# CHAPTER 3

# MATERIALS AND METHODS

## Materials

### 1. Plant material

The fresh leaves of *Piper sarmentosum* Roxb. (40 kg) were collected from Ranod district, Songkla Province, Thailand in March 2005. The plant material was identified by Assist. Prof. Choatip Purintaworakul, Botany Section, Department of Biology, Prince of Songkla university. All Leaves were cleaned with tap and distilled water, respectively and dried at room temperature (air-dried). The 40 kg fresh leaves gave 9 kg air-dried leaves. The dried leaves were pulverized to give powder of weighing 6 kg by the electric blender, and stored in an airtight container.

### 2. Extraction procedure

*Piper sarmentosum* Roxb. leaves powder (6 kg) was extracted using cold extraction by macerating in 20 liters of methanol for 7 days at room temperature and repeated for 2 times. The total extract was filtered through Whatman® No. 40 filter paper with suction. After filtration, the extract was evaporated by water bath at 40 ºC and Rotavac evaporator (Buchi®) at 40-45 ºC, under reduced pressure condition at about 200-300 mbar, which produced by B169 Water Pump (Buchi®), respectively. The evaporated extract gave the 480 ml black viscous, oil-like mixture. The mixture obtained was lyophilized by freeze-dried to give a solid residue of 270 g (4.5 % w/w yield). Methanol extract of *Piper sarmentosum* leaves (MEPS) was stored in a bottle and kept away from moisture and kept in a refrigerator at below 4 ºC. Just prior to the use of the extract for the pharmacological experiments, the extract was suspended in distilled water to give a desired concentration for testing (see appendix 1 and 2).

### 3. Animals

Male albino mice with weight ranging from 28-40 g were used for analgesic activities test; hot plate test, writhing test and formalin test. Male Wistar rats with weight ranging from 140-170 g were used for another analgesic activity test, tail flick test; and for anti-inflammation and antipyretic activity studies. The test animals were kept in the room maintained

environmentally controlled conditions of 26 ºC and 12 h light/dark cycle. Food and water were given *ad libitum* unless otherwise specified. All animals used in this study were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkla, Thailand. All procedures described were reviewed and approved by the Institutional Committee for Ethical Use of Animals, Prince of Songkla University, Hat Yai, Thailand. (ศธ. 0521.11/328)

**4. Chemicals and Instruments**

The following drugs were used : Morphine sulfate (Sigma), Naloxone hydrochloride dihydrate (Sigma), Brewer's yeast (Sigma), Carrageenan (Sigma), Acetylsalicylic acid or Aspirin (Sigma), Acetic acid and 40% Formaldehyde (A.R. grade, J.T., Baker) and Methanol for extraction (Commercial grade, KSP).

Tail flick apparatus (Socrel model DS20) and Plethysmometer manufactured by Ugo Basile, Italy; the hot plate (Harvard) and digital thermometer (9SK-1250 MC, Sato Keiryoki mfg Co., Ltd. Japan) were used in this experiment.

**5. Drug and Extract Administration** (Agent preparation see appendix B)

The distilled water, extract and standard drug aspirin were administered by oral gavage. Animals were divided into 5-8 groups, and each group comprised of 10 animals of mice or rats.

| Group | Name group | Details |
|---|---|---|
| 1 | Control | Distilled water at the amount of 10 ml/kg (mice), 5 ml/kg (rats) |
| 2, 3 | Standard drug | 1) Aspirin at the dose of 200 mg/kg (writhing test, formalin test, anti-inflammation test and antipyretic test)<br>2) Morphine sulfate at the dose of 5 mg/kg, subcutaneously (hotplate test, tail flick test and formalin test)<br>3) Naloxone at the dose of 2 mg/kg, intraperitoneally (hot plate test and tail flick test) |
| 4, 5, 6 | MEPS | Methanol Extract of *Piper sarmentosum* from leaves (MEPS) at doses of 50, 100, 200 mg/kg, respectively. |
| 7, 8 | Antagonist effect | 1) Naloxone at the dose of 2 mg/kg given intraperitoneally before administration of morphine at dose of 5 mg/kg, subcutaneously (hot plate test and tail flick test) |

| | | |
|---|---|---|
| | | 2) Naloxone at the dose of 2 mg/kg given intraperitoneally before administration of MEPS at doses of 200 mg/kg (hot plate test and tail flick test) |

## Methods

### 1. Acute toxicity

The up-and-down method was used to study the acute toxicity which described by Bruce (1985). The dose was adjusted by a constant multiplicative factor, viz. 1.5, for the experiment. The methanol extract of *Piper sarmentosum* leaves at the dose of 5 g/kg was orally administered to a group of both male and female mice (each sex consists of 10 mice). Behavior parameters were observed during a period of 8 hours and 7 days after administration such as convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration. Food and water was given *ad libitum*.

### 2. Antinociceptive Activity test

#### 2.1. Hot plate test

The hot plate test was carried out according to the method described by Woolfe and MacDonald (1944). Male albino mice weighting 28-40 g were divided into 8 groups (each group consisted of 10 mice).

Group 1 : Control group : distilled water (10 ml/kg, p.o.)

Group 2 : Morphine (5 mg/kg, s.c.)

Group 3 : Naloxone (2 mg/kg, i.p.)

Group 4-6 : MEPS (50, 100 and 200 mg/kg, p.o. respectively)

Group 7 : Naloxone (2 mg/kg, i.p.) 10 minutes before Morphine (5 mg/kg, s.c.)

Group 8 : Naloxone (2 mg/kg, i.p.) 10 minutes before MEPS (200 mg/kg, p.o.)

After 30 minutes (15 minutes for morphine and 10 minutes for naloxone), mice were placed on a hot plate maintained the temperature at 55 ºC ± 1 ºC. Latency of nociceptive responses such as licking, flicking of a hind limb or jumping was measured with cut-off time of 45 seconds. The nociceptive responses were measured at 30, 45, 60, 75 and 90 minutes after agent

administration. Only the mice that showed nociceptive responses within 15 seconds were selected to use for the experiments.

**2.2. Tail flick test**

The tail flick test used in this experiment was described by D'Amour and Smith (1941). Male Wistar rats weighting 140-170 g were divided into 8 groups (each group consisted of 10 rats).

Group 1 : Control group : distilled water (5 ml/kg, p.o.)

Group 2 : Morphine (5 mg/kg, s.c.)

Group 3 : Naloxone (2 mg/kg, i.p.)

Group 4-6 : MEPS (50, 100 and 200 mg/kg, p.o. respectively)

Group 7 : Naloxone (2 mg/kg, i.p.) 10 minutes before Morphine (5 mg/kg, s.c.)

Group 8 : Naloxone (2 mg/kg, i.p.) 10 minutes before MEPS (200 mg/kg, p.o.)

After 30 minutes (15 minutes for morphine, 10 minutes for naloxone), rats were placed on the tail flick apparatus (Socrel model DS20, Ugo basile), held gently by the experimenter with their tails smoothed into the tail groove. The reaction time was measured by focusing an intensity controlled beam of light on the distal one-third portion of the animal tail (about 4 centimeters from tip). When the rat felt pain and flicked its tail, the time was stopped with cut-off time was 10 seconds. The nociceptive responses were measured at 30, 45, 60, 75 and 90 minutes after agent administration. Only the rats that showed nociceptive responses within 3 seconds were used for the experiments. The schematic plan of the hot plate and tail flick test experiments were shown by the below diagram in Figure 14.

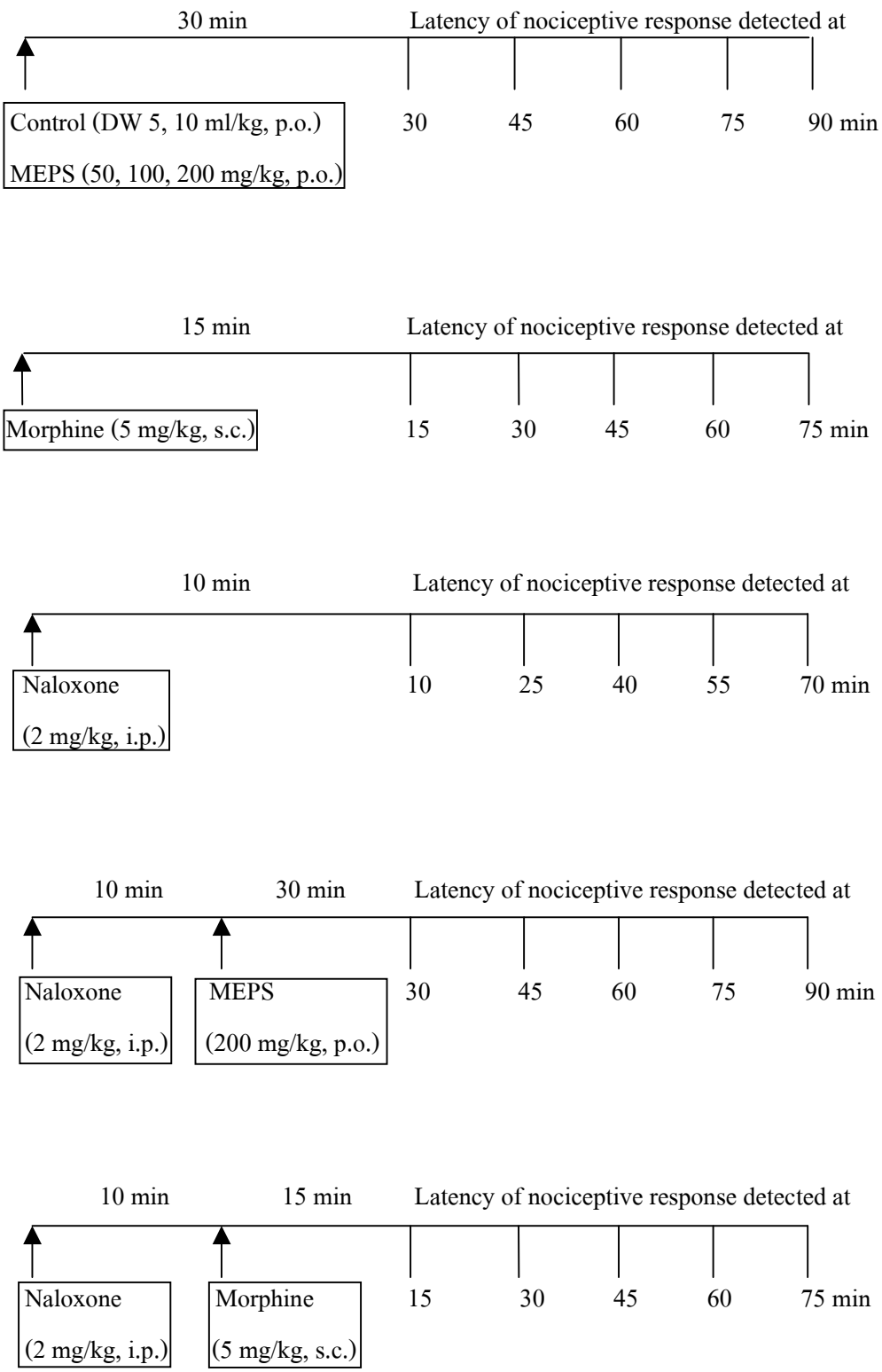


**Figure 14. Schematic plan of the hot plate and tail flick tests**

**2.3. Writhing test**

The method was described by Koster *et al.* (1959). Male albino mice weighing 28-40 g were divided into 5 groups (each group consisted of 10 mice).

Group 1 : Control group : distilled water (10 ml/kg, p.o.)

Group 2 : Aspirin (200 mg/kg, p.o.)

Group 3-5 : MEPS (50, 100 and 200 mg/kg, p.o. respectively)

After 30 minutes, 0.6% acetic acid in 0.9% normal saline (10 ml/ kg bodyweight) was injected intraperitoneally and the number of writhing and stretching was counted over a 20-minute period. The schematic plan of the writhing test was illustrated in Figure 15.

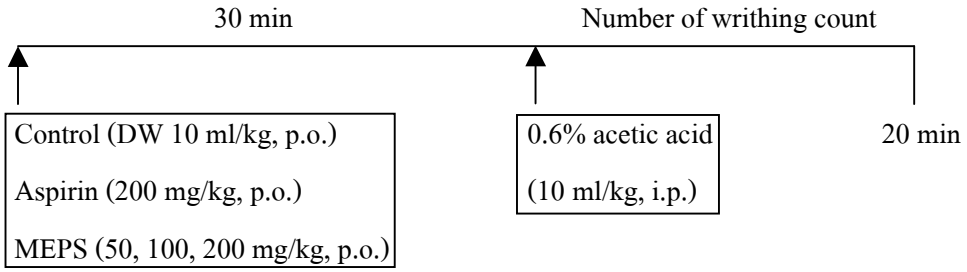


**Figure 15. Schematic plan of the writhing test**

**2.4. Formalin test**

The formalin test was performed according to the method described by Hunskaar *et al.* (1985). Male albino mice weighing 28-40 g were divided into 6 groups (each group consisted of 10 mice).

Group 1 : Control group : distilled water (10 ml/kg, p.o.)

Group 2 : Morphine (5 mg/kg, s.c.)

Group 3 : Aspirin (200 mg/kg, p.o.)

Group 4-6 : MEPS (50, 100 and 200 mg/kg, p.o. respectively)

After 30 minutes, 20 µl of 2.5% formalin in 0.9% normal saline was injected subcutaneously to a hindpaw. The time spent licking the injected paw was recorded and the data were expressed as total licking time in the early phase (0-5 minutes) and the late phase (15-30 minutes) after formalin injection. The schematic plan of the formalin test experiment was shown in Figure 16.

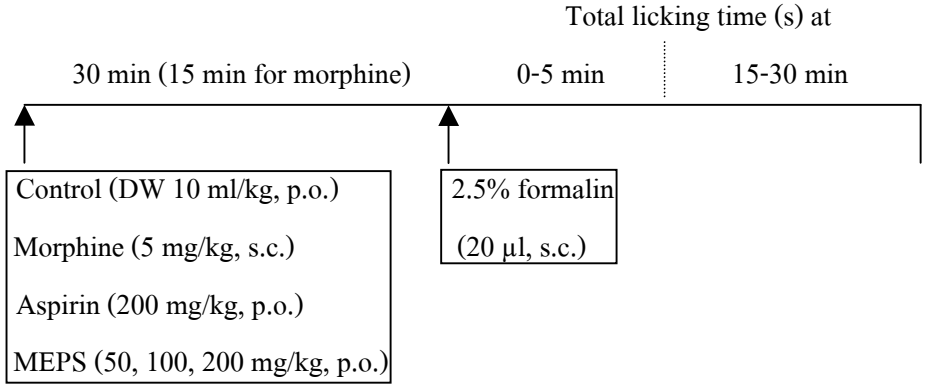


**Figure 16. Schematic plan of the formalin test**

**3. Anti-inflammatory activity**

The anti-inflammatory activity was studied according to the method described by Winter *et al.* (1962). The initial right hind paw volume of the rats was measured and recorded using a Plethysmometer (Ugo Baseline). Male Wistar rats weight ranging from 140-170 g were divided into 5 groups (each group consisted of 10 rats).

Group 1 : Control group : distilled water (5 ml/kg, p.o.)

Group 2 : Aspirin (200 mg/kg, p.o.)

Group 3-5 : MEPS (50, 100 and 200 mg/kg, p.o.)

After 30 minutes, 0.1 ml of 1 % (w/v) carrageenan in 0.9% normal saline was subcutaneously injected into the subplantar region of the right hindpaw. The volume of right hind paw was measured at 0.5, 1, 2, 3, 4 and 5 hour after carrageenan injection and hindpaw volume was determined. The data were expressed as percentage of swelling compared with the initial hind paw volume of each rat. The schematic plan of the carrageenan-induced paw edema test was illustrated in Figure 17.

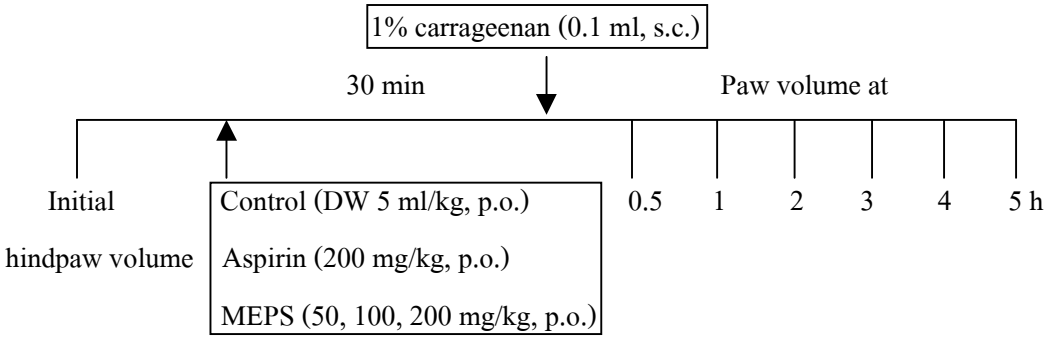


**Figure 17. Schematic plan of the carrageenan-induced paw edema test**

**4. Antipyretic activity**

The antipyretic activity was performed by minor modifying method described by Adam *et al.* (1968). Male Wistar rats were fasted overnight with water *ad libitum* before experiments. Pyrexia was induced by subcutaneously injecting 20% (w/v) brewer's yeast suspension (10 ml/kg) into animal's dorsal region. Seventeen hours after injection, the rectal temperature of each rat was measured using a digital thermometer. The probe was attached to a digital display and was inserted 2 cm into the rectum. Only rats that showed an increased in temperature of at least 0.7 ºC were used for the experiments, an initial rectal temperature was recorded. Male Wistar rats weighing 140-170 g were divided into 5 groups (each group consisted of 10 rats).

Group 1 : Control group : distilled water (5 ml/kg, p.o.)

Group 2 : Aspirin (200 mg/kg, p.o.)

Group 3-5 : MEPS (50, 100 and 200 mg/kg, p.o. respectively)

The rectal temperature was measured at 1, 2, 3, 4 and 5 hour after agent administration. The schematic plan of antipyretic study was shown in Figure 18.

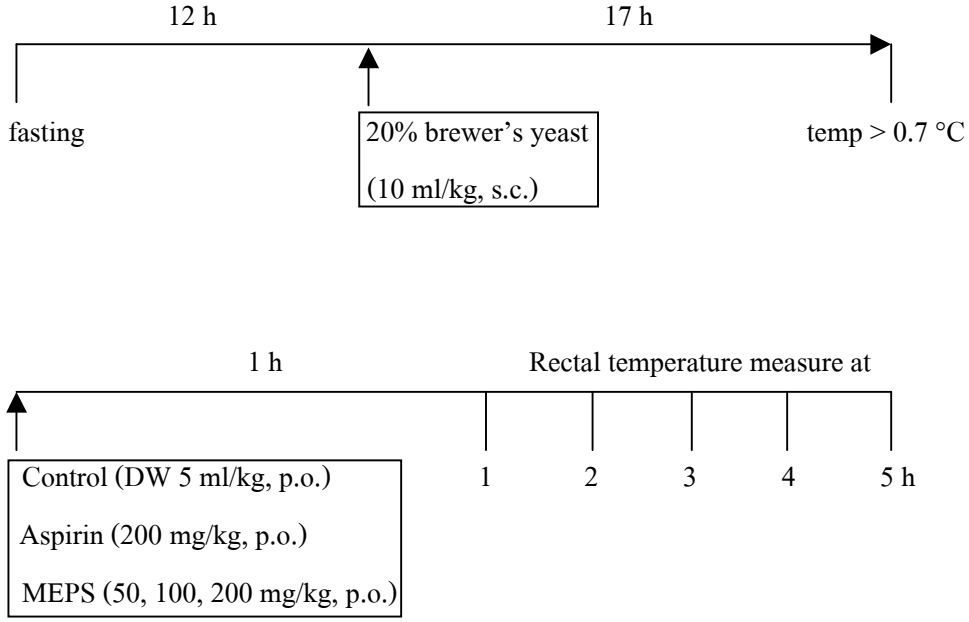


**Figure 18. Schematic plan of the brewer's yeast-induced pyrexia test**

**Statistical analysis**

All experimental groups were consisted of 10 animals. The data obtained using SPSS software version 10.0 and expressed as mean ± S.E.M. Statistical significance between groups was performed by the application of analysis of variance (ANOVA) followed by Bonferroni's test and independent *t*- test for compared between 2 groups. P values less than 0.05 ($P$ <0.05) were used as the significant level.