# CHAPTER 2
# MATERIALS AND METHODS

## 2.1 Preparation of the extract

*Garcinia wallichii* was collected from Koh Chang, Trat province in March, 2005. The methanol extract of *G. wallichii* (GW extract) is kindly provided by Professor Dr.Vichai Reutrakul, Director of the Center for Innovation in Chemistry: PPERCH-CIC. The method of extraction was briefly as follows: Dried, grounded leaf and twig of *G. wallichii* (500 g) was percolated with methanol (3.2 L). The percolated was evaporated using rotary evaporator, freezen dried and yielded the methanol extract 16.54 g (3.31% 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 obtained from the National Laboratory Animal Center, Salaya, Mahidol University, Nakornpathom, Thailand, were used. All animals were kept in a room maintained under environmentally control conditions of 24±1 ºC, relative humidity 50±10 % and 12 h light-12 h dark cycle. All animals had free access to water and standard diet. 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.

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**2.3 Preparation of test drugs**

All test drugs were dissolved in distilled water, except in ear edema model, they were dissolved in 5% DMSO in acetone.

**2.4 Drugs administration**

For the anti-inflammatory, analgesic experiments and acute toxicity test, 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 tail-flick test in rats, test drugs were intraperitoneally administered, whereas in the ear edema model a local application of the test drug to the outer and the inner surfaces of the ear was performed.

**2.5 Experimental models**

**2.5.1 Anti-inflammatory study**

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

Topical anti-inflammatory activity of the GW extract was assessed by the method described by Brattsand *et al* (1982) [53]. This model was used for screening and evaluation of the inhibitory activity of test substance on the ear edema formation response to inflammation induced by ethyl phenylpropiolate (EPP). Edema was the cardinal sign of acute inflammation. Male rats at of 40-60 g body weight were divided into 3 groups of 3 animals per group (6 ears).

Group 1 control group, received vehicle (5% DMSO in acetone) 20 σL/ear

Group 2 reference group, received phenylbutazone 1 mg/ear

Group 3 test group, received the GW extract 1 mg/ear

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Ear edema was induced by topical application of EPP to the inner and outer surfaces of both ears of each rat using an automatic microliter pipet. Each ear received EPP 1 mg/20 µL, immediately after application of the vehicle, phenylbutazoe or the GW extract. The thickness of the ear was measured using a digital vernier calipers before and 15, 30, 60 and 120 minutes after application of EPP. The increase in the ear thickness was compared with that of the control group and percent inhibition was calculated as follows:

$$ED_X = ET_X - ET_O$$

$$\%EDI = \frac{ED_C - ED_T}{ED_C} \times 100$$

where,

$ED_X$ = edema thickness (σm) at time X

$ET_X$ = ear thickness (σm) at time X

$ET_O$ = ear thickness (σm) before application of EPP

%EDI = percent edema inhibition of test compound at time X

$ED_C$ = edema thickness (σm) of control group at time X

$ED_T$ = edema thickness (σm) of test group at time X

The diagram illustrating the procedure of the EPP-induced ear edema in rats is shown in Figure 4.

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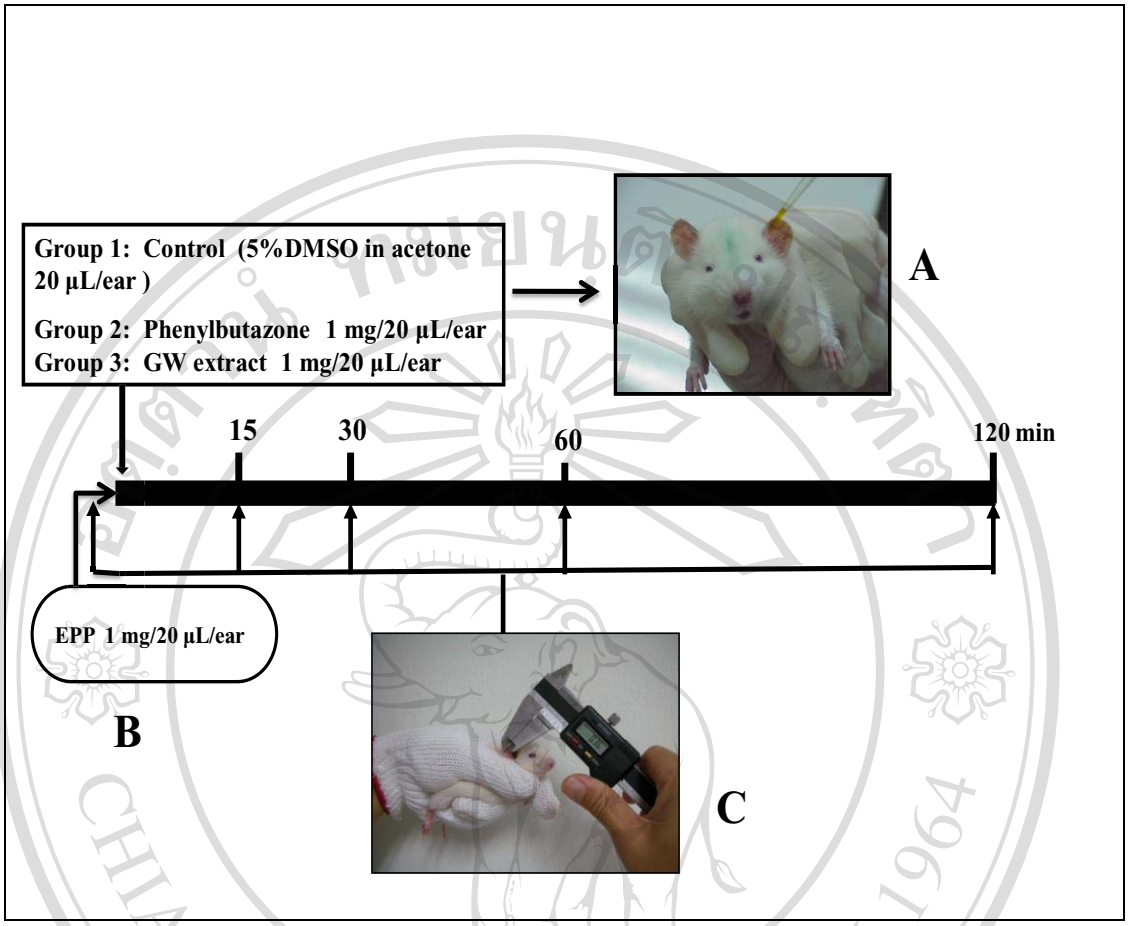


**Figure 4. Diagram illustrating the procedure of the EPP-induced ear edema in rats**.

A. Topical application of the substance to the inner and outer surfaces of the ear by using an automatic microliter pipette.

B. The rats were treated locally to outer and inner surfaces of both ears with EPP using an automatic microliter pipet.

C. Measurement of the thickness of the ear by using the digital vernier calipers.

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**2.5.1.2 Carrageenin-induced hind paw edema in rats.**

The GW extract was assessed by the method described by Winter *et al* (1962) [54]. This experiment was performed to investigate the inhibitory effect of test agents on the hind paw edema formation induced by carrageenin. The test is known to be sensitive to cyclooxygenase inhibitors. Male rats of 100-120 g body weight were divided into 5 groups of 6 animals per group.

Group 1 control group, received distilled water

Group 2 reference group, received 10 mg/kg of diclofenac

Group 3-5 test groups, received 75, 150 and 300 mg/kg of the GW extract

The rats were pretreated orally with distilled water, diclofenac or the various doses of the GW extract 1 h prior to carrageenin injection. Acute inflammation was produced by subplantar injection of 0.05 mL of 1% carrageenin in sterile normal saline solution (NSS) into the right hind paw of the rats. Paw volume was determined by means of a volume displacement technique using a plethysmometer (model 7150, Ugo Basile, Italy). The paw volume was measured prior to and at 1, 3 and 5 h after carrageenin injection. The edema volume of the paw and the percent edema inhibition of each test compound were obtained by the following calculation:

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$$EV_X = PV_X - PV_O$$

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

where,

$EV_X$ = edema volume (mL) at time X

$PV_X$ = paw volume (mL) at time X

PVo = paw volume (mL) measured before carrageenin injection

$\% EI_X$ = percent edema inhibition of test compound at time X

The diagram illustrating the procedure of the carrageenin-induced hind paw edema in rats is shown in Figure 5.

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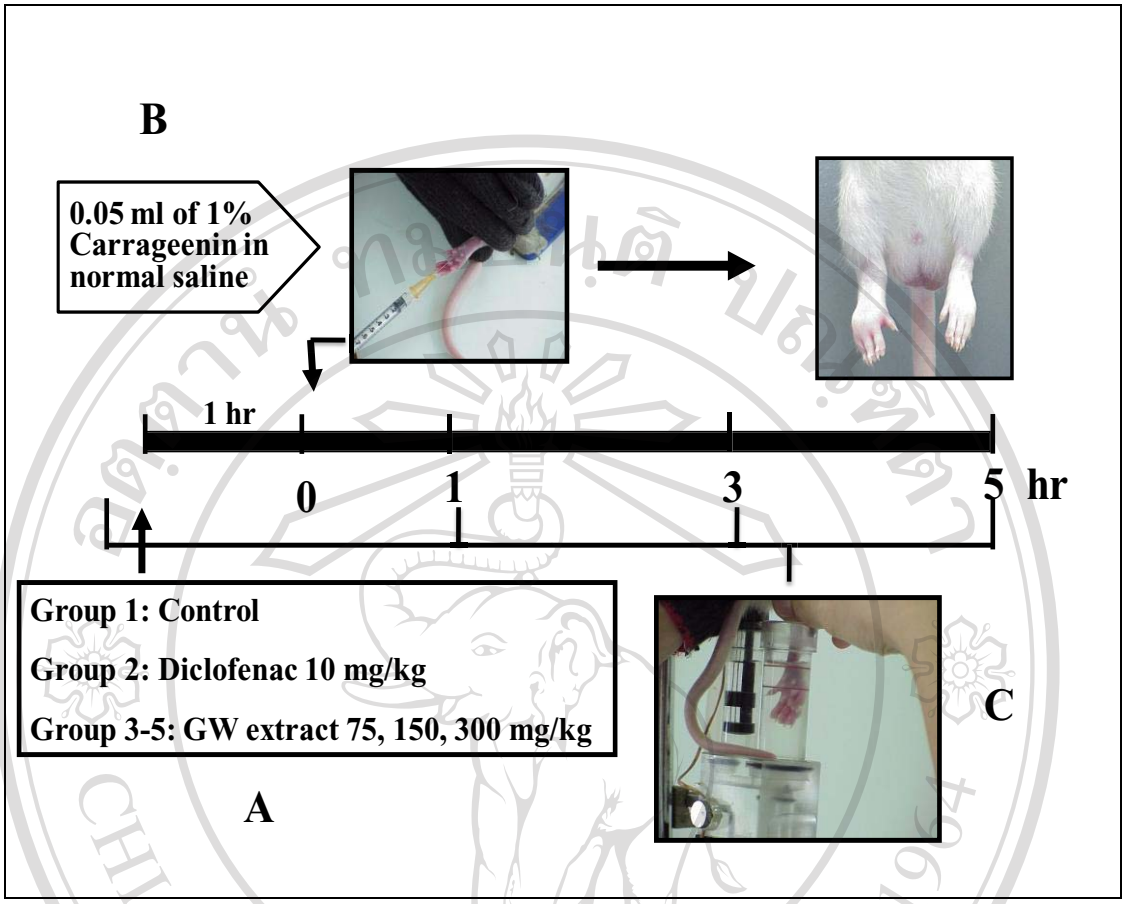


**Figure 5. Diagram illustrating the procedure of the carrageenin-induced hind paw edema in rats**.

A. The rats were pretreated orally with distilled water, diclofenac or the various doses of the GW extract 1 h prior to carrageenin injection.

B. Injection of 0.05 mL of 1% carrageenin in NSS into the right hind paw of the rats.

C. The paw volume was measured prior to and at 1, 3 and 5 h after carrageenin injection.

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**2.5.1.3 Arachidonic acid-induced hind paw edema in rats.**

The method was described by Di Martino *et al* (1987) [55]. This experiment was performed to investigate the inhibitory effect of test agents on the edema formation induced by AA. AA-induced hind paw edema in rats was a widely used method for evaluating the anti-inflammatory activity of LOX inhibitors and other agents with a mechanism of action different from COX inhibitors. 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 distilled water

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 the GW extract

Distilled water, diclofenac, prednisolone and various doses of the GW extract were administered orally 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 of the rats. Paw volume was measured using a plethysmometer (model 7150, Ugo Basile, Italy ) before and at 1 h after AA injection. Paw volume, edema volume and the percent edema inhibition of each test compound were determined similarly to the method described in the model of carrageenin-induced hind paw edema.

The diagram illustrating the procedure of the arachidonic acid-induced hind paw edema in rats is shown in Figure 6.

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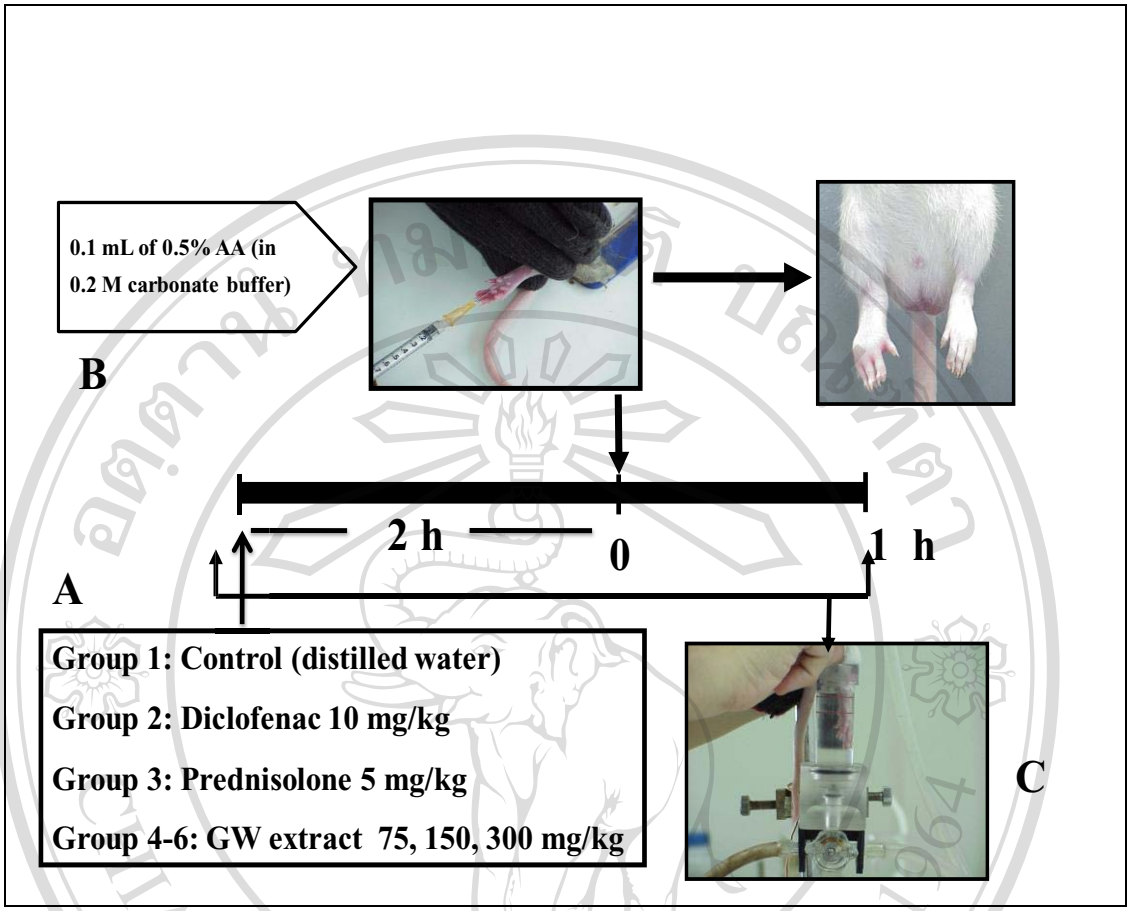


**Figure 6. Diagram illustrating the procedure of the arachidonic acid-induced hind paw edema in rats.**

A. The rats were pretreated orally with distilled water, diclofenac, prednisolone or the various doses of the GW extract 2 h prior to AA injection.

B. Injection of 0.1 mL of 0.5% AA (in 0.2 M carbonate buffer) into the right hind paw of the rats.

C. The paw volume was measured before and at 1 h after AA injection.

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**2.5.1.4 Cotton pellet-induced granuloma formation in rats**

The method was described by Swingle and Shideman (1972) [56]. This experiment was performed for investigation of the ability of an agent to inhibit the transudative and proliferative components of chronic inflammation. 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°C for 2 h. Male rats of 180-210 g body weight were used and divided into 4 groups of 6 animals.

Group 1 control group, received distilled water

Group 2 reference group, received 2.5 mg/kg/day of diclofenac

Group 3 reference group, received 5 mg/kg/day of prednisolone

Group 4 test group, received 300 mg/kg/day of GW extract

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 anesthesia and sterile technique. GW extract and prednisolone as well as diclofenac were administered orally in a once daily dosage regimen throughout the experimental period of 7 days whereas the control group was received distilled water only.

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**A. Measurement of the body weight gain**

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

**B. Measurement of the alkaline phosphatase activity in serum**

This method was described by Bessey *et al* (1946) [57]. The animals in the cotton pellet-induced granuloma formation model were used. On the 8$^{th}$ day after implantation, rats were anesthetized by a large dose of thiopental sodium (50 mg/kg, intraperitoneally). Blood was collected into a tube by cardiac puncture technique. Samples of serum were sent to Lanna Lab (129/255 Mahidol Rd., Muang, Chiang Mai) for determination of the alkaline phosphatase and the total protein. Measurement of the alkaline phosphatase activity in serum was calculated as follows:

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

**C. Measurement of the thymus weight [56]**

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

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**D. Measurement of granuloma weight and transudative weight [56]**

The implanted pellets were dissected out and carefully removed from the surrounding tissues and weighed immediately for the wet weight. Cotton pellets were dried at 60$^{o}$C for 18 h and their dry weight were determined. The changes in granuloma weight and transudative weight of the test group were compared with those of the control

group and the reference groups. The percent granuloma inhibition of the extract was calculated according to the following formulae:

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

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

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

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 determined 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 7.

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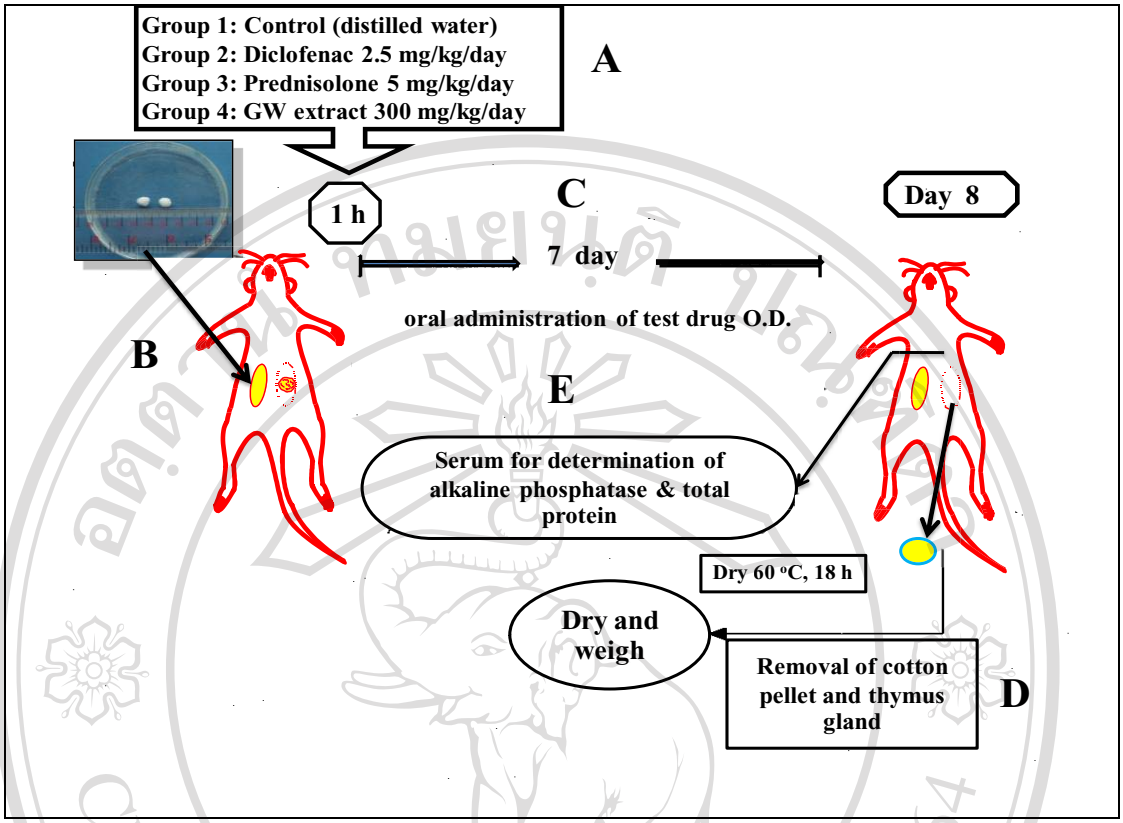


**Figure 7. Diagram illustrating the cotton pellet-induced granuloma formation in rats.**

A. The rats were pretreated orally with distilled water, diclofenac, prednisolone or 300 mg/kg of the GW extract 1 h prior to implanted cotton.

B. Two pellets of cotton wool weighing 20±1 mg were implanted subcutaneously, one on each side of the abdomen of the animal.

C. Test drugs were administered once daily throughout the experimental period of 7 days.

D. On the 8$^{th}$ day, the cotton pellets and thymus gland were removed.

E. Blood was collected into a tube for determination of the alkaline phosphatase and the total protein.

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### 2.5.2 Analgesic study

#### 2.5.2.1 Acetic acid-induced writhing response in mice

The method was described by Collier *et al* (1968) and modified by Nakamura *et al* (1986) [58, 59]. Acetic acid-induced writhing response in mice is used to screen for both peripherally and centrally acting analgesic activity. Male mice weighing 30-40 g were used and divided into 5 groups of 6 animals.

Group 1 control group, received distilled water

Group 2 reference group, received 10 mg/kg of diclofenac

Group 3-5 test groups, received 75, 150 and 300 mg/kg of the GW extract

Each mouse was given an injection of 0.75% acetic acid aqueous solution in a volume of 0.1 mL/10 g body weight into the peritoneal cavity and the animals were then placed in a transparent plastic box. The number of writhes, a 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 the acetic acid injection. Test drugs and control vehicle were administered orally 1 h before the acetic acid injection. Percentage of inhibition of writhing response was calculated according to the following formula:

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$$\%IW = \frac{NW\,of\,control\,group - NW\,of\,test\,group}{NW\,of\,control\,group} \times 100$$

%IW = Percent inhibition of writhing response

NW = Number of writhes

The diagram illustrating the procedure of the acetic acid-induced writhing response in mice is shown in Figure 8.

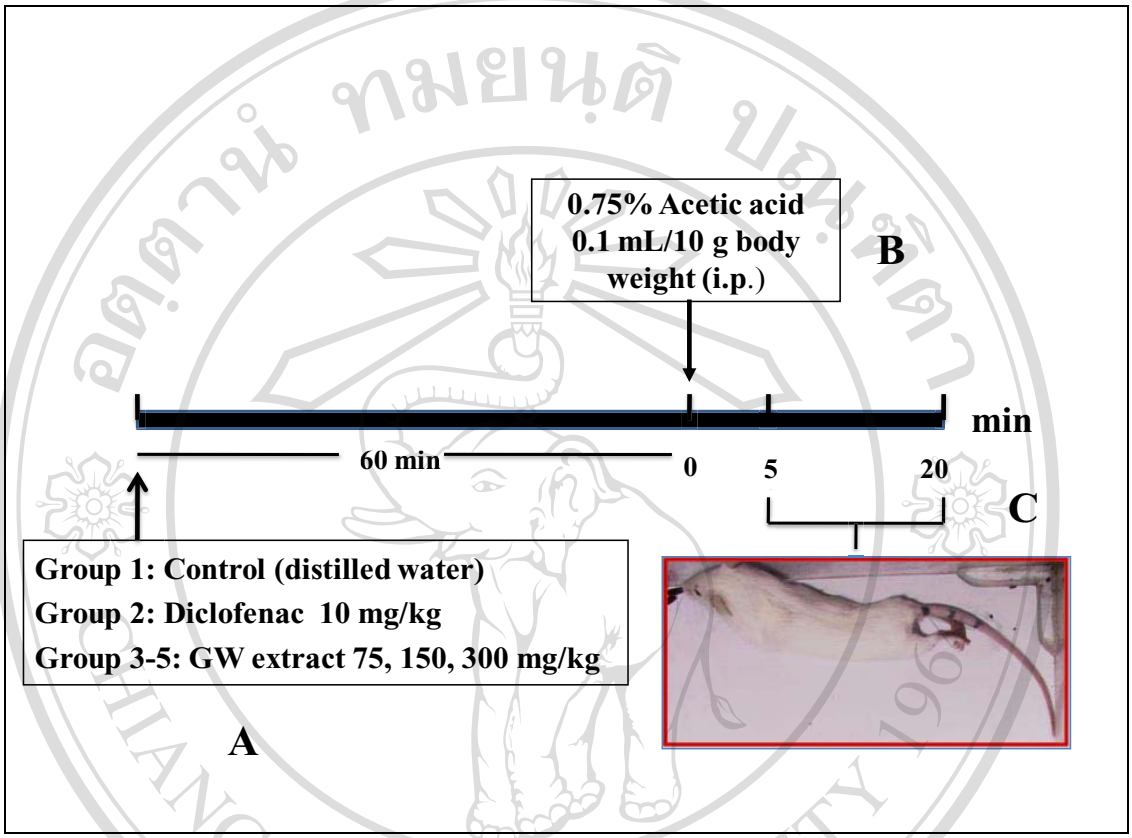


**Figure 8. Diagram illustrating the procedure of the acetic acid-induced writhing response in mice**.

A. The rats were pretreated orally with distilled water, diclofenac or the various doses of the GW extract 1 h prior to 0.75% acetic acid injection.

B. Injection of 0.1mL/10 g body weight of 0.75% acetic acid into the peritoneal cavity of the rats.

C. The number of writhes was counted during continuous observation for 15 min beginning from 5 min after the acetic acid injection.

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**2.5.2.2 Tail-flick test in rats**

The method was described by D'Amour and Smith (1941) and modified by Gray *et al* (1970) [60, 61]. The tail-flick test is widely used to investigate the centrally acting analgesic activity. Male rats weighing 180-200 g were used and divided into 6 groups of 6 animals.

Group 1 control group, received distilled water

Group 2 reference group, received 10 mg/kg of diclofenac

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

Group 4-6 test groups, received 100, 200, 300 mg/kg of the GW extract

The rat's tail was placed to cover 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. The apparatus was arranged so that when the operator turn on the lamp a timer was activated. When the rat felt pain and flicked its tail, light fell on the photocell then the timer was automatically stopped. The light intensity was adjusted to give a normal reaction time of 2-4 second. The cut-off time of 10 second was the maximum time which an unflicked tail can be exposed to the heat without damage. The control reaction time was first determined. The extract or drug was administered intraperitoneally immediately after this step, and 30 min later, the post drug reaction time was measured. The analgesic response was calculated as a percentage of the maximum possible response time.

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$$\% \text{ inhibition} = \frac{Tt - Tc}{10 - Tc} \times 100$$

Tt = test drugs reaction time (second)

Tc = control reaction time (second)

10 = cut-off time (second)

The diagram illustrating the procedure of the tail-flick test in rats is shown in Figure 9.

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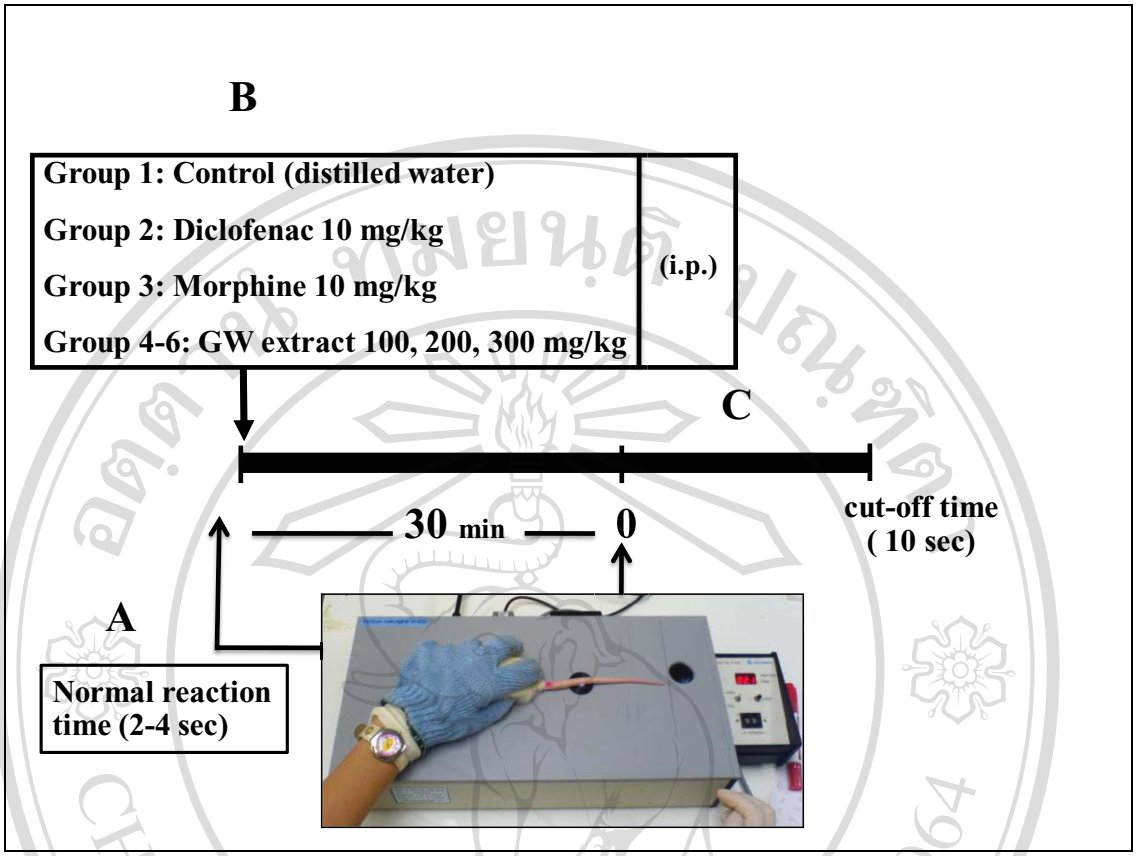


**Figure 9. Diagram illustrating the procedure of the tail-flick test in rats**.

A. The control reaction time was adjusted to give a normal reaction time of 2-4 sec.

B. The extract or drug was administered intraperitoneally immediately thereafter.

C. The post drug reaction time was measured at 30 min after drugs administration.

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### 2.5.3 Acute toxicity

The procedure was conducted according to the Organization of Economic Co-operation and Development (OECD) guidelines for testing of chemicals with slight modification [62]. Assessment of the acute toxicity is the first step in the toxicological investigations of an unknown substance. Female rats weighing 180-200 g were used and divided into 2 groups of 5 animals.

Group 1 control group, received distilled water

Group 2 test group, received 3000 mg/kg of the GW 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 and then once daily for 14 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 experimental period were autopsied and gross pathological changes of the internal organs (heart, lungs, liver, spleen, kidneys, adrenals, ovaries, uterus, thymus, brain, eyes, stomach, intestines, etc.) were recorded. The surviving rats were sacrificed on the 15$^{th}$ 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.

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## 2.6 Drugs and chemicals

### Drugs

1. Diclofenac (Sigma Chemical Company, St. Louis, U.S.A.)
2. Morphine (T.P. Drug Laboratories, Thailand) Soi Sukhumvit 62, Prakhanong 10110, Bangkhae, Bangkok, Thailand
3. Phenylbutazone (Sigma Chemical Company, St. Louis, U.S.A.)
4. Prednisolone (Scherisone$^{\supset}$, Schering, Bangkok Ltd, Thailand)

### Chemicals

1. AA (Sigma Chemical Company, St. Louis, U.S.A.)
2. Acetic acid (The Government Pharmaceutical Organization, Bangkok, Thailand)
3. EPP (Fluka Chemicals Co., Ltd., Japan)
4. Lambda carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

## 2.7 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 using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test and the *P* values of less than 0.05 were considered significant.

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