CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemical substances

- Ethyl acetate (Lab-scan Asia, Thailand)
- Formaldehyde (FORMALIN®, Vidhyasom, Thailand)
- Hexane (Lab-scan Asia, Thailand)
- Methanol (Lab-scan Asia, Thailand)
- Polyoxyethylene (20) sorbitan monolaurate (TWEEN 20®, Ajax fine chem, Australia)
- Sodium pentobarbital (NEMBUTAL®, SANOFI, France)

1.2 Instrument

- Battalian
- Black silk no. 3-0 (B/BRAUN AESCULAP AG Co.)
- Hot plate 3.5 x 4.6 cm (width x length)
- Surgical blade no. 23
- Tensiometer (EZ-TEST I 30804100798, SHIMADZU Corporation, Japan)
- Thermometer
- Transparent polythene paper (graph paper)
- Vernier caliper
- Others: operation set, syringes, needles, plaster

1.3 Experimental animals

A total of 112 male Sprague Dawley rats weighing 250-300 grams purchased from The National Laboratory Animal Center, Salaya, Mahidol University, Bangkok were used in this study. The animals were divided into two groups of 56 animals for incision wound and burn wound models. In each group the animals were subdivided into seven subgroups of eight animals each. The rats were caged in the air-conditioned room maintained temperature at $25\pm1^{\circ}C$. They were fed with commercial pellet diet CP mice fed, Pokphand Animal Fed Co, Ltd. Bangkok, Thailand. They were provided with food and water *ad libitum*. The rats were used after acclimatization to the laboratory environment for a 7-day period.

2. Methods

2.1 Preparation and extraction of CA

2.1.1 Plant materials

The trunks of CA were collected from Nongdindang district, Nakornpathom province. It was cleaned, dried, powdered and used for sequential extraction. The extract was prepared into 4 fractions performed by Associate Professor Dr. Rutt Suttisri and Mr. Chutichot Mungmee, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The plant extracts were tested for quality specification using thin layer chromatography (TLC).

2.1.2 Extracts and standard used

The plant was shade dried and powdered. Then the powdered plant materials were used for extraction. First, the powdered plant materials were soaked in hexane 3 times for 5 days each to yield the hexane extract (Fraction 1, F_1). Next, the rubbish from the first step was soaked in ethyl acetate 3 times for 5 days each to yield the ethyl acetate extract (Fraction 2, F_2). Then, the rubbish from the second step was soaked in methanol 3 times for 5 days each to yield the methanol extract (Fraction 3, F_3). Finally, the rubbish from the third step was soaked in water 3 times for 5 days each to yield the water extract (Fraction 4, F_4). The steps of extraction are shown in the following diagram (Figure 3.1).

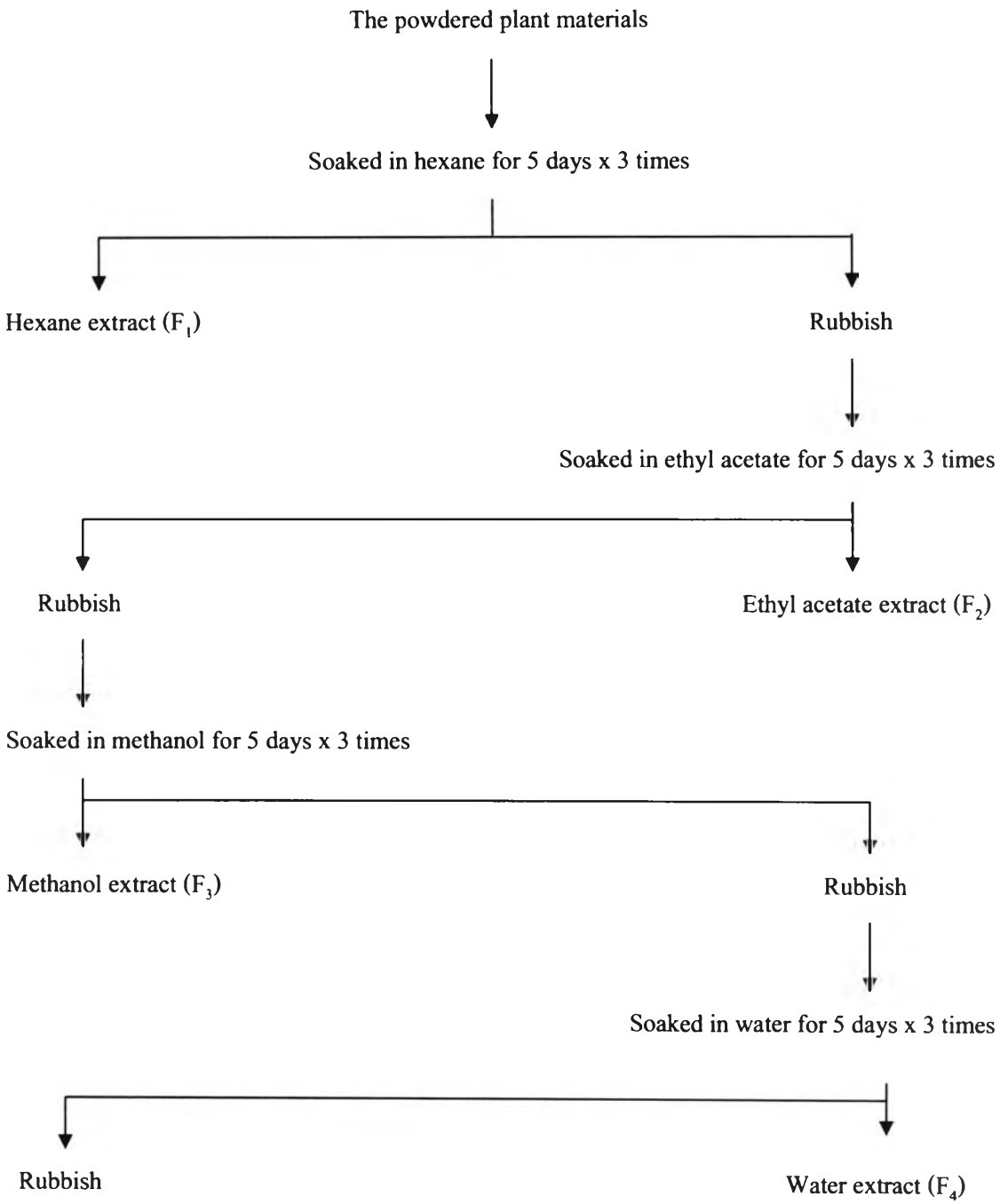

Figure 3.1 Diagram illustrating steps of extraction

2.1.3 Characteristic feature of CA extracts

Characteristic features of CA extracts were tested by thin layer chromatography using silica gel 60 F_{254} plate (GF_{254}). The solvent system of each extract are as follows:

- Hexane extract: chloroform : acetone (9 : 1)
- Ethyl acetate extract: chloroform : methanol (9 : 1)
- Methanol extract: ethyl acetate : methanol : water (4 : 0.4 :0.2)
- Water extract: ethyl acetate : methanol : water (4 : 0.4 : 0.3)

TLC plate of each extract was sprayed with 10% sulfuric acid in ethanol and then heated by hot plate which maintained temperature at 100 °C for 15 min.

2.1.4 Preparation of CA

The concentration of CA extracts used in this study was 10% (w/v) in 10% Tween 20® (v/v) in freshly prepared solution. First, 1 ml of Tween 20® was dissolved in 9 ml of distilled water to obtain 10% (v/v) Tween 20, and then 1 mg of the extract was dissolved in 9 ml of prepared 10% Tween 20 volume was adjusted to obtain 10% (w/v) of the extract for using topically in the experiment.

2.2 Evaluation of wound healing

2.2.1 Tensile strength test for incision wound

The tensile strength of incision wounds was measured on day 7 post wounding. The sutures were removed and the skin tissue was cut in size of 1 cm away from each side of wound as shown in figure 3.2. The isolated wound tissues were used to measure the load (force) to break the tissue with a computerized tensiometer (Figure 3.3 and 3.4) and tensile strength was calculated by the following formula (Saringat and Wasim, 1995).

$$\text{Tensile strength } (N/cm^2) = \text{Breaking load (force)} / \text{Area } (cm^2)$$

$$\text{Area } (cm^2) = \text{Thickness (cm)} \times \text{Width (cm)}$$

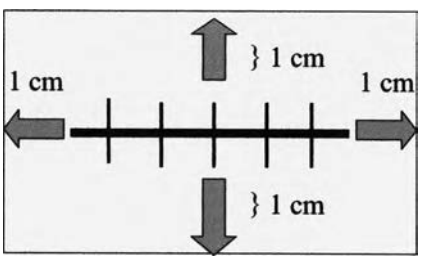

Figure 3.2 Diagram illustrating the preparation of tissue for the determination of tensile strength.

Figure 3.3 The tensiometer

Figure 3.4 The tissue holder

2.2.2 Gross pathology evaluation for burn wound

2.2.2.1 Wound lesion

The lesion of wounds was grossly examined on day 3, 7, 10, and 14 post burning. The wounds were examined by the following criteria such as wound bed, color, exudates, swelling of wound surface and the consistency of surrounding wound tissue.

2.2.2.2 Wound area

On day 3, 7, 10, and 14 post burning the color photographs of the wounds were taken by digital camera. The areas of wound were measured by tracing the wound boundaries using a millimeter scale transparent graph paper with permanent marker and the degree of wound healing were calculated using the following formula (Reddy et al., 2002):

The degree of wound healing (%) = 1- [wound area on corresponding day (cm^2) / wound area on zero day (cm^2)] x 100

2.2.3 Histopathological evaluation

The specimen of skin, 0.5x0.5 cm in size, was taken from the middle of burn area. The tissues were preserved in the fresh fixative aqueous 10% neutral buffered solution of formaldehyde for at least 24 hrs. Sections were stained with hematoxylin and eosin dyes. The light microscope (Nikon 516609) with x20 and x40 objective lens was used.

3. Experimental protocol

There were two experimental protocols as follows:

3.1 Effects of the CA extract on healing of incision wound

The effect of CA extract on incision wound was investigated using the model of Baie and Shiekh (2000). The animals were anesthetized with sodium pentobarbital 60 mg/kg body weight, intraperitoneally. The right side on the back of each animal was shaved and depilated. Then, a midline incision 3 cm was made through the whole skin thickness with a sharp scalpel. Each incised wound was closed with 0.5 cm spaced interrupted silk sutures to secure the edges of the incision wound (Figure 3.5, 3.6). After wounding, test substances as described below were topically applied to the animals once daily. They were housed for a week with free access to water and standard laboratory chow. On the day 7 post wounding the animals were sacrificed with sodium pentobarbital 100 mg/kg body weight, intraperitoneally. Then the sutures were removed and the tissue was isolated from the healed wound for the measurement of tensile strength. Fifty-six animals were included in this experiment and divided into seven groups of eight animals each as follows:

- Group 1, untreated group	no treatment
- Group 2, NSS- treated group	0.5 ml of NSS
- Group 3, Tween 20- treated group (TW) (vehicle control group)	0.5 ml of 10% Tween 20 (v/v)
- Group 4, hexane extract- treated group (HE)	0.5 ml of 10% hexane extract (w/v)
- Group 5, ethyl acetate extract- treated group (EE)	0.5 ml of 10% ethyl acetate extract (w/v)
- Group 6, methanol extract- treated group (ME)	0.5 ml of 10% methanol extract (w/v)
- Group 7, water extract- treated group (WE)	0.5 ml of 10% water extract (w/v)

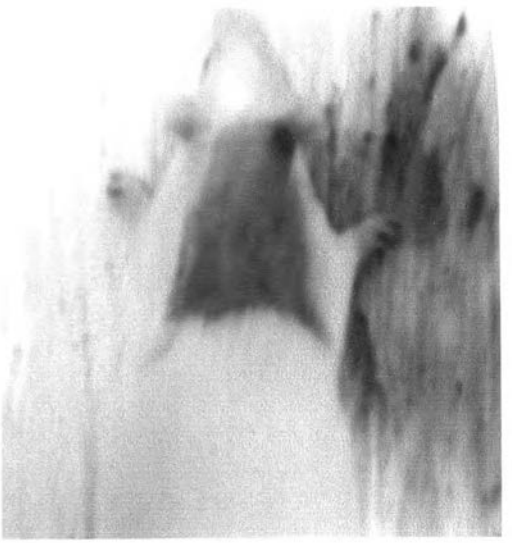

Figure 3.5 The area prepared for wounding

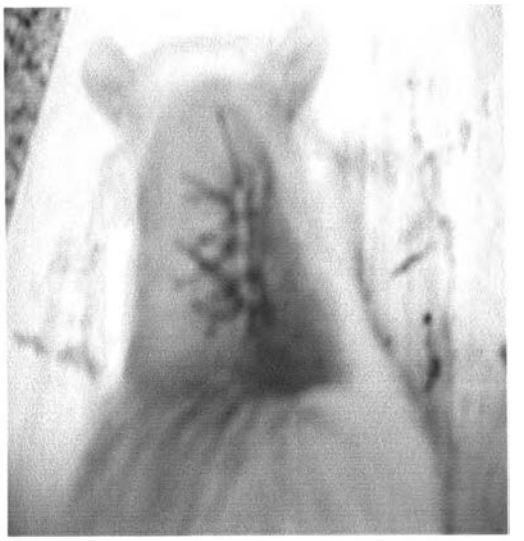

Figure 3.6 The sutured skin

3.2 Effects of the CA extract on healing of burn wound

The effect of CA extract on burn wound was investigated using the method described by Somboonwong et al. (2000) which was modified from Zawacki (1974). The animals were anesthetized with sodium pentobarbital 60 mg / kg body weight, intraperitoneally. The back of animal between the lower parts of both scapulas was shaved and depilated. Then second degree burn was made by putting the hot plate [3.5 x 4.6 cm (width x length)] (Figure 3.7) with temperature of 75 ^{o}C on the prepared area for 10 seconds. The burned area was about 10% of total body surface area. The burned area of each animal was measured immediately after burning and on day 3, 7, 10, 14 post burning using a millimeter scale graph paper and degree of wound healing was calculated as previously mentioned. The animals were housed with free access to water and standard laboratory for two weeks in which the wounds were treated daily with one of the test substances topically. On day 14 post burning, the animals were sacrificed with sodium pentobarbital 100 mg/kg body weight, intraperitoneally. Then a specimen sample of tissue in the healed wound (0.5 x 0.5 cm) was isolated from each animal for histopathological examination. Fifty-six animals were included in this experiment and they were divided into seven groups of eight animals each as follows:

- Group 1, untreated group	no treatment
- Group 2, NSS- treated group	0.5 ml of NSS
- Group 3, Tween 20- treated group (TW) (vehicle control group)	0.5 ml of 10% Tween 20 (v/v)
- Group 4, hexane extract- treated group (HE)	0.5 ml of 10% hexane extract (w/v)
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- Group 7, water extract- treated group (WE)	0.5 ml of 10% water extract (w/v)

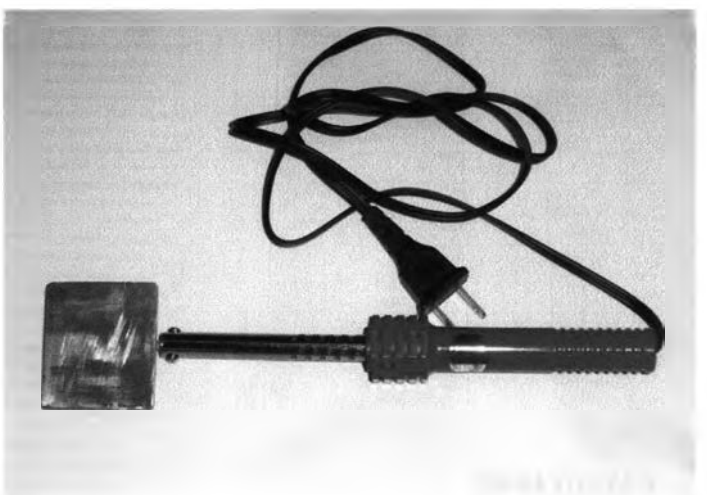

Figure 3.7 The hot plate

4. Statistical analysis

Results are presented as mean ± S.E.M. The differences among experimental groups were compared by one-way ANOVA followed by Least significant different test (LSD) and were considered statistically significant when P was less than 0.05.