CHAPTER III

RESEARCH METHODOLOGY

Samples

1. Plant collection and identification

Tuber roots of *B. superba* were collected from Phrae provinces in March, 2001 and Phayao provinces in May, 2001, Thailand and identified by Associate Professor Yuthana Smitasiri, School of Science, Mae Fah Luang University, Thailand.

2. Plant extraction

The roots were washed and air-dried at room temperature. Bark and root core were separated and sliced into small and thin pieces. Some of fresh bark and root core were dried in a hot air oven at 50°C and grinded into fine powder. Then the fresh and dried bark or root core were extracted 3 times with ethanol at 1:4 ratio w/v and stirred overnight at room temperature. The ethanolic extract was evaporated under reduced pressure at 45°C using a rotary evaporator. The extracts were stored at –50°C until used.

3. Characterization of *B. superba* extract

Ingkaninan et al [115] characterized *B. superba* extracts collected from Phrae and Phayao province by thin layer chromatography (TLC; silica gel 60 F254). TLC chromatogram showed that *B. superba* extracts collected from both Phrae and Phayao provinces contain similar chemical components. On the other hand, the chemical components of the extracts prepared from fresh and dried *B. superba* are different. Moreover, the chemical components of the extracts prepared from bark and root of *B. superba* are slightly different.

4. Animals

Sprague-Dawley rats (age 8 wks) and ICR mice (age 8 wks) were obtained from the National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. The animals were housed under a 12:12-h light-dark cycle and maintained at 24 ± 1°C. They were used at the age of 20-24 wks for the ICP study. The protocol had been approved by the Naresuan University Ethical Committee.

Materials

Chemicals and instruments used in this study are listed below:

1. Chemicals

- 95% ethanol (Merck, Germany)
- ethylenediaminetetra-acetic acid (Merck, Damstadt, Germany)
- pentobarbital sodium (Sanofi, France)
- heparin (Leo, Denmark)
- phenylephrine (Sigma, St Louis, MO, USA)
- isobutyl-methylxanthine (Sigma, St Louis, MO, USA)
- cGMP (Sigma, St Louis, MO, USA)
- formalin solution (Merck, Germany)
- formaldehyde (Scharlau, Spain)
- hematoxylin (Sigma, St Louis, MO, USA)
- eosin (Sigma, St Louis, MO, USA)
- TCM199 medium modified with Earle's salt and L-glutamine (Life Technologies, USA)
- bovine serum albumin (Sigma, St Louis, MO, USA)
- NaCl (Sigma, St Louis, MO, USA)
- KCl (Sigma, St Louis, MO, USA)
- $CaCl_2$ (Sigma, St Louis, MO, USA)

- $MgCl_2$ (Sigma, St Louis, MO, USA)
- glucose (Sigma, St Louis, MO, USA)
- HEPES-buffer (Sigma, St Louis, MO, USA)

2. Instruments

- automatic hematological analyzer (Sysmex KX-21 Corporation, Kobe, Japan)
- automatic chemistry analyzer (Roche Diagnostics, Switzerland)
- neubauer hemocytometer (Fischer Scientific, Germany)
- balance (A & D, Japan)
- pH meter (Precisa, Dietikon, Switzerland)
- rotatory evaporator (Eyela, Tokyo Rikakihai, Japan)
- light microscope (Leica, Germany)
- 5% CO_2 incubator (Sanyo Electric, Japan)
- microtome (Shandon, UK)
- MacLab data acquisition system (MacLab Software V 4.0, ADI Instruments, MA)
- macintosh computer (ADI Instruments, MA, U.S.A)
- force transducer (FTO3, Quincy, MA, U.S.A)

Methods

1. Study of *B. superba* effects on ICP

This study aimed to screen for the most effective parts of *B. superba* alcoholic extract by using an ICP model. The extracts used were prepared from dried bark (DB), dried root (DR), fresh bark (FB), and fresh root (FR) of *B. superba* collected from Phrae and Phayao provinces. The extract preparation method is mentioned in 1.2.

1.1 Surgical method

The alcoholic extracts dissolved in water (or water in the case of control) were orally administered to the animals at various doses. One hour after the administration, an anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (Nembutal®, 35 mg/kg). The method of Quinlan et al [116] with some modification was employed. The penile skin was incised and the prepuce was degloved to completely expose the corpora cavernosa. A 23 gauge needle connected to polyethylene tubing (Clay-Adams PE-50) filled with physiological saline with 100 IU of heparin/ml was inserted into the corpus cavernosum on one side to measure intracavernous pressure. A femoral artery was cannulated in a similar fashion with polyethylene tubing to permit continuous monitoring of mean arterial pressure (MAP). Both polyethylene tubings were connected to blood pressure transducers, which were, in turn, connected via transducer amplifiers to a data acquisition board. Real-time display and recording of pressure measurements were performed on a Macintosh computer. The pressure transducers were calibrated in millimeter of mercury (mmHg) before each experiment.

1.2 Induction of penile erection [115]

Penile erection was electrically induced and ICP was recorded. The major pelvic ganglion, pelvic and cavernous nerves were exposed through a midline abdominal incision. Stainless steel bipolar hook wire electrodes were used to stimulate the cavernous nerve. The exposed ends of the electrodes were hooked around the cavernous nerve to be stimulated with the positive electrode positioned proximally and the negative electrode 2-3 mm positioned distally. Stimulus parameters were 5 volts, frequency of 20 Hertz and duration of 5 milliseconds. Each animal received stimulation

three times. The animals were allowed to rest until the intracavernous pressure decreased to the baseline values before the subsequent stimulations were conducted.

2. Screening for the most active part of *B. superba* extracts

Male Sprague-Dawley rats (age 20-24 wks) were used. The animals were divided into 2 groups: control and treatment groups. The control group was orally administered 1 ml of distilled water. The treatment groups were subdivided into four groups treated with water suspension of alcoholic extracts of fresh root (FR), dried root (DR), fresh bark (FB) and dried bark (DB) at the dose of 1, 10 or 1,000 mg/kg body weight (BW). One hour after administration, an anesthesia was induced by an intraperitoneal injection of pentobarbital sodium. Then, the ICP was recorded after the operation as mentioned in 3.1.1.

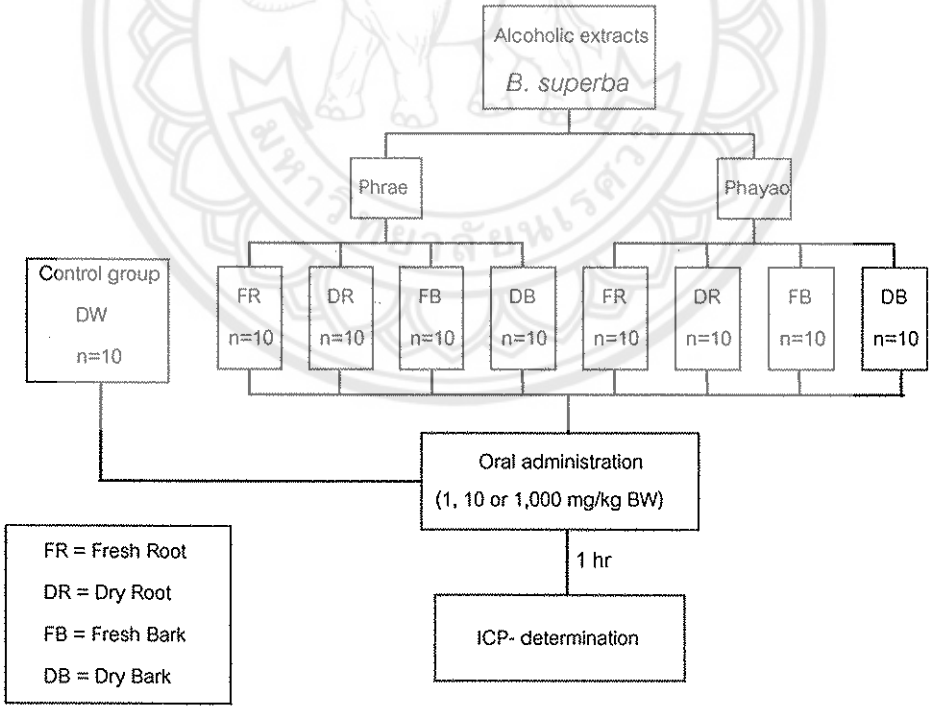


Figure 7 Screening diagram of *B. superba* alcoholic extracts.

3. Dose-response relationship study of *B. superba* extracts

Once the most effective part of *B. superba* extract had been obtained from the previous screening of a dose-response relationship study (3.3.2). Male Sprague-Dawley rats (age 20-24 wks) were orally treated with water suspension of alcoholic extracts of DRPr at the doses of 0.1, 0.5, 1, 10 or 1,000 mg/kg body weight (BW). One hour after the administration of the extracts, an anesthesia was induced by an intraperitoneal injection of pentobarbital sodium. Then, the ICP was recorded after the operation as mentioned in 3.3.1.

4. Effects of *B. superba* on cavernosal smooth muscle tone

This study aimed to investigate the effect of DRPr on cGMP/cAMP-mediated cavernosal smooth muscle tone.

4.1 Tissue preparation

Sprague-Dawley male rats (8 wks) were sacrificed by cervical dislocation. The entire penis was surgically removed and the corpus cavernosum was carefully dissected from the corpus spongiosum, tunica albuginea and surrounding connective tissue.

4.2 Smooth muscle tone determination

To assess the relaxation responses of cavernosal strips, the excised tissue was immediately placed in 100% oxygen-saturated HEPES-buffered physiological salt solution (HPSS: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 11 mM glucose, pH 7.4) and evaluated within 1 hr. The strips of rat cavernosal smooth muscle were trimmed to the size of about 0.2x0.2x0.5 cm. To record isometric tension, the strip was attached by a silk tie to a fixed support on one end and to a wire connected force transducer. Changes in isometric force were then measured using a MacLab data acquisition system. The tissue was placed in a 10-ml organ chamber containing HPSS bubbled with 100% O_2 and maintained at 37^{o}C, pH 7.4. The strip was equilibrated for 2 hr with several changes of HPSS. The resting tension was adjusted for each strip by stimulating with 10^{-6} M phenylephrine. The relaxation was induced by the addition of

isobutyl-methylxanthine (IBMX), a non-selective phosphodiesterase inhibitor, or cGMP at various concentrations. Effects of various concentration *B. superba* extract on the smooth muscle tone were observed in the presence of the extract alone or the extract with IBMX or with cGMP.

Two strips were taken from each animal and only one concentration-response study and response to a combination of the tested compounds were conducted on each preparation. After constructing a concentration-response curve of each agent or combination, the strip was washed at least three times with fresh HPSS over 1 hr and the muscle were allowed to relax to the baseline level before the subsequence experiment.

5. Effect of *B. superba* extract on male reproductive system

Male rats and mice were randomly divided into four groups: one control group and three different treatment groups. The control rats and mice were orally given distilled water daily for 6 months. The treatment groups received water suspension of alcoholic extract of *B. superba* dried root from Phrae at the doses of 0.1, 1.0 or 10.0 mg/kg BW daily for 6 months.

5.1 Preparation of culture drop

Motility assay was performed in TCM199 medium modified with Earle's salt and L-glutamine and supplemented with 3 mg/ml bovine serum albumin. To prepare the culture drop for sperm motility evaluation, 100-μl droplet under 10 ml paraffin oil were placed in a 60-mm petri dish and equilibrated overnight in high humidified atmosphere with 5% CO_2 before further analysis.

5.2 Sperm motility assay

Sperm motility was assessed by the method of Bavister and Andrews [117]. The distal cauda epididymis was dissected out and placed in 2 ml of equilibrated medium in a 35-mm tissue culture dish. A needle was used to release the spermatozoa from the cauda epididymis into the medium. The spermatozoa were allowed to swim out into the medium for 3 min in the 5 % CO_2 incubator at 37^{o}C. The sperm concentration

was then determined. A suitable volume was added to the culture drop for sperm motility assay, providing a final concentration of 2×10^6 spermatozoa/ml. The tissue culture dish was placed under an inverted microscope and an observation was performed at a magnification of 20X within 1 min. The motility of sperm was observed at various time intervals up to 6 hr.

5.3 Sperm count and morphology

The cauda epididymis was cut and weighed, finely minced and filtered through a piece of gauze. The sperms were counted using a Neubauer hemocytometer. An aliquot of the epididymal sperm suspension was smeared and stained with hematoxylin and eosin and then examined under a light microscope at magnification of 100X. The head and tail abnormalities (200 sperms per animal) were recorded.

5.4 Testicular histopathology

The testis was dissected out, cut into small slices and fixed in 10% formaldehyde buffer for 24 hr. The tissues were washed in buffer free of 10% formaldehyde and stored in 70% alcohol until being embedded. The tissues were dehydrated in alcohol series and embedded in paraffin. Tissue sections of 5 μm thickness were prepared and placed on glass slides. The sections were stained with hematoxylin and eosin and mounted in mounting medium. Slides were examined under the light microscope. For each testis several cross sections composing of 20-50 tubule sections were examined for signs of interstitial edema, seminiferous tubule degeneration and congestion.

6. Toxicity study

6.1 Acute toxicity study

Acute toxicity study was performed according to OECD guideline [118]. Thirty rats and thirty mice of each sex were randomly divided into 2 groups. The first group was the control group and composed of 7 rats and 7 mice of each sex. They were orally given 1 and 0.5 ml of distilled water, respectively (Figure 8). The second group

was composed of 8 rats and 8 mice of each sex and orally given 1 and 0.5 ml, respectively, suspension of DRPr at the dose of 5,000 mg/kg BW as a single dose. The treated animals were observed for lethality during the first 24 hr. The survived animals were kept for 14 days. During this period all recovering abnormal signs were recorded. After 14 days, all the remaining animals were sacrificed and examined for organ histopathology. The organ examined included liver, spleen, kidney, adrenal gland, heart, testis, epididymis, seminal vesicle, prostate gland, penis, ovary and uterus.

6.2 Chronic toxicity study

Male and female rats (8 wks) and mice (8 wks) were divided into control and treatment groups (Figure 10). The control animals were orally administered distilled water daily for 6 months. Whereas the treatment groups orally received the DRPr extracts at the doses of 0.1, 1 or 10 mg/kg BW daily for 6 months. All abnormal signs and mortality occurring during the 6-month period were recorded. At the end of the 6-month period, the animals were fasted for 18 hrs and then blood was collected under light ether anesthesia. The blood samples were anticoagulated with EDTA and then used for hematological and biochemical examinations.

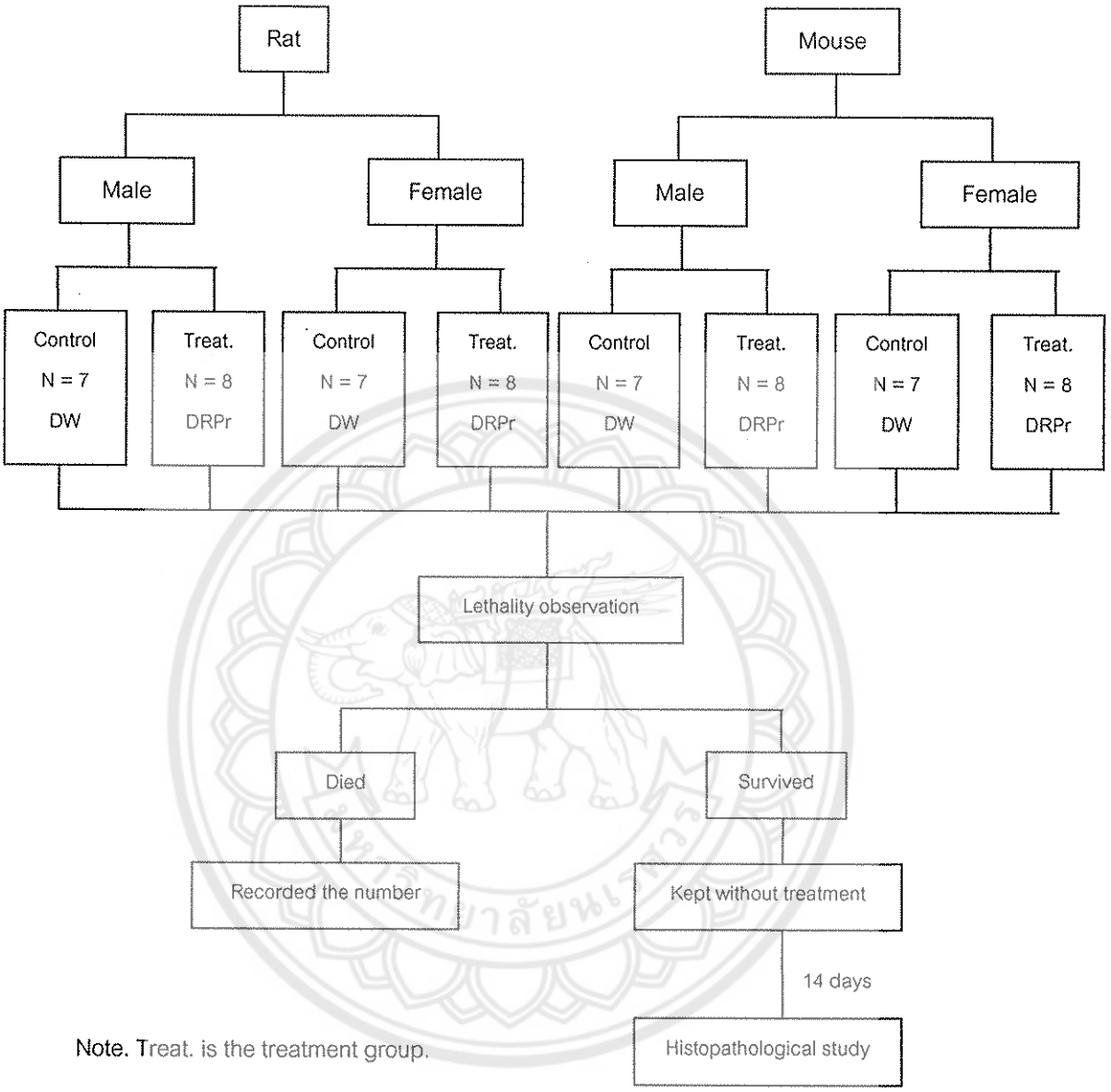
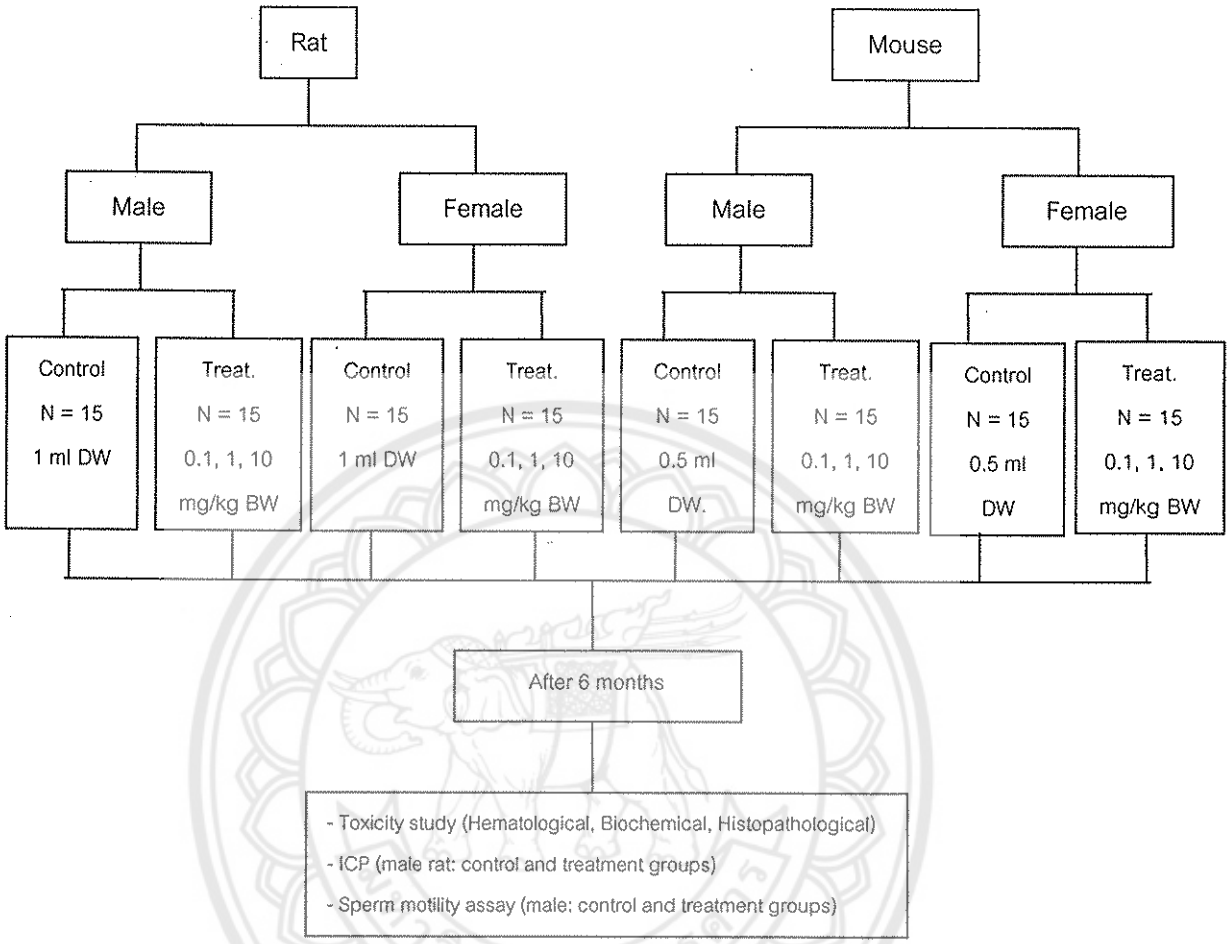


Figure 8 Summary diagram of acute toxicity study of *B. superba*. The treated animals were given DRPr at the dose of 5,000 mg/kg BW as a single dose.



Note. Treat. is the treatment group.

Figure 9 Summary diagram of chronic toxicity study of *B. superba*. The treated animals were given DRPr at the dose of 0.1, 1 or 10 mg/KgBW/day for 6 months.

6.2.1 Hematological analysis

Blood was collected from the abdominal aorta and anticoagulated with EDTA. Hematological parameters including white blood cell (WBC), neutrophil, lymphocyte, monocyte, eosinophil, basophil, red blood cell (RBC), platelet, hemoglobin and hematocrit (Hct) were determined by an automatic hematological analyzer.

6.2.2 Biochemical analysis

Blood was collected from the abdominal aorta and anticoagulated with EDTA. Biochemical parameters including alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid, creatinine and cholesterol were determined by an automatic chemistry analyzer.

6.2.3 Histopathological analysis

The position, shape, size and color of the internal organs were visually observed for any signs of gross lesions. The studied organs were brain, heart, lung, kidney, adrenal gland, spleen, prostate gland, seminal vesicle, epididymis, and testis and penis in the male or uterus and ovary in the female. After being collected, the organs were weighed and preserved in 10% buffered formalin solution. Then, section preparations made in conventional paraffin embedding were stained with hematoxylin and eosin (HE) and were observed under light microscope with 100X magnification.

Statistical data analysis

Statistical analysis was performed with one way analysis of variance (ANOVA). Where a significant treatment effect was found, differences among individual group means were then tested by the least significant difference (LSD) test. The differences were considered statistically significant at $P<0.05$.