inserted into the rectums about 5 cm depth. The basal rectal temperature was recorded at 1 h after probe insertion. At 18 h after yeast injection, the rectal temperature was measured again and those rats that showed rises in rectal temperature ≥ 1 °C were used. The test substances were administered orally, at the volume of 0.2 mL/100 g body weight, and the rectal temperatures were then recorded at 30, 60, 90, 120, and 180 min.

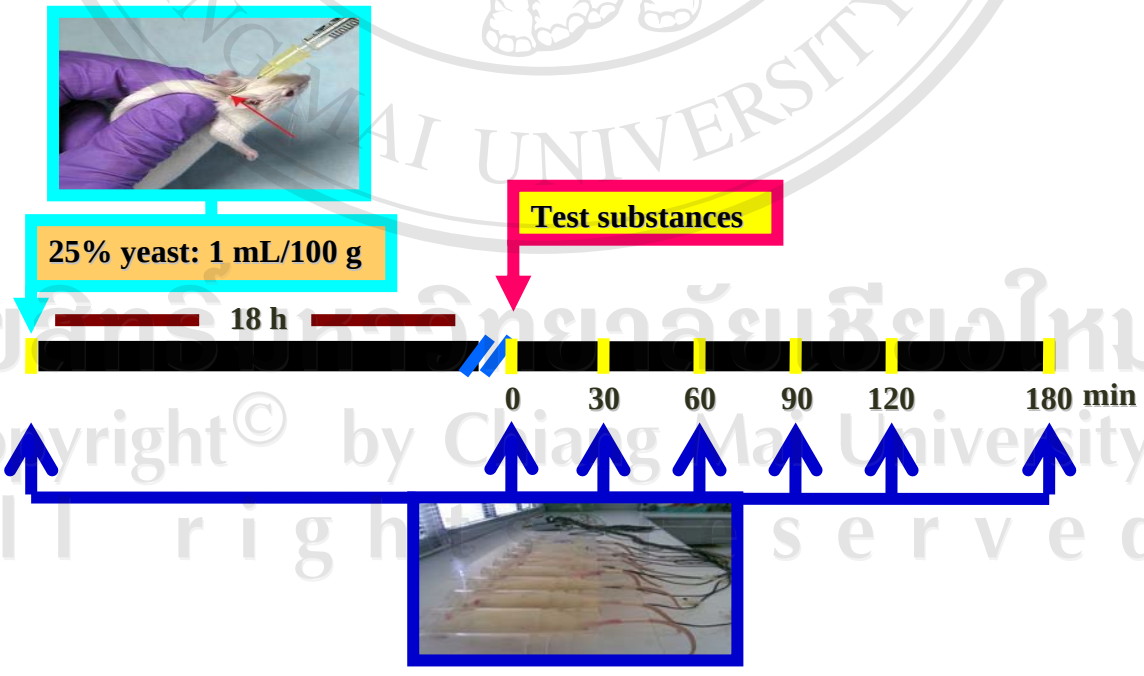

Figure 10 Method of yeast-induced hyperthermia in rats.

2.5 Statistical analysis

The data are expressed as mean ± standard deviation (S.D.). Statistical comparison between groups were analyzed using one-way analysis of variance (ANOVA) followed by post hoc least-significant difference (LSD) test, whereas the comparison between each time-point in the same group was analyzed using paired samples *t* test. The *p* values of less than 0.05 were considered significant.

2.6 Drugs and chemicals

2.6.1 Drugs

A. Diclofenac Sodium (Sigma Chemical Company, St. Louis, U.S.A.)

B. Prednisolone (Schering, Bangkok Ltd., Thailand)

C. Codeine Phosphate (FDA)

2.6.2 Chemicals

A. EPP (Fluka Chemicals Co., Ltd., Japan)

B. Lambda carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

C. AA (Sigma Chemical Company, St. Louis, U.S.A.)

D. Acetic acid (The Government Pharmaceutical Organization, Bangkok, Thailand)

E. Brewer's yeast (Fluka Chemicals Co., Ltd., Japan)