CHAPTER 2

MATERIALS AND METHODS

2.1 PREPARATION OF THE EXTRACT

The methanol extract of *Bauhinia siamensis*, designated as BS 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). *B. siamensis* was collected from Phu Miang, Phitsanulok, Thailand and was identified by Mr. Narong Nuntasaen. A voucher specimen (BKF. 140460) has been deposited at The Forest Herbarium National Park, Wildlife and Plant Conservation Department, Bangkok, Thailand. BS extract was prepared as follows: air-dried and finely powdered of the aerial part of *B. siamensis* (4 Kg) was extracted with methanol (113 L). The solvent was evaporated to dryness under reduced pressure and trace of solvent was removed by freeze-dried to give methanol extract (395 g).

2.2 EXPERIMENTAL ANIMALS

Male Sprague-Dawley rats weighing 40-60 g, 100-120 g, 180-220 g, and 200-250 g as well as male Swiss albino mice weighing 30-40 g, purchased from the National Laboratory Animal Center, Nakorn Pathom, were used. All animals were kept in a room maintained under environmentally control condition of 24±1 ^{o}C and 12-h light and 12-h dark cycle. All animals had free access to water and standard diet (Pokaphan Animal Feed Co., Ltd., Samutpragarn, Thailand). They were acclimatized for at least one week before starting the experiments. All animal experiments were approved by the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University.

2.3 PREPARATION OF TEST DRUGS

All test drugs were dissolved in distilled water, except in the ear edema model, test drugs were dissolved in 5% DMSO (dimethyl sulfoxide) in acetone.

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. In the rat ear edema model, test drugs were given topically to the outer and inner surfaces of the ears in equivalent volume of 20 µL/ear.

2.5 EXPERIMENTAL PROTOCALS

2.5.1. ANTI-INFLAMMATORY STUDY

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

This experiment was used to screen anti-inflammatory effect of the test compounds. The method described by Brattsand *et al.* (79) was performed as follow:

Male rats weighing 40-60 g were divided into 3 groups of 3 animals (6 ears per group).

Group 1: Control group, received 5% DMSO in acetone (20µL/ear)

Group 2: Reference group, received diclofenac (0.6 mg/20µL/ear)

Group 3: Test group, received BS extract (1 mg/20µL/ear)

Rat ears were applied topically on the inner and outer surfaces by means of an automatic microliter pipette with either 5% DMSO in acetone, diclofenac, or the BS extract. Immediately after application of test drugs, ear edema was induced by topical application of EPP dissolved in acetone at a dose of 1 mg/20µl/ear. Ear thickness was recorded using digital vernier calipers before and at 15, 30, 60, 90, and 120 min after EPP application. The scheme of protocol is shown in Figure 3.

The ear edema volume and the percent edema inhibition of the test drugs were calculated as follows:

$$ED_x = ET_x - ET_0$$

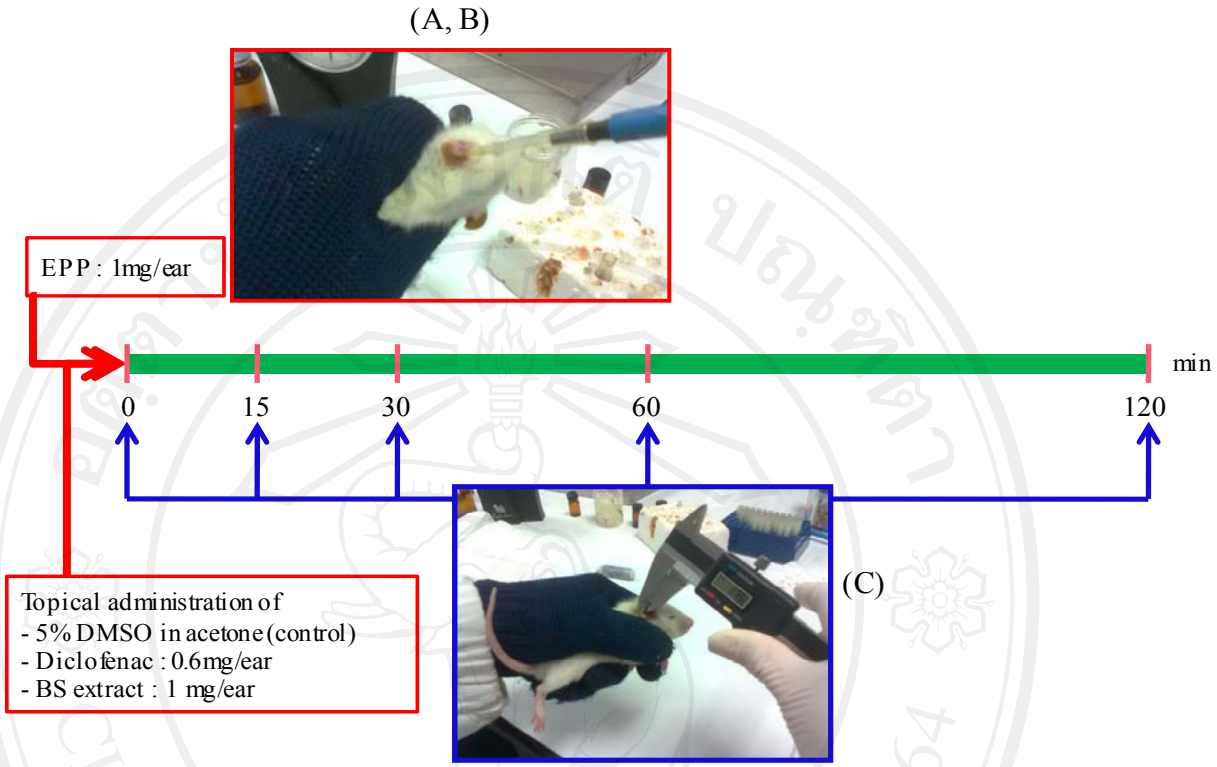

Figure 3 Diagram illustrating the method of EPP-induced ear edema in rats

Topical application of test drugs (A); and followed immediately by topical application of EPP to the inner and outer surfaces of the ear, using an automatic microliter pipette (B); Measurement of the thickness of the ear, using digital vernier calipers before and at 15, 30, 60 and 120 min after EPP application (C).

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

Where,

ED_x = ear edema thickness (μm) at time x

ET_x = ear thickness (μm) at time x

ET_0 = ear thickness (μm) at time 0

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

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

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

2.5.1.2 Carrageenin-induced hind paw edema in rats

This experiment was performed to investigate anti-inflammatory activity of test compounds mediated via COX pathway. The edema was produced in the right hind paw of rats by intradermal injection of carrageenin. The method described by Winter *et al.* (80) was performed as follow:

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

Group 1: Control group, received distilled water

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

Group 3-5: Test groups, received 75, 150 and 300 mg/kg of BS extract, respectively

Rats were pretreated with orally distilled water, diclofenac or various doses of BS extract for 1 h and then 0.05 mL of 1% carrageenin in normal saline solution was injected intradermally into the right subplantar of each rat. Paw volume was measured by means of a volume displacement technique using a plethysmometer (model 7150, Ugo Basile, Italy) (Figure 4). The right hind paw was immersed into the measuring chamber containing 0.05% NaCl in distilled water, exactly to anatomical hair line. The paw volume was measured before and at 1, 3 and 5 h after carrageenin injection. The scheme of protocol is shown in Figure 5.

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

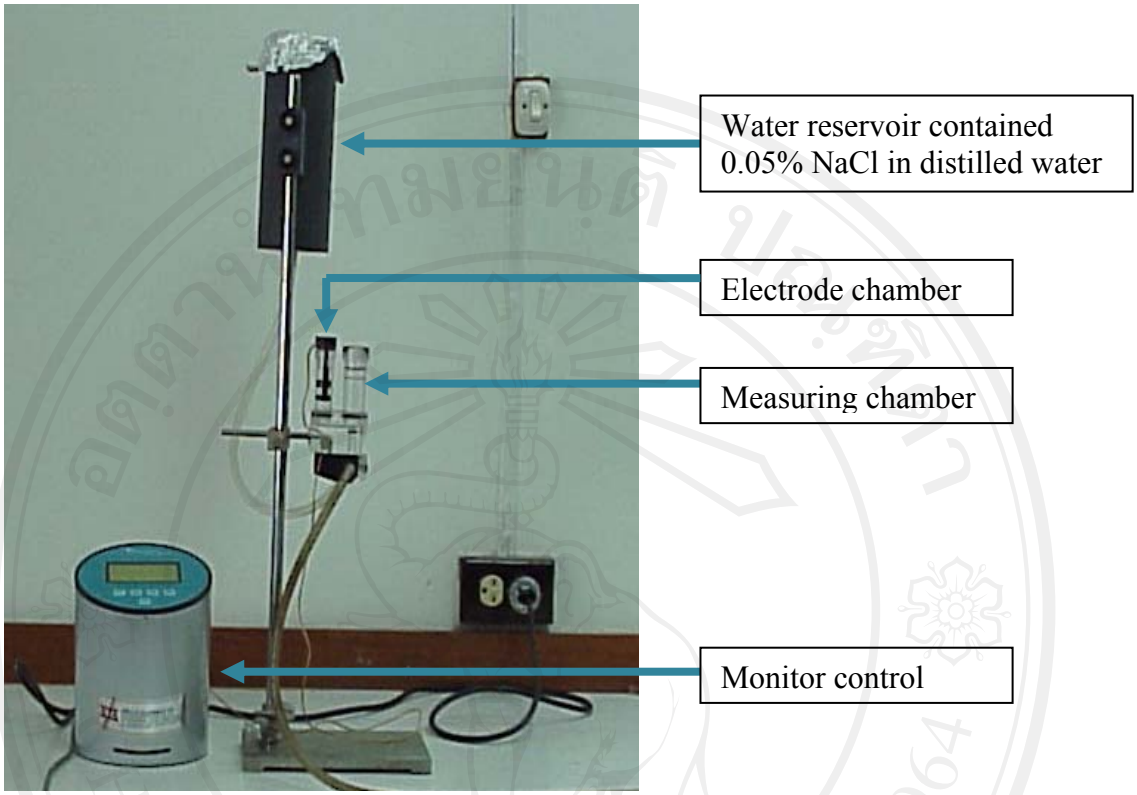

Figure 4 The illustration of plethysmometer

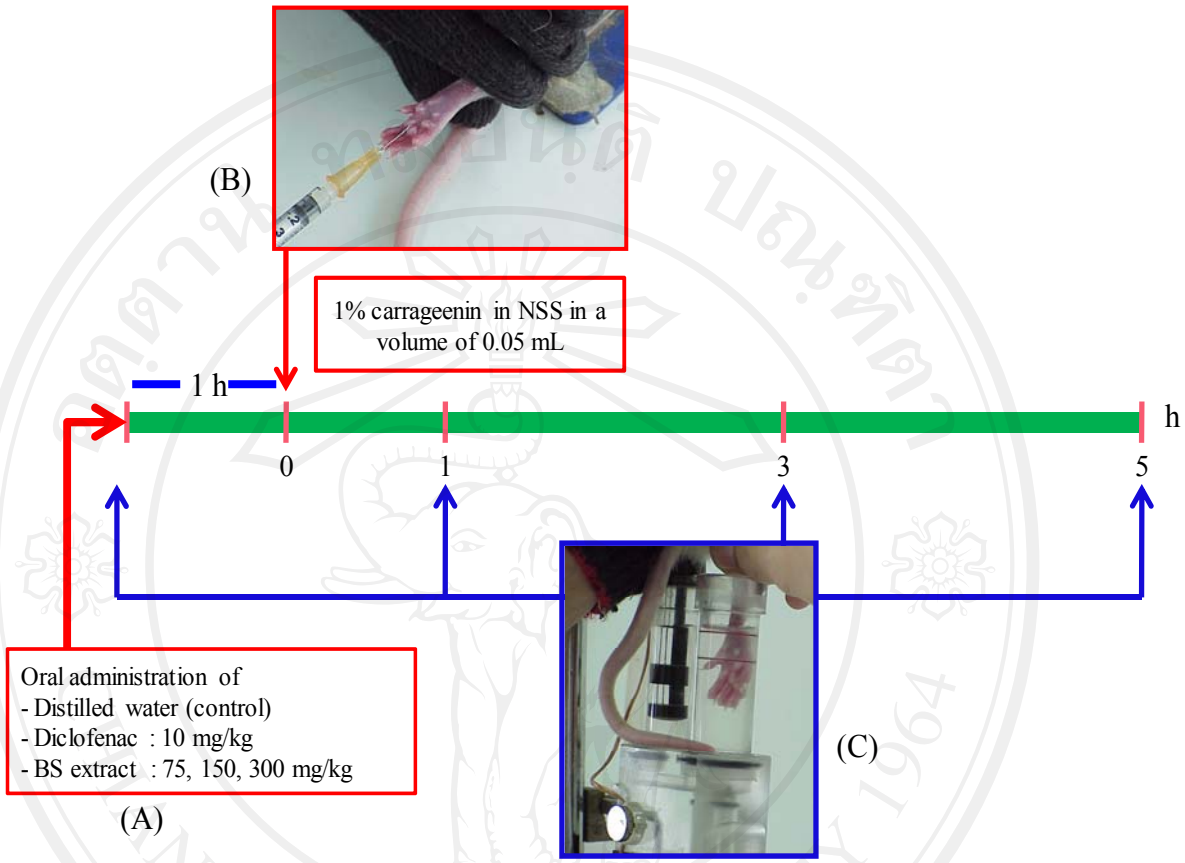

Figure 5 Diagram illustrating the water displacement method for measuring rat paw volume (carrageenin-induced edema)

Oral administration of test drugs 1 h before injection of carrageenin (A); Injection of 1% carrageenin into right subplantar 1 h after test drug administration (B); Measurement of the paw volume by immersion into the measuring chamber (C).

$$EV_x = PV_x - PV_0$$

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

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 test drug at time x

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

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

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

This experiment was performed to investigate anti-inflammatory activity of compounds mediated via LOX pathway. The edema was produced in the right hind paw of rats by intradermal injection of AA. The method described by Di Martino *et al.* (81) was performed as follow:

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

Group 1: Control group, received distilled water

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

Group 3: Reference group, received prednisolone (5 mg/kg)

Group 4-6: Test groups, received 75, 150, 300 mg/kg of BS extract, respectively

Rats were pretreated orally with distilled water, diclofenac, prednisolone or various doses of BS extract for 2 h and then 0.1 mL of 0.5% AA in 0.2 M carbonate buffer (pH 8.4) was injected intradermally into the right subplantar of each rat. Paw volume was measured using a plethysmometer (model 7150, Ugo Basile, Italy) before and at 1 h after AA injection. Edema volume of paw and the percent edema inhibition of each compound were calculated by the same way as described in carrageenin-induced hind paw edema. The scheme of protocol is shown in Figure 6.

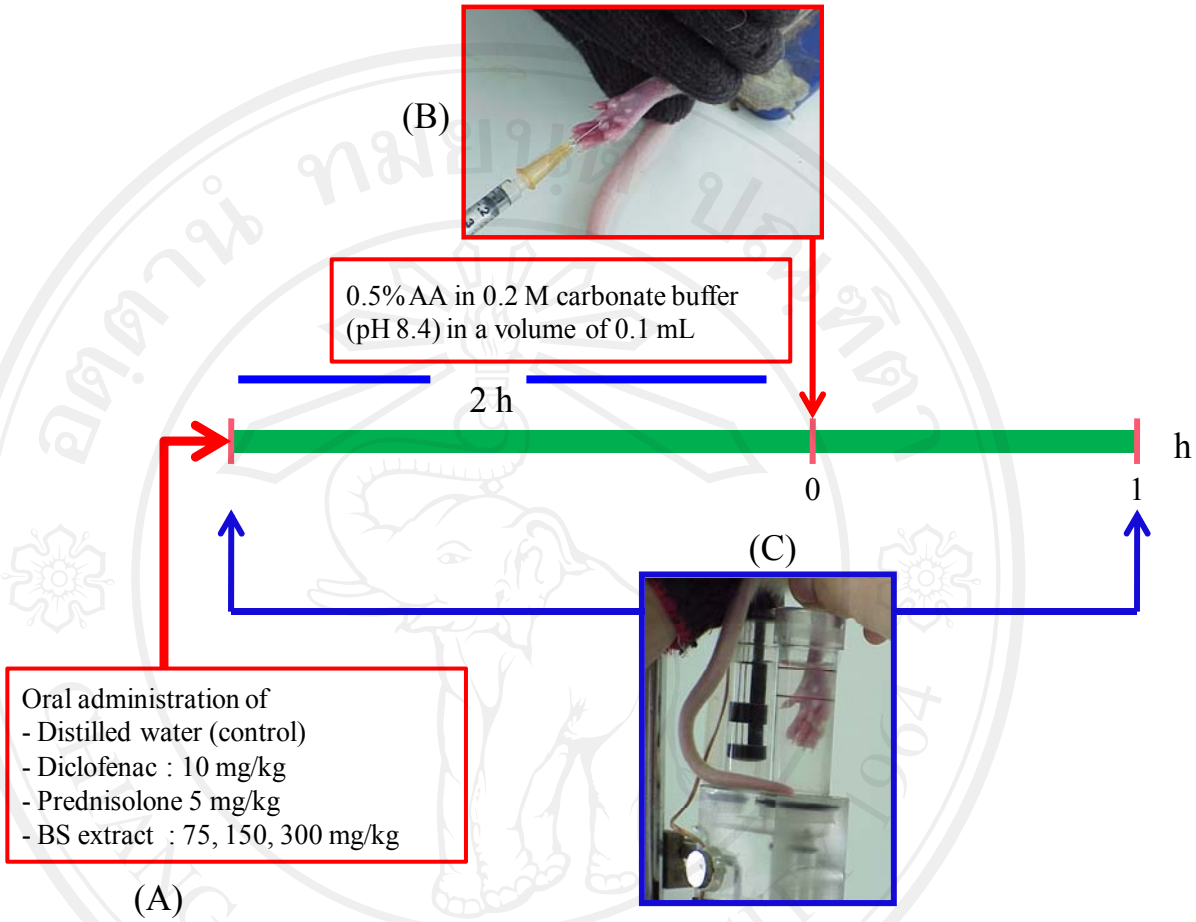

Figure 6 Diagram illustrating the water displacement method for measuring rat paw volume (AA-induced edema)

Oral administration of test drugs 2 h before injection of AA (A); Injection of 0.5% AA into right subplantar 2 h after test drug administration (B); Measurement of the paw volume by immersion into the measuring chamber (C).

2.5.1.4 Cotton pellet-induced granuloma formation in rats

This experiment was used for investigation of the ability of the compounds to inhibit the proliferative components of the chronic inflammatory processes by the method described by Swingle and Shideman (82) with slight modification as follow:

Male rats weighing 180-200 g were divided into 4 groups of 6 animals.

Group 1: Control group, received distilled water

Group 2: Reference group, received diclofenac (2.5 mg/kg)

Group 3: Reference group, received prednisolone (5 mg/kg)

Group 4: Test group, received BS extract (300 mg/kg)

A. Measurement of the granuloma formation and transudation

Adsorbent cotton wool was cut into pieces weighing 20±1 mg and made into a small pellet. 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 (82).

On the first day, test drugs were administered orally to each group of rats. One hour thereafter, the abdominal skin of rats was shaved and disinfected with 70% alcohol. Two cotton pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light anesthesia with pentobarbital sodium (35 mg/kg, intraperitoneally) under sterile technique. The suture was then made and the rat was allowed to recover. Test drugs were administered once daily throughout the experimental period of 7 days.

On the 8th day, rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally) and the abdominal skin was opened. The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for their wet weight. Cotton pellets were dried at 60 ^{o}C for 18 h and weighed for dry weight. The scheme of protocol is shown in Figure 7.

The granuloma weight and transudative weight from the extract treated rats were compared with those of the control and the reference groups. The percent granuloma inhibition of each test drug was calculated according to the following formula:

$$TW = Wt_w - Wt_d$$

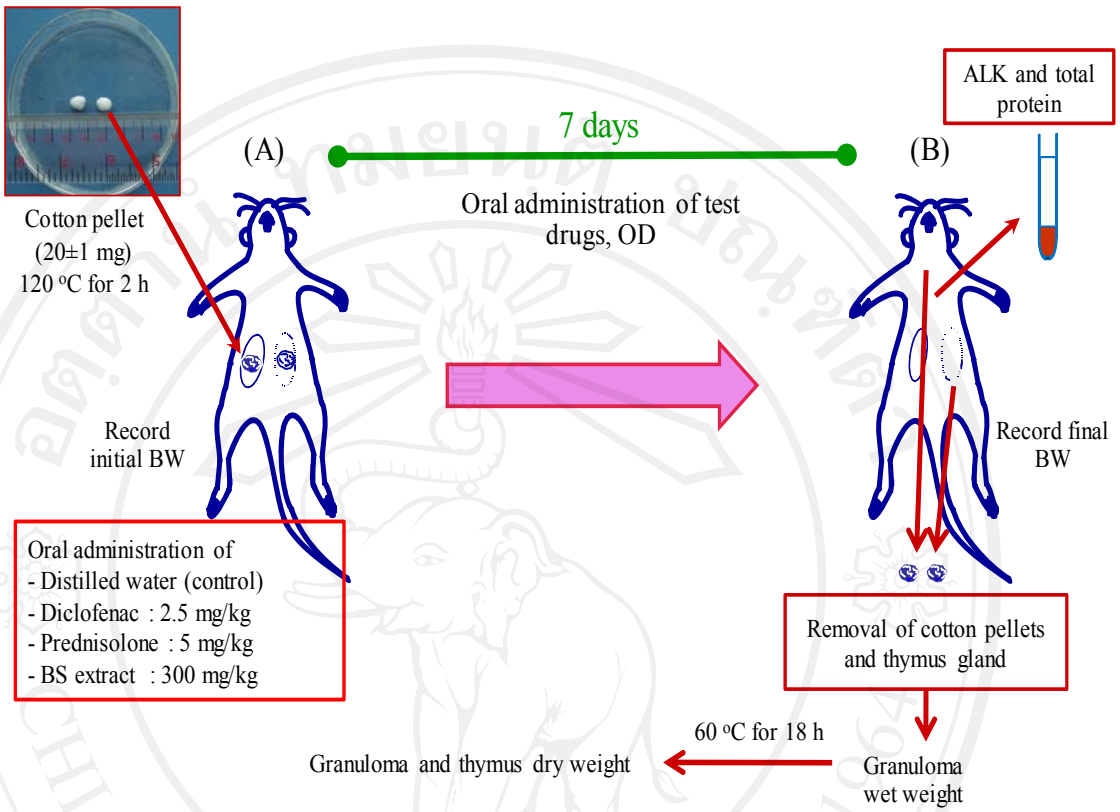

Figure 7 Diagram illustrating the method of cotton pellet-induced granuloma formation in rats

Test drugs were orally administration, then rats were anesthetized and two cotton pellets were implanted subcutaneously one on each side of the abdomen (A); On the 8th day, rats were anesthetized, the implanted pellets and the thymus gland were then dissected out, the blood was collected for determination of alkaline phosphatase activity (B).

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

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

Where,

TW = transudative weight (mg)

GW = granuloma weight (mg/mg cotton)

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)

% GI = percent granuloma inhibition

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

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

B. Measurement of the alkaline phosphatase activity in serum

This method was described by Bessey *et al.* (83). The animals from the cotton pellet-induced granuloma formation model were used. After the implanted cotton pellets were dissected out, the chest wall of rats was opened. Blood was collected by cardiac puncture technique. Samples of serum were sent to the medical laboratory for determination of alkaline phosphatase (ALP) activity and total protein. The ALP activity of the rats from treated groups was compared with that of control group and non-implanted group (normal group). The ALP activity of the each test compound was calculated according to the following formula:

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

C. Measurement of thymus weight and body weight gain

This method was described by Swingle and Shideman (82). The animals from the cotton pellet-induced granuloma formation model were used. Immediately after collection of the blood, the thymus gland was dissected out. The thymuses were dried

at 60 ^{o}C for 18 h and weighed for their dry weight. The change in body weight from the first and last day of experiment was also recorded. The body weight gain and the dry thymus weight of the extract treated rats were compared with those of the control and the reference groups. Thymus weight and body weight gain of the each test compound were calculated according to the following formula:

$$\text{Thymus weight} = \frac{\text{Dry thymus weight (mg)}}{\text{100 g body weight}}$$

$$\text{Body weight gain} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

2.5.2. ANALGESIC STUDY

2.5.2.1 Acetic acid-induced writhing response in mice

This experiment was used as a basis for investigation of analgesic effect of the test compounds (84-85). The method described by Collier *et al.* (86) and modified by Nakamura *et al.* (87) was performed as follow:

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

Group 1: Control group, received distilled water

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

Group 3-5: Test groups, received 75, 150, 300 mg/kg of BS extract, respectively

Male mice were pretreated with distilled water, diclofenac, or various doses of BS extract 60 min before intraperitoneal (i.p.) injection of 0.75% acetic acid in normal saline in a volume of 0.1 mL/10 g body weight. The animals were placed in a transparent plastic box. The number of writhes, a response consists of contraction of an abdominal wall, pelvic rotation followed by hind limb extension, was counted during continuous observation for 15 min, beginning 5 min after the acetic acid injection. The scheme of protocol is shown in Figure 8.

The percent writhing response inhibition of the each test drug was calculated according to the following formula:

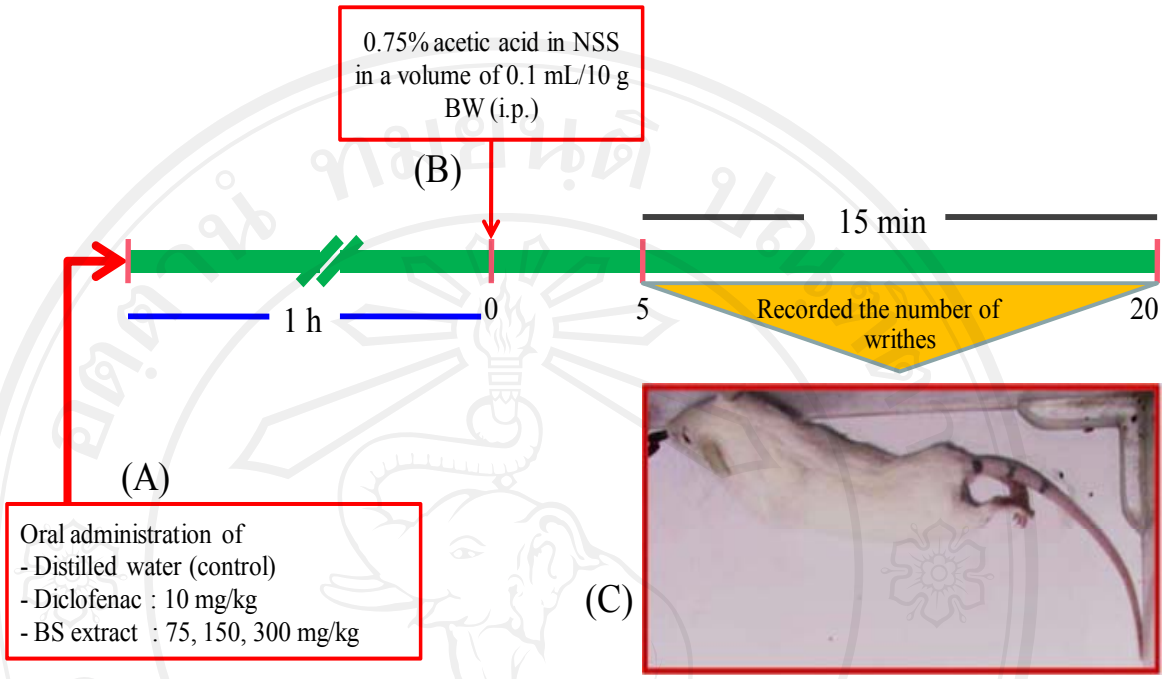

Figure 8 Diagram illustrating the method of acetic acid-induced writhing response in mice

Oral administration of test drugs 1 h before injection of acetic acid (A); Injection of 0.75% acetic acid (i.p.) (B); The number of writhes (contraction of an abdominal wall, pelvic rotation and hind limb extension) were counted for 15 min, beginning at 5 min after acetic acid injection(C).

$$\% \text{ WI} = \frac{WR_c - WR_t}{WR_c} \times 100$$

Where,

% WI = percent writhing response inhibition

WR_c = number of writhing response of control group

WR_t = number of writhing response of test group

2.5.2.2 Tail-flick test in rats

This experiment was used for investigation of analgesic activity mediated via central nociceptive mechanism. The method described by Gray *et al.* (88) was performed as follow:

Male rats weighing 180-200 g were divided into 4 groups of 6 animals.

Group 1: Control group, received distilled water

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

Group 3: Reference group, received codeine (75 mg/kg)

Group 4: Test group, received BS extract (300 mg/kg)

At first, rats were screened for the basal reaction time. The rat's tail was placed to cover a flush mounted photocell window of the Tail Flick Apparatus (model 7360 tail flick, Ugo Basile, Italy). Heat was applied by the infrared lamp (50 W bulb) mounted in a reflector. The light intensity was adjusted to give a normal reaction time of 2–4 s. The timer was activated when the lamp was turned on. Rats flicked its tail when it felt pain, the light then fell on the photocell and automatically stopped the timer.

Rats with control reaction time of 2-4 s were used. Test drugs were administered orally 1 h before re-exposure to the heat. The cut-off time of 10 s was the maximum time which an unflicked tail could expose to the heat without damage. The scheme of protocol is shown in Figure 9.

The analgesia was quantified as the percentage of maximum possible response time by the following calculation:

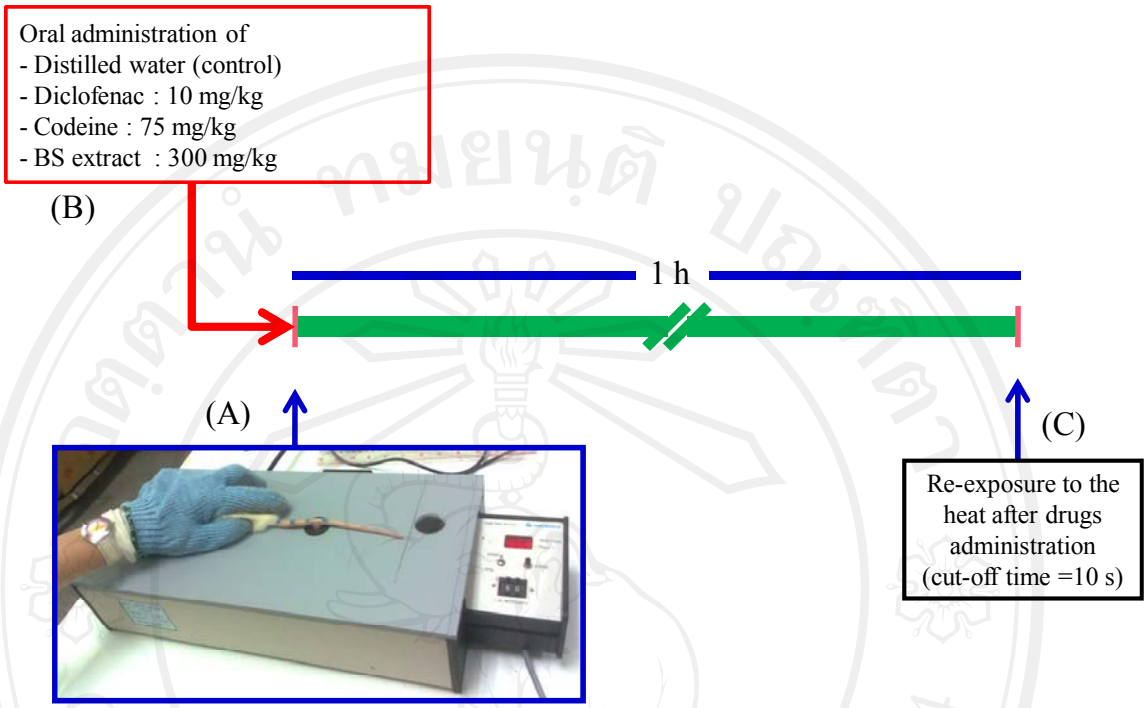

Figure 9 Diagram illustrating the method of tail-flick test in rats

Screening for normal reaction time and those with reaction time of 2-4 s were used (A); Oral administration of test drugs (B); Post drug reaction time was recorded 1 h after drugs administration (C).

$$\% \text{ Inhibition} = \frac{T_t - T_c}{10 - T_c} \times 100$$

Where,

T_t = reaction time after recieved test drugs

T_c = control reaction time

2.5.3. ANTIPYRETIC STUDY

2.5.3.1 Yeast-induced hyperthermia in rats

This experiment was used to investigate antipyretic agents. The method described by Teotino *et al*. (89) was performed as follows:

Male rats weighing 200–250 g were divided into 4 groups of 6 animals.

Group 1: Control group, received distilled water

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

Group 3-4: Test groups, received 150 and 300 mg/kg of BS extract, respectively

Rat was restrained in plastic cages, and the initial rectal temperature was recorded using a 12-channel electric thermometer (model TPM 812, Letica, Spain) connected with probes that were inserted into rat rectum about 5 cm depth. The basal rectal temperature was 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. The rectal temperature was again recorded 18 h after yeast injection. Those animals which showed a rise in rectal temperature of more than 1 ^{o}C were used. Distilled water, diclofenac and BS extract were administered orally and the rectal temperature was recorded at 30 min interval for 2 h after drug administration. The scheme of protocol is shown in Figure 10.

2.6 DRUGS AND CHEMICALS

2.6.1. Drugs

(1) Codeine (The Government Pharmaceutical Organization (GPO), Bangkok, Thailand)

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

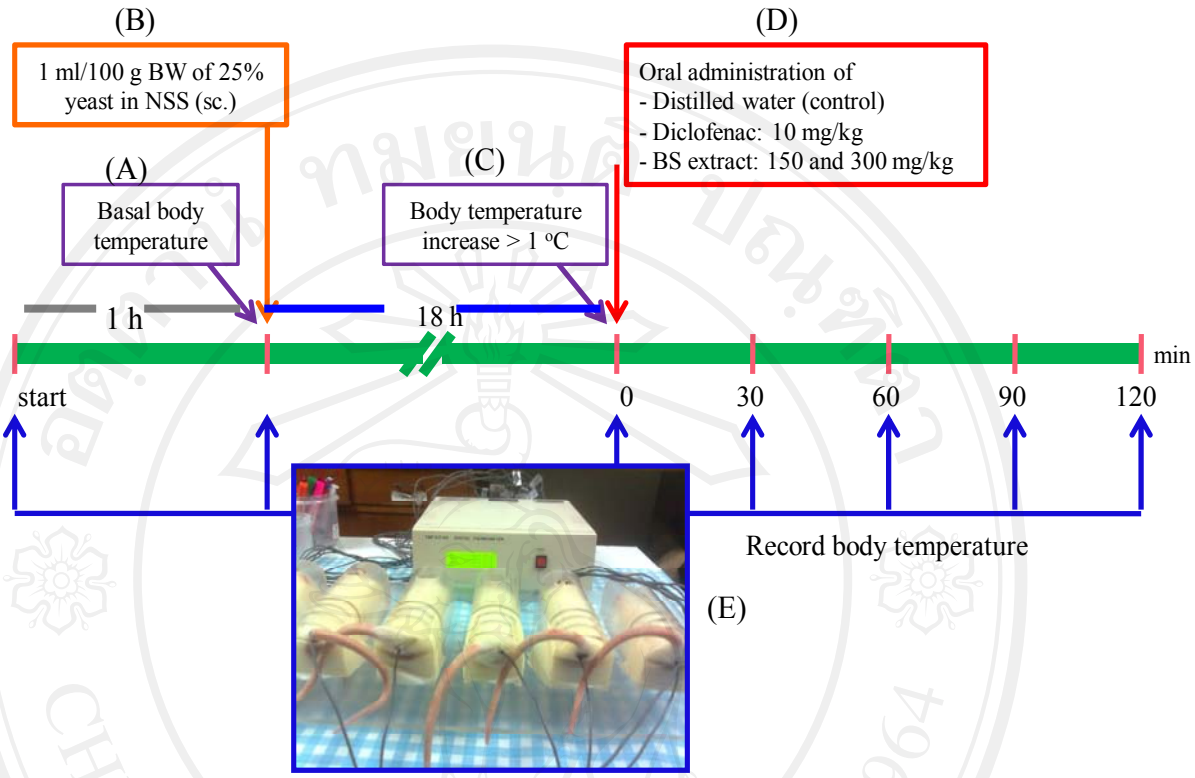

Figure 10 Diagram illustrating the protocol for testing antipyretic effect using yeast-induced hyperthermia in rats

Record of the basal body temperature (°C) (A); Subcutaneous injection of 25% yeast in NSS (B); Rats with an increase in body temperature ≥1°C at 18 h after yeast injection were used (C); Oral administration of test drugs (D); Measurement of body temperature (°C) at 30, 60, 90 and 120 min after administration of test drugs using 12-channel electric thermometer (E).

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

(4) Prednisolone (Scherisone®, Schering, Bangkok Ltd., Nonthaburi, Thailand)

2.6.2. Chemicals

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

(2) Brewer's yeast (Sigma Chemicals Company, St. Louis, U.S.A.)

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

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

(5) Acetic acid (GPO, Bangkok, Thailand)

2.7 STATISTICAL ANALYSIS

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