CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material and preparation of extract

Methanolic extract of *A. purpurata*, designated as AP extract, was prepared and kindly provided by Professor Dr. Vichai Reutrakul, Director of the Center for Innovation in Chemistry (PERCH-CIC). The rhizomes of *A. purpurata* were collected in 2008 by Mr. Narong Nuntasaen, a botanist from The Forest Herbarium National Park, Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Bangkok, Thailand.

The method of extraction was briefly summarized as follows: the AP extract was obtained by maceration of the dry rhizome powder with methanol overnight (repeated 3 times), filtration, evaporation and lyophilization. The yield obtained was 3% w/w.

2.2 Experimental animals

Male Swiss albino mice weighing 30-40 g, male Sprague Dawley rats weighing 40-60 g, 100-120 g and 180-210 g as well as female Sprague Dawley rats weighing 180-200 g were obtained from the National Laboratory Animal Center, Salaya, Mahidol University, Nakhorn Pathom, Thailand. All animals were kept in room maintained under environmental control conditions of 24 ± 1 ^{o}C, relative humidity 50 ± 10%, and a 12 h light - 12 h dark cycle. The animals had free access to water and food and were acclimatized for at least one week before starting the experiments. All animals (except for the EPP-induced ear edema) were fasted overnight but with water *ad libitum* prior to the day of experiment. All animal experiments were approved by the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University (Registration No: 24/2553).

2.3 Preparation of test drugs

All test drugs were dissolved in distilled water, except in ear edema model where AP extract and diclofenac were dissolved in absolute ethanol.

2.4 Drug administration

For most experiments, all test drugs were orally administered in an equivalent volume of 0.5 mL/100 g body weight of rats and 0.1 mL/10 g body weight of mice. In the ear edema model, test drugs were applied topically to the outer and the inner surfaces of the ears in equivalent volume of 20 µL/ear.

2.5 Experimental protocols

For analgesic activity study, acetic acid-induced writhing response in mice and tail-flick test in rats were performed. Anti-inflammatory activity of the AP extract on both acute and chronic inflammation was assessed by using three models of inflammation, i.e., ethyl phenylpropiolate (EPP)-induced ear edema in rats, carrageenin- and AA-induced hind paw edema in rats as well as cotton pellet-induced granuloma formation in rats.

2.5.1 Analgesic study

2.5.1.1 Acetic acid-induced writhing response in mice

This experiment was done as described by Collier *et al*. (1968) (47) and modified by Nakamura *et al*. (1986) (48). Male mice weighing 30-40 g were divided into 5 groups of 6 mice and administered orally of test drugs as follows:

Group 1: Control group, received distilled water
Group 2: Reference group, received 10 mg/kg of diclofenac
Groups 3-5: Test groups, received 150, 300 and 600 mg/kg of AP extract, respectively

At 1 h after administration, mice were injected intraperitoneally in a volume of 0.1 mL/10 g body weight of 0.75% aqueous acetic acid. After acetic acid injection, mice were placed in a transparent plastic box for observation of the writhing responses for 15 min starting at 5 min after injection. The writhes consist of abdominal wall contractions, pelvic rotation and hind limb extension. Mean values of the treated groups were compared with those of the control group. The percent inhibition of writhing response was calculated according to the following formula:

$$\% \text{ IW} = \frac{\text{NW of control group - NW of test groups}}{\text{NW of control group}} \text{ X } 100$$

where,

% IW = inhibition of writhing response (%)

NW = number of writhe

The diagram illustrating the procedure of the acetic acid-induced writhing response in mice is shown in Figure 5.

2.5.1.2 Tail-flick test in rats

The method was described by D'Amour and Smith (1941) (49) and modified by Gray *et al.* (1970) (50). Male rats weighing 180-200 g were used. They were screened for the basal reaction time and then divided into 4 groups of 6 rats.

Group 1: Control group, received distilled water

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

Group 3: Reference group II, received 200 mg/kg of codeine

Group 4: Test group, received 600 mg/kg of AP extract

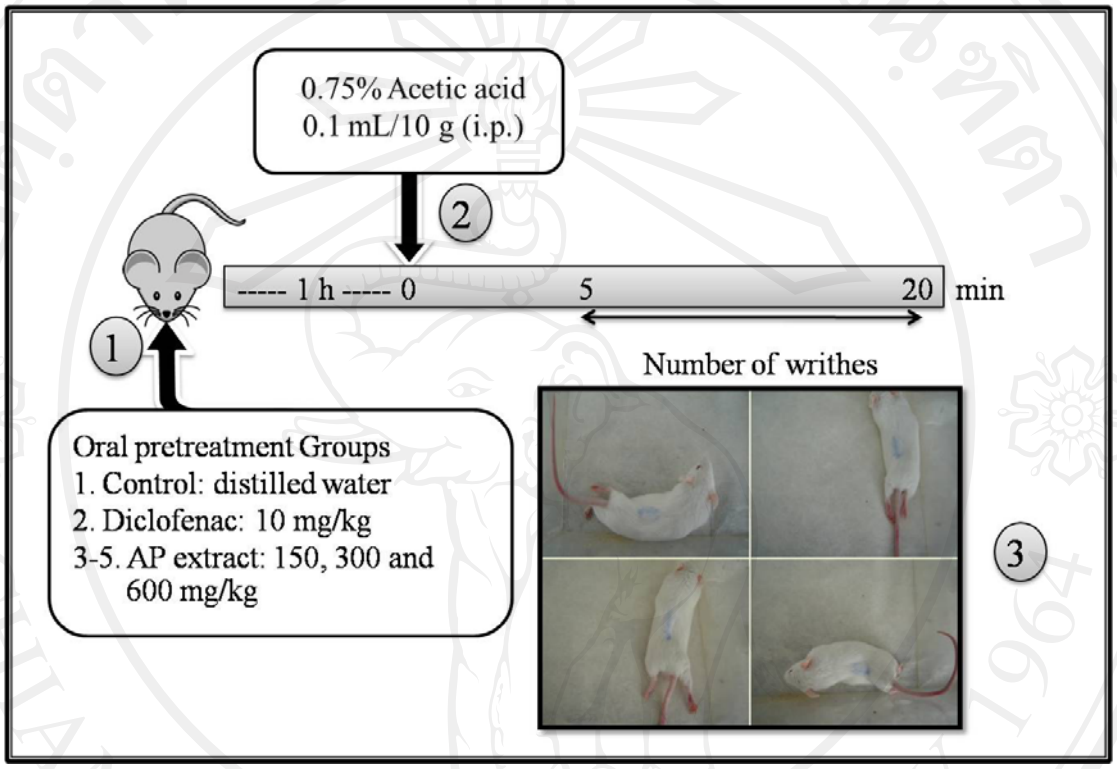

Figure 5 Diagram illustrating the procedure of the acetic acid-induced writhing response in mice

Rat's tail was placed to cover a flush mounted photocell window of the Tail Flick Apparatus (Ugo Basile, Italy). Heat was applied by infrared lamp (50 W bulb) mounted on a reflector. The timer was activated when the lamp was turned on and stopped when rat flicked its tail away from the photocell window. The rat's tail was exposed to the infrared lamp and the length of time taken for the rat to flick its tail away from the heat was recorded. The light intensity was adjusted to give a normal reaction time of 2-4 sec. The cut-off time of 10 sec was the maximum time which an unflicked tail was allowed to expose to the heat without causing tissue damage. The reaction time was measured before and at 1 h after drug administration. The analgesic response was calculated as a percentage maximum possible response time.

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

where,

T_t = reaction time after recieved test drugs

T_c = control reaction time

10 = cut-off time of 10 sec

The diagram illustrating the procedure of the acetic acid-induced writhing response in mice is shown in Figure 6.

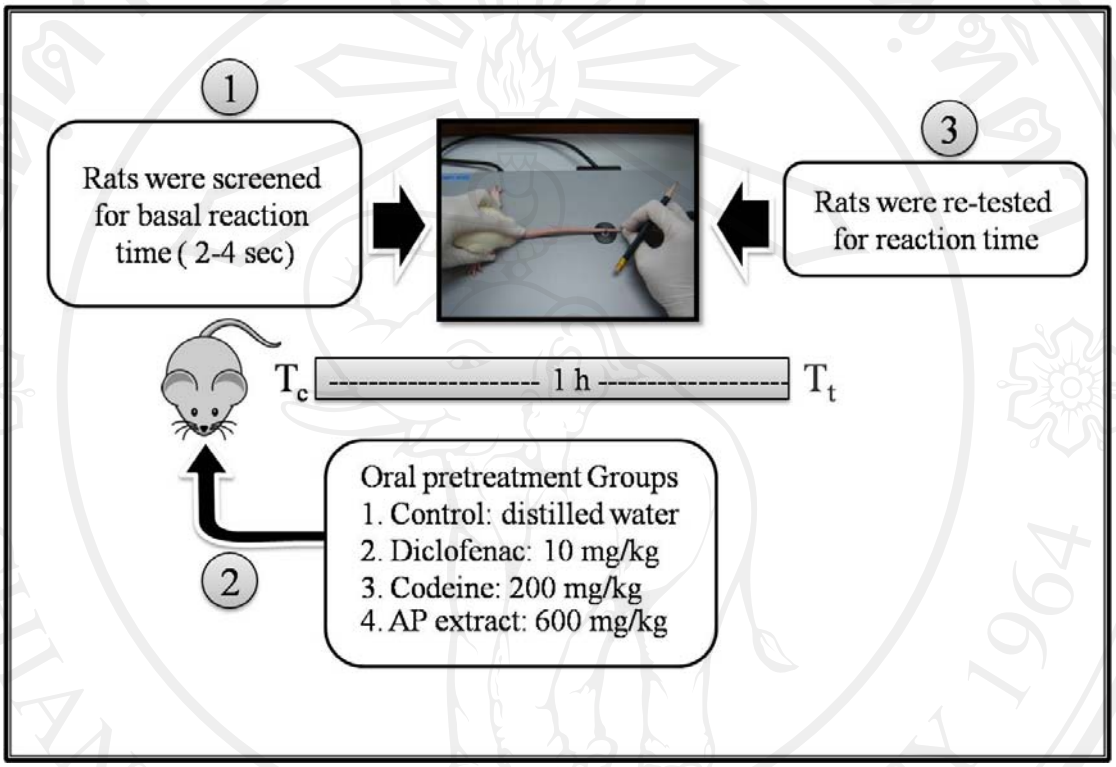

Figure 6 Diagram illustrating the procedure of the tail-flick test in rats

2.5.2 Anti-inflammatory study

2.5.2.1 EPP-induced ear edema in rats

Topical anti-inflammatory activity of AP extract was assessed by the method of Brattsand *et al.* (1982) (51). Male rats weighing 40-60 g were divided into 3 groups of 3 rats (6 ears). Test drugs were applied topically in a volume of 20 µL just before the irritant (EPP).

Group 1: Control group, received vehicle (ethanol)
Group 2: Reference group, received 3 mg/ear of diclofenac
Group 3: Test group, received 3 mg/ear of AP extract

Ear edema was induced by topical application of EPP immediately after application of the reference or test drug. EPP at a dose of 1 mg/20 µL/ear was applied to the inner and outer surfaces of both ears using an automatic microliter pipet. Ear thickness was measured by automatic vernier calipers before and at 15, 30, 60 and 120 min after edema induction. Ear thickness of the tested groups was compared with the vehicle-treated group. The percent inhibition of the edema formation of test substances was calculated using the formulae as follows:

$$ED_x = ET_x - ET_o$$

$$\% ED = \frac{ED_c - ED_t}{ED_c} X\ 100$$

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
% ED = percent edema inhibition by test compound at time x

The diagram illustrating the procedure of the EPP-induced ear edema in rats is shown in Figure 7.

2.5.2.2 Carrageenin-induced hind paw edema in rats

Carrageenin-induced hind paw edema in rats was carried out as described by Winter *et al.* (1962) (52). Male rats weighing 100-120 g were divided into 5 groups of 6 rats. The rat was pretreated orally with distilled water or test drugs 1 h before carrageenin injection.

Group 1: Control group, received distilled water
Group 2: Reference group, received 10 mg/kg of diclofenac
Groups 3-5: Test groups, received 150, 300 and 600 mg/kg of AP extract, respectively

One hour later, the rats were challenged by an intradermal injection of 0.05 mL of 1% solution of carrageenin in sterile normal saline solution (NSS) into sub-plantar side of the right hind paw. The paw was marked with ink at the level of the lateral malleolus. The paw volume was measured using plethysmometer (model 7150, Ugo Basile, Italy) by immersing the paw into the measuring chamber containing 0.05% NaCl in distilled water before and at 1, 3 and 5 h after carrageenin injection. Each paw volume was obtained from the average of 3 readings. The increase in paw volume was compared with the basal volume. The difference of average values between the treated rats and the control group was calculated for each time interval and statistically evaluated. The percent inhibition was calculated using the formulae as follows:

$$EV_x = PV_x - PV_o$$

$$\% EI_x = \frac{EV_x \text{ of control group} - EV_x \text{ of test group}}{EV_x \text{ of control group}} \times 100$$

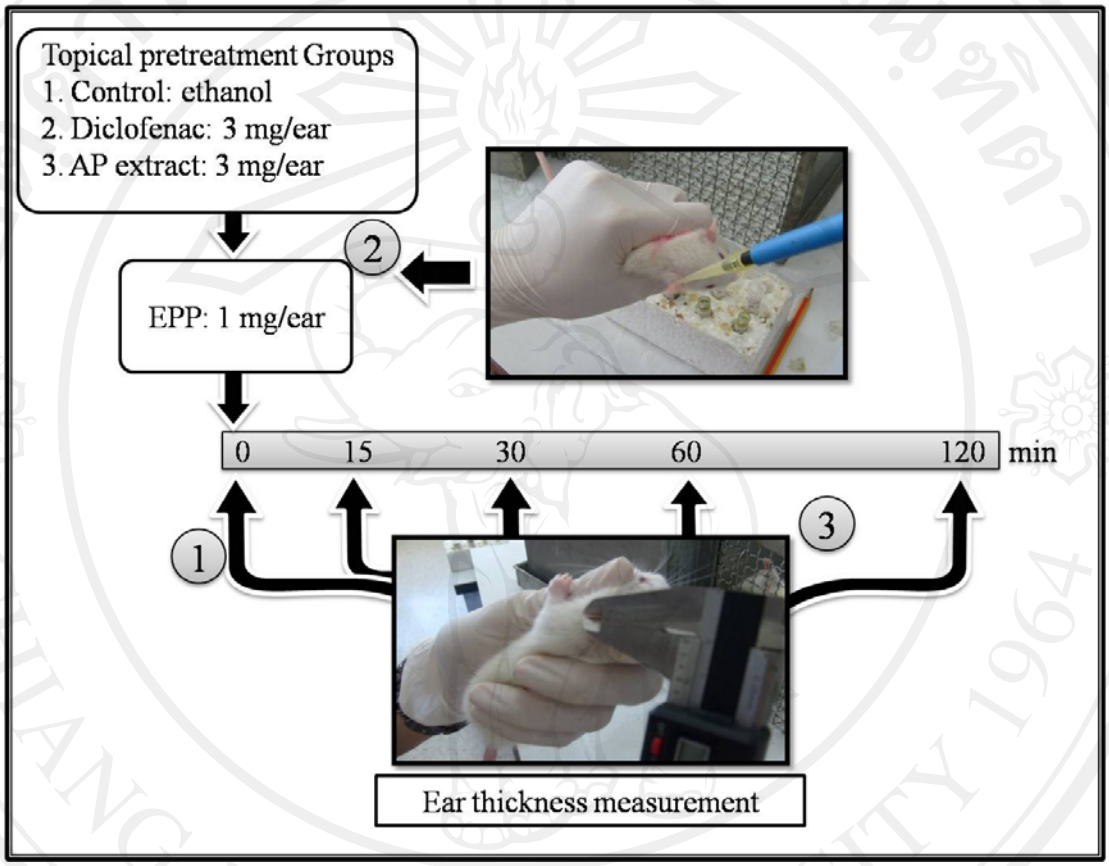

Figure 7 Diagram illustrating the procedure of the EPP-induced ear edema in rats

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 by test compound at time x

The diagram illustrating the procedure of the carrageenin-induced hind paw edema in rats is shown in Figure 8.

2.5.2.3 AA-induced hind paw edema in rats

AA-induced hind paw edema in rats was performed according to the method of Di Martino *et al*. (1987) (53). Male rats weighing 100-120 g were divided into 6 groups of 6 rats. Test drugs were administered orally 2 h prior to AA injection.

Group 1: Control group, received distilled water

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

Group 3: Reference group II, received 5 mg/kg of prednisolone

Groups 4-6: Test groups, received 150, 300 and 600 mg/kg of AP extract, respectively

Paw edema was induced by an intradermal injection of AA (0.5% in 0.2 M carbonate buffer, pH 8.4) into plantar surface of the right hind paw of the rat, at a volume of 0.1 mL. The edema volume was determined using a plethysmometer (model 7150, Ugo Basile, Italy) prior to and 1 h after AA injection. Paw volume and edema volume measurements and the calculation of percent inhibition were similar to the method of carrageenin-induced hind paw edema in rats as described above.

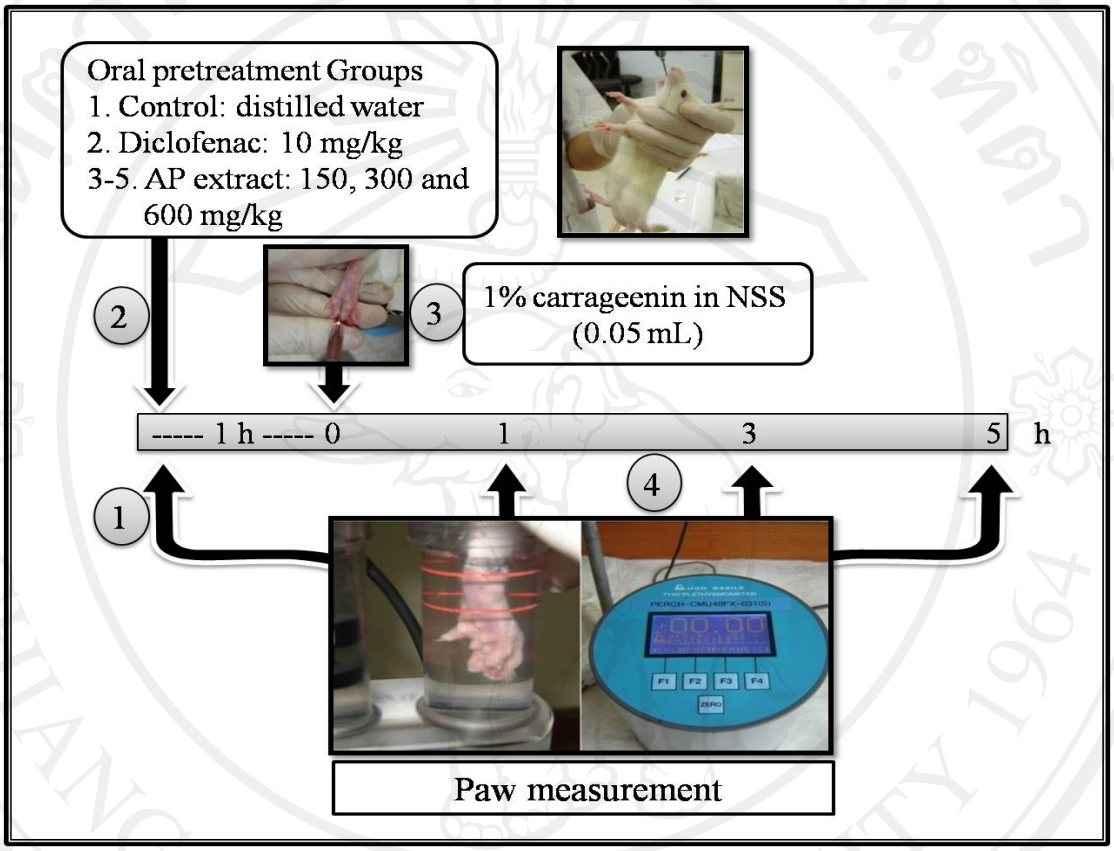

Figure 8 Diagram illustrating the procedure of the carrageenin-induced hind paw edema in rats

2.5.2.4 Cotton pellet-induced granuloma formation in rats

This experiment was carried out using the method of Swingle and Shideman (1972) (54) with slight modification. Adsorbent cotton wool was cut into pieces weighing 20 ± 1 mg and made into pellets. The pellets were sterilized in a hot air oven at 120 ^{o}C for 2 h. Male rats weighing 180-210 g were divided into 5 groups of 6 rats.

Group 1: Normal group, received distilled water
Group 2: Control group, received distilled water
Group 3: Reference group I, received 5 mg/kg/day of diclofenac
Group 4: Reference group II, received 5 mg/kg/day of prednisolone
Group 5: Test group, received 600 mg/kg of AP extract

The abdominal skin of rat was shaped and disinfected with 70% alcohol. All rats, except those in the normal group, were implanted subcutaneously with two sterilized cotton pellets, one on each side of abdomen under light anesthesia (pentobarbital sodium, 40 mg/kg, intraperitoneally) with sterile technique. The AP extract, reference drugs and distilled water were administered orally once daily for 7 days.

2.5.2.4.1 Measurement of body weight gain

The change in body weight between the first and the last day of experiment was recorded. The body weight gain of AP extract treated rats was compared with those of the control and reference groups.

2.5.2.4.2 Measurement of alkaline phosphatase activity in serum

The animals in the cotton pellet-induced granuloma formation model were used for determination of alkaline phosphatase (ALP) activity as described by Bessey *et al* (55). On the 8th day after cotton pellets implantation, rats were anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally). Blood was collected into a glass tube by cardiac puncture technique and serum was separated. Samples of serum were

sent to the Associated Medical Sciences Clinical Services Center, Faculty of Associated Medical Sciences, Chiang Mai University for determination of ALP and total protein. Measurement of the alkaline phosphatase activity in serum was calculated as follows:

$$\text{ALP activity (U of enz/mg of serum protein x } 10^{-4}) = \frac{\text{ALP (U/L)}}{\text{Total protein (g/dL)}}$$

2.5.2.4.3 Measurement of thymus weight

After blood collection, the chest of the rats was opened and the thymuses were dissected out. The thymuses were dried at 60 ^{o}C for 18 h and weighed for their dry weight. The thymuses dry weight of AP extract treated rats were compared with those of the control and the reference groups.

2.5.2.4.4 Measurement of granuloma weight and transudative weight

The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for wet weight. Cotton pellets were dried at 60 ^{o}C for 18 h and their dry weights were 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 (% GI) by AP extract was calculated according to the following formulae:

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

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

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

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

The diagram illustrating the procedure of the cotton pellet-induced granuloma formation in rats is shown in Figure 9.

2.5.2.4.5 Evaluation of ulcerogenic effect (56)

The stomach of the rat from the cotton pellet-induced granuloma formation experiment was removed, 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 millimeter (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

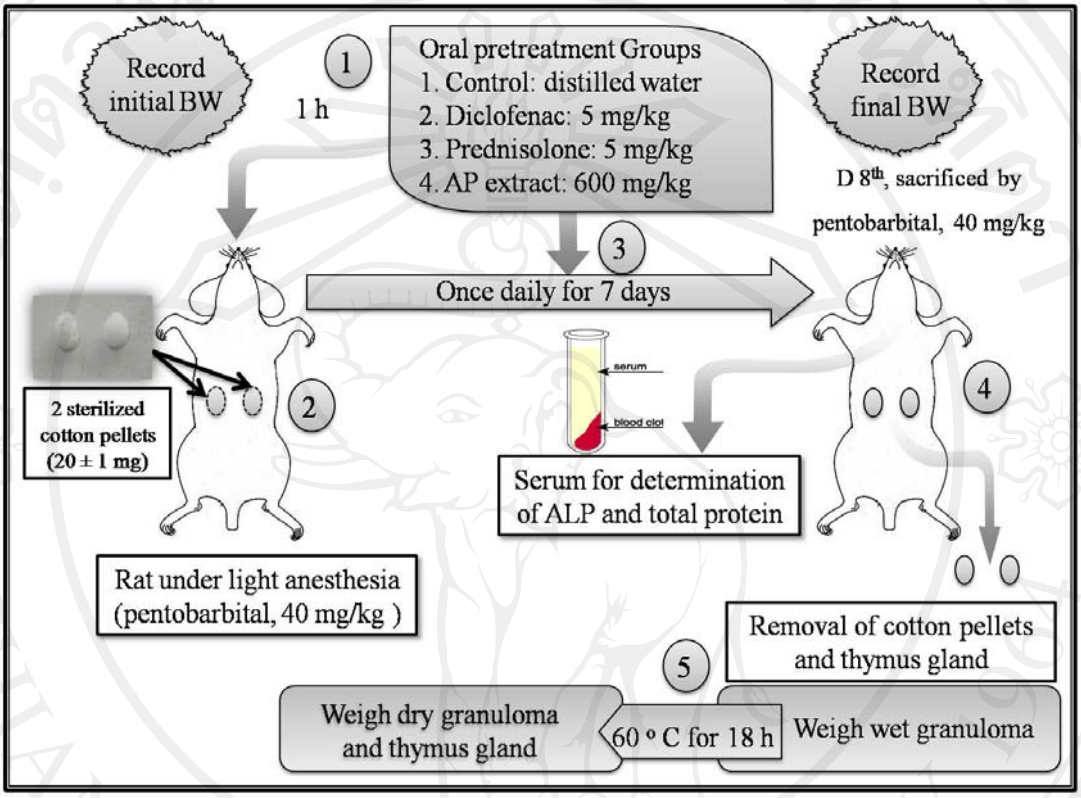

Figure 9 Diagram illustrating the procedure of the cotton pellet-induced granuloma formation in rats

2.5.3 Acute toxicity

The procedure was conducted according to the Organization of Economic Cooperation and Development (OECD) guideline (Test No. 420) for testing of chemicals (57) with slight modification. Adult (7 weeks) female Sprague Dawley rats weighing 180-200 g were randomly divided into 2 groups of 5 rats each. Rats were deprived of food but not water for 16-18 h before administration of test substances. Distilled water was given to the rats in vehicle control group. In test group, AP extract was administered by oral gavage at a dose of 2,000 mg/kg body weight. Visual observation of signs and symptoms such as changes in the skin, fur, eyes, and mucous membrane were made and recorded at 1, 2, 4, 6 h and then once daily for 14 days after the administration of test substances. Survival rats were sacrificed on the 15^{th} day to examine any gross pathological changes of the internal organs. Any changes of the intestinal organs compared with those of the control group were recorded.

2.6 Drugs, chemicals and equipments

2.6.1 Drugs

1. Codeine (the Government Pharmaceutical Organization [GPO], Bangkok, Thailand)
2. Diclofenac (Sigma Chemical Company, St. Louis, U.S.A.)
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. AA (Sigma Chemical Company, St. Louis, U.S.A.)
2. Absolute ethanol (Merck, Darmstadt, Germany)
3. Acetic acid (GPO, Bangkok, Thailand)
4. Acetone (Merck, Darmstadt, Germany)
5. EPP (Fluka Chemicals Co., Ltd., Japan)
6. Lambda carrageenin (Fluka Chemicals Co., Ltd., Japan)

2.6.3 Equipments

1. Tail Flick Apparatus (Ugo Basile, Italy)
2. Plethysmometer (model 7150, Ugo Basile, Italy)

2.7 Statistical analysis

The results obtained for evaluation of ulcerogenic effect were expressed as median. The other results were expressed as mean ± standard error of mean (S.E.M.). Statistical comparisons between groups were analyzed by using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. *P* values less than 0.05 were considered significant.