CHAPTER 2
MATERIALS AND METHODS

2.1 Plant material and plant extract

C. serratum was collected from Phujong–nayoy national park, Ubonratchathani province in April, 2006. The methanol extract of *C. serratum* was kindly prepared and 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). The method of extraction was briefly as follows: Dried, grounded whole plant of *C. serratum* (5.5 kg) was percolated with methanol (10 x 13 L). After filtration and solvent was removed using rotary evaporator yielded the methanol extract 697 g (12.7% w/w).

2.2 Experimental animals

Male Swiss albino mice weighing 30–40 g and male Sprague-Dawley rats weighing 40–60 g and 100–120 g were used. All animals were kept in a room maintained under environmental control conditions of 24 ± 1 ºC, relative humidity 50 ± 10 %, and a 12 h light–dark cycle. All animals had free access to water and food. 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.

In the *in vivo* testing, all test drugs were dissolved in normal saline solution (NSS), except in the ear edema model, where they were dissolved in acetone. In the *in vitro* experiment, test drugs were dissolved in Krebs' solution.

2.4 Drugs administration

For the anti-inflammatory and analgesic experiments, all test drugs were orally administered in an equivalent volume of 0.5 mL/100 g body weight of the rats and all test drugs were intraperitoneal (i.p.) administered in an equivalent volume of 0.1 mL/10 g body weight of the mice, except in the ear edema model when a local application of test drug to outer and inner surfaces of the ear was performed. In the *in vitro* experiment, test drugs were added into the organ bath in the volume not exceeded 10% of the physiological solution in the chamber.

2.5 Experiment protocol

Part I

Anti-inflammatory and analgesic activity of the methanol extract from *C. serratum* Linn.

2.5.1 Anti-inflammatory model

2.5.1.1 Ethyl phenylpropiolate (EPP)–induced rat ear edema

The method described by Brattsand *et al* (1982) [48] was performed. 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 EPP. Edema was the cardinal sign of acute inflammation. Male rats of 40-60 g body weight were used and divided into 4 groups of 3 animals per group (6 ears).

Group 1 The control group, received acetone

Group 2 The reference group, received 5 mg/20 µL/ear of diclofenac

Group 3 The reference group, received 4 mg/20 µL/ear of Daflon®

Group 4 The test group, received 2 mg/20 µL/ear of the CS extract

EPP was used as an irritant and was dissolved in acetone at a concentration of 50 mg/mL. 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. The CS extract as well as reference drugs, diclofenac and Daflon®, were dissolved in acetone and applied topically in a volume of 20 µL to the inner and outer surfaces of the ear by means of an automatic microliter pipet just before the irritant. The control group received only vehicle in the same volume. The thickness of each ear was measured with the vernier caliper before and at 15, 30, 60

and 120 min after edema induction. The increase in the ear thickness was compared with 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 at time X

ET_X = ear thickness (μm) at time X

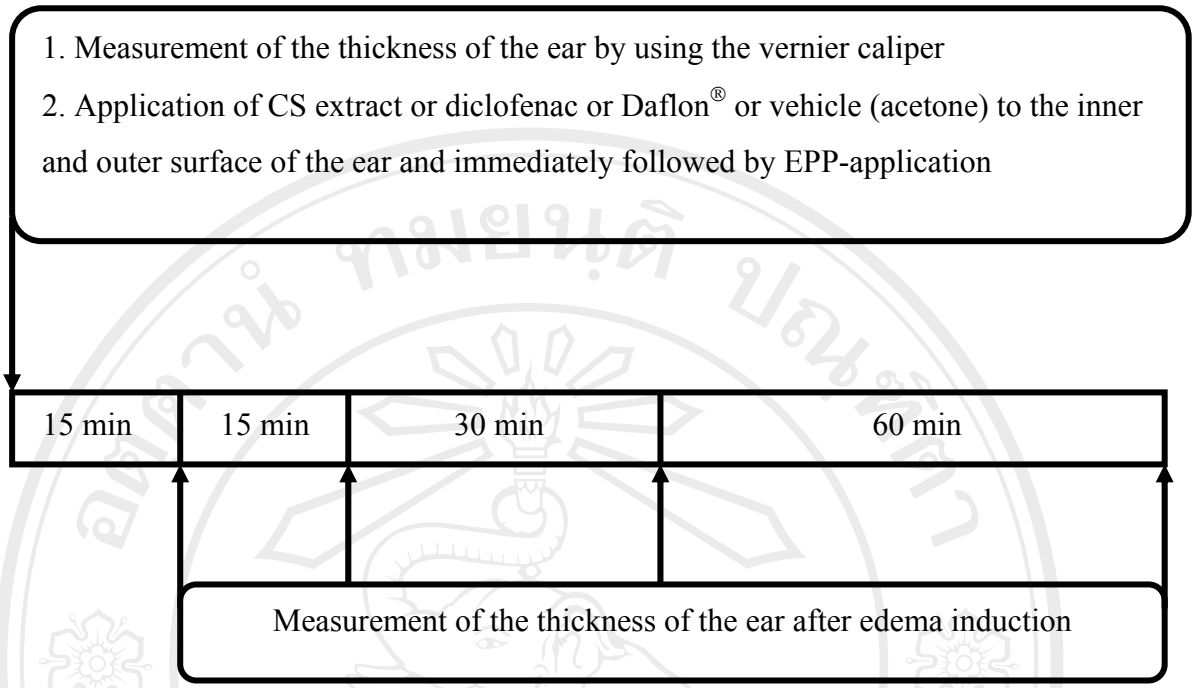
ET_O = ear thickness (μm) before application of EPP

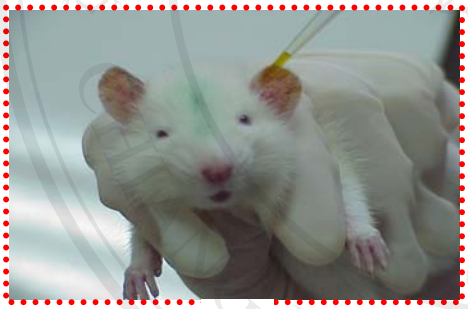
ED_C = edema thickness (μm) of control group at time X

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

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

The diagram illustrating the procedure of the EPP-induced ear edema is shown in Figure 3 below.





A

B

C

Figure 3. 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: Measurement of the thickness of the ear by using the vernier caliper.

C: Vernier caliper.

2.5.1.2 Carrageenin–induced hind paw edema in rats

The method described by Winter *et al* (1962) [49] was used. This method was performed to investigate the inhibitory effect of agents on the edema formation of the rat paw induced by carrageenin. The edema was produced by a sequential release of pharmacological mediators; histamine, serotonin, bradykinin and PGs. The test was excellent for detecting inhibitors of COX. Male rats of 100-120 g body weight were used and divided into 6 groups of 6 animals per group.

Group 1	The control group, received NSS
Group 2	The reference group, received 10 mg/kg of diclofenac
Group 3	The reference group, received 300 mg/kg of Daflon®
Group 4–6	The test groups, received 40, 80 and 160 mg/kg of the CS extract, respectively

Groups of rat were pretreated orally with vehicle (NSS), diclofenac, Daflon® or the various doses of the CS extract 1 h prior to the carrageenin injection. Acute inflammation was produced by subplantar administration of 0.05 mL of 1% carrageenin in sterile NSS into the right hind paw of the rat. Paw volume of animal was determined by means of a volume displacement technique using a plethysmometer (model 7150, Ugo Basile, Italy). The right hind paw was immersed into the measuring chamber containing 0.05% NaCl in distilled water, exactly to an ink mark at anatomical hair line. Each paw volume was obtained from the average of 3 readings. The paw volume was measured prior to and at 1, 3 and 5 h after carrageenin injection. The edema volume by the paw and the percent edema inhibition of each test compound were obtained by the following calculation:

$$EV_X = PV_X - PV_O$$

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

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

Figure 4 shows the diagram of the water displacement method for measuring rat paw volume.

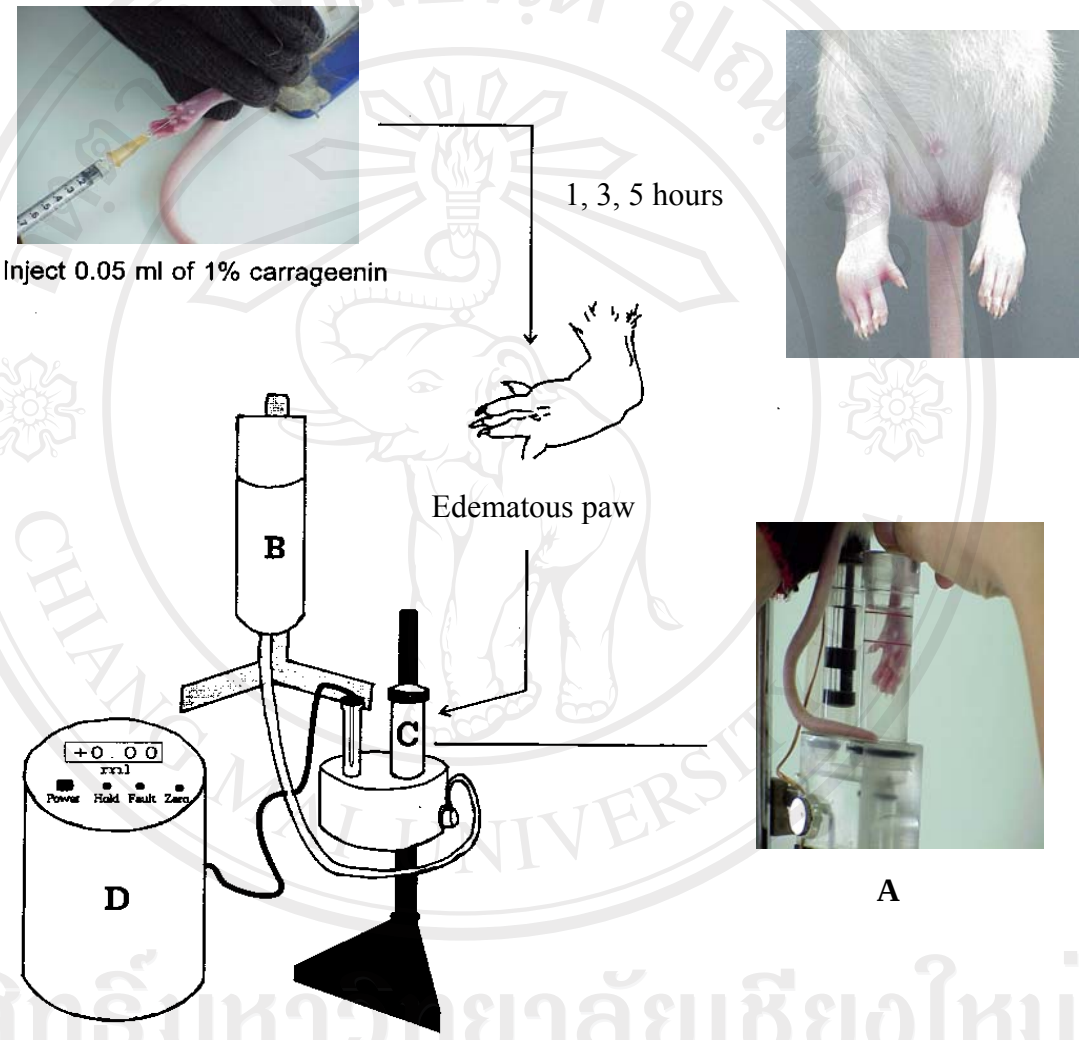


Figure 4. Diagram illustrating the water displacement method for measuring rat paw volume.

A: Measurement of paw volume with line indicated at anatomical hair line mark

B: Water reservoir containing 0.05% NaCl in distilled water

C: Measuring chamber

D: Plethysmometer

2.5.1.3 AA-induced hind paw edema in rats

The method described by Di Martino *et al* (1987) [52] was carried out. This method was used for investigation of the inhibitory effect of agents on the edema formation induced by AA. AA-induced hind paw edema in rat 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 inhibitor. Male rats of 100-120 g body weight were used and divided into 7 groups of 6 animals per group.

Group 1	The control group, received NSS
Group 2	The reference group, received 10 mg/kg of diclofenac
Group 3	The reference group, received 5 mg/kg of prednisolone
Group 4	The reference group, received 300 mg/kg of Daflon®
Group 5-7	The test groups, received 40, 80 and 160 mg/kg of the CS extract, respectively

Daflon®, prednisolone, diclofenac, CS extract and vehicle (NSS) were administered orally 2 h prior to AA injection. AA was dissolved in 0.2 M carbonate buffer (pH 8.4) in the concentration of 0.5% and a volume of 0.1 mL was injected into the plantar side of the right hind paw.

The paw volume of rat was measured prior to and at 1 h after AA injection. Paw volume, edema volume and the percent edema inhibition of each test compound was determined similarly to the method described in the model of carrageenin-induced hind paw edema.

2.5.2 Analgesic study

2.5.2.1 Formalin test in mice

The method described by Hunskaar and Hole (1987) [59] was followed. The formalin test comprises the early phase and the late phase assessment of the analgesic effect. Two sets of male Swiss albino mice weighing 30-40 g were used and divided into 7 groups of 6 animals per group in each set.

Group 1	The control group, received NSS
Group 2	The reference group, received 10 mg/kg of morphine
Group 3	The reference group, received 5 mg/kg of diclofenac
Group 4	The reference group, received 150 mg/kg of Daflon®
Group 5-7	The test groups, received 20, 40 and 80 mg/kg of the CS extract, respectively

In the early phase assessment, 20 μL of 1% formalin in NSS was injected subcutaneously into the right dorsal hind paw of the mice 60 min after i.p. administration of the test drug. Then between 0-5 min after formalin injection, the time in seconds the mice spent intensively licking the right dorsal hind paw was determined.

In the late phase assessment, another set of mice was used. The formalin was injected 40 min after test drug treatment and the licking time was determined between 20-30 min after the formalin injection. The licking time and the percent pain inhibition of each test compound were obtained by the following calculation:

$$\% \text{ Inhibition} = \frac{L_C - L_T}{L_C} \times 100$$

where,

L_C = Licking time (sec) of control group

L_T = Licking time (sec) of test compound group

The diagrams showing the protocol of the early and late phases of the formalin test are illustrated in Figures 5 and 6, respectively.

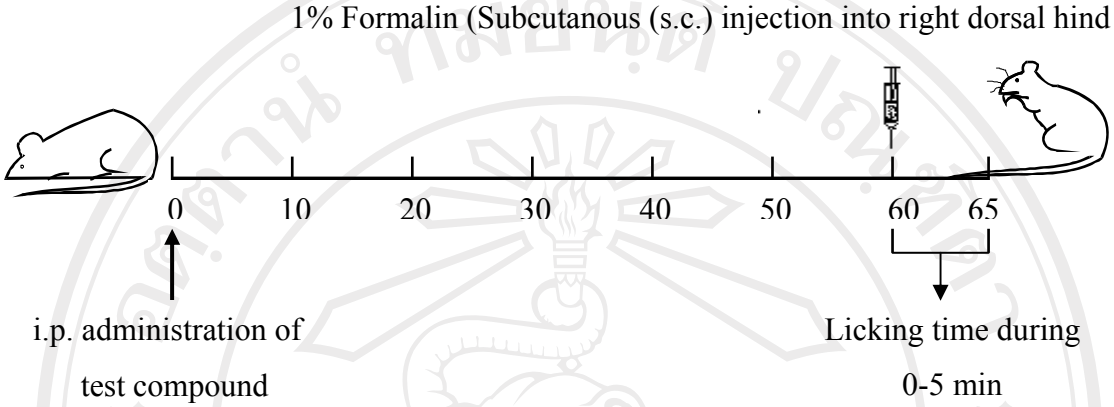


Figure 5. Diagram illustrating the method of the early phase of the formalin test in mice.

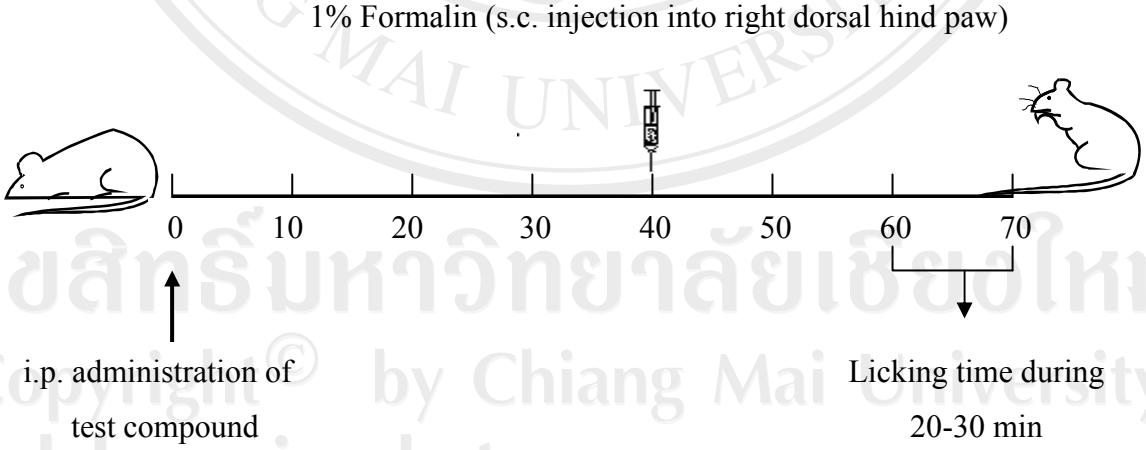


Figure 6. Diagram illustrating the method of the late phase of the formalin test in mice.

Part II

2.5.3 Study of the vascular effect of the methanol extract from *C. serratum* Linn. using isolated human umbilical vein.

The experiment protocol was prepared using the method modified from those of Altura (1972) [60], Gant and Dyer (1971) [78] and Sardi *et al* (1977) [79].

The umbilical cord was cut into segments, each segment was about 15-25 cm in length. The umbilical vein was dissected out from the cord, and cut into rings of 3-4 mm in length. Each vein ring, deprived of endothelium layer, was mounted in an isolated organ bath containing Krebs' solution (NaCl 6.9, KCl 0.35, $MgSO_4$, $7H_2O$ 0.29, $CaCl_2$ 0.28, KH_2PO_4 0.16, $NaHCO_3$ 2.1 and glucose 2.1 g/L). One end of the ring was attached to a fixed hook at the bottom of the bath and the other end was attached to a force-displacement transducer (Grass FT 03B) which was connected to a Grass model 7D polygraph. The vein ring was equilibrated for at least 2.5-3 h under a resting tension of 1 g. The bathing fluid was maintained at 37 ^oC, and continuously aerated with 95% oxygen. During the equilibration period of 120 min, the bathing fluid was changed at 30 min-interval to prevent the accumulation of metabolic products.

The CS extract and Daflon® at the doses of either 0.1, 0.2 and 0.4 mg/mL was added into the isolated organ bath. Norepinephrine (NE) at a dose of 15 μM was used to test the responsiveness of the human umbilical vein. The picture showing the procedure of preparing umbilical vein ring for recording venocontraction are show in Figures 7 and 8 below.

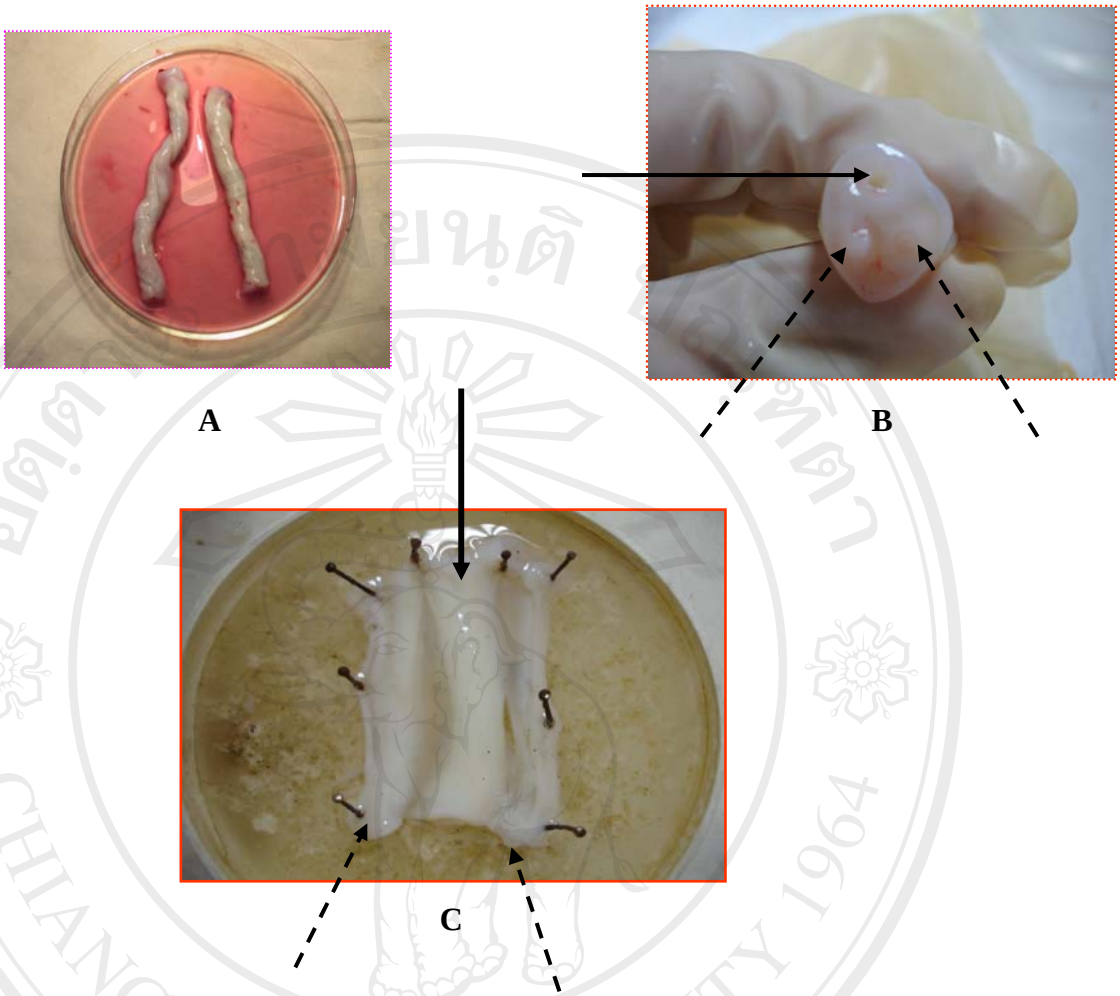


Figure 7. Pictures showing the procedure of preparing human umbilical vein

A: Human umbilical cord

B: Human umbilical cord showing artery (- - -) and vein (——)

C: Cutting longitudinally the umbilical cord

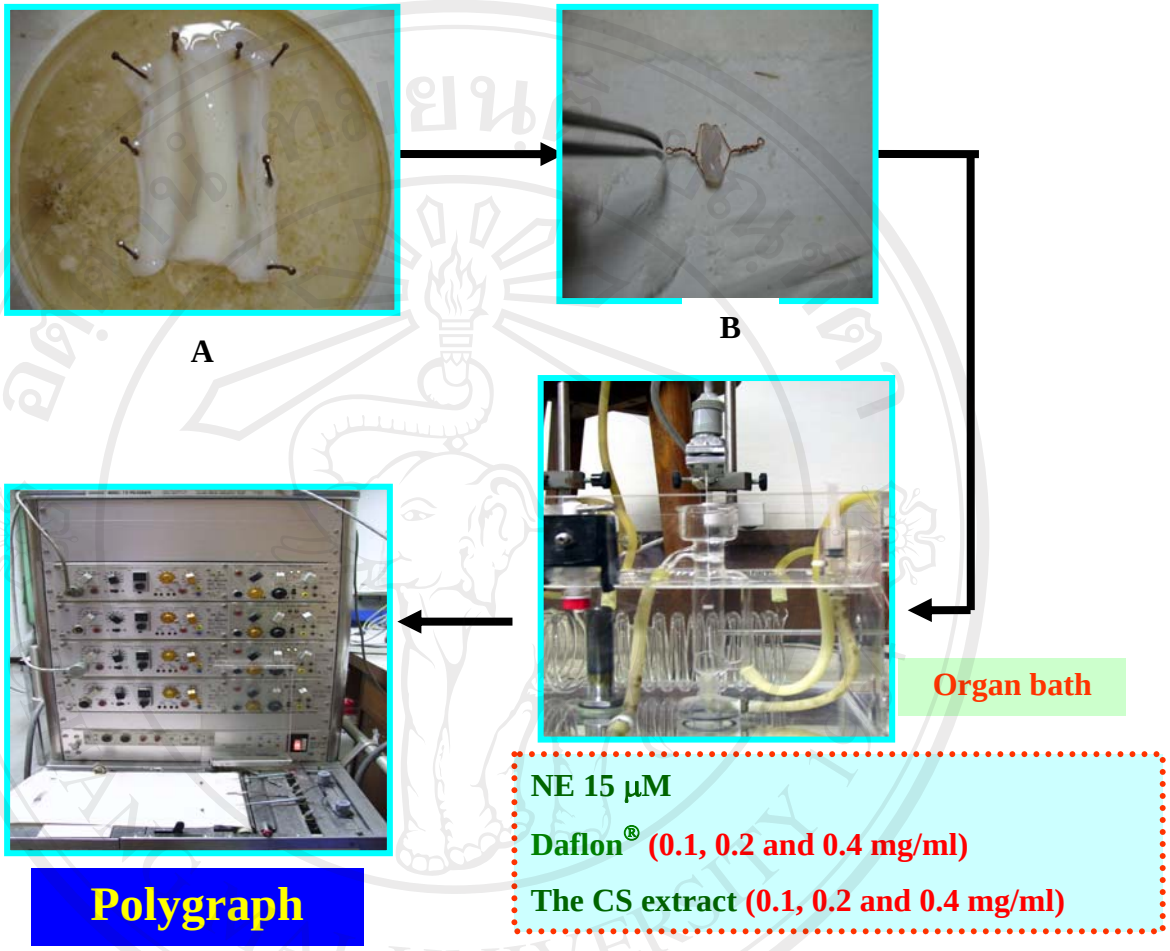


Figure 8. Illustration of the procedure of human umbilical vein for contraction recording

A: Cutting longitudinally the umbilical cord

B: Vein dissection out from the cord, and cutting into rings of 3-4 mm in length

C: Attachment of vein ring to a fixed hook at the bottom of the bath and to a force-displacement transducer (Grass FT 03B)

D: Connection of vein ring to a Grass model 7D polygraph

2.6 Statistical Analysis

The data from 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 the *P* values of less than 0.05 were considered significant.

2.7 Drugs and chemicals

2.7.1 Drugs

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

2.7.1.2 Daflon® (Les Laboratories Servier, France)

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

2.7.1.4 Morphine (T.P. Drug Laboratories, Thailand) Soi Sukhumvit 62, Prakhanong 10110, Bangkhae, Bangkok

2.7.1.5 Norepinephrine (Abbott Laboratories, North Chicago, IL 60064, USA)

2.7.2 Irritants

2.7.2.1 EPP (Fluka Chemicals Co., Ltd., Japan)

2.7.2.2 λ-Carrageenin (Sigma Chemical Company, St. Louis, U.S.A.)

2.7.2.3 AA (Sigma Chemical Company, St. Louis, U.S.A.)

2.7.2.4 Formalin (Chiang Mai Winner R.O.P. Co., Ltd., Thailand)

2.7.3 Vehicles

2.7.3.1 Acetone (Merck, Darmstadt, Germany)

2.7.3.2 Carbonate buffer (pH 8.4)