CHAPTER III

MATERIALS AND METHODS

1. Animals

Adult female Wistar rats with regular estrous cycle (4-5 days) for at least 3 consecutive cycles, age 60 days, weighing 200-250 g, and adult male Wistar rats , age 60 days, weighing 250-300 g from National Laboratory Animal Centre, Mahidol University were used in this study. They were housed 5 animals/cage in a room with controlled lighting (lights on 06.00-20.00 h) in which the temperature maintained at 25 $\pm$ 1°C at Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. Food and water were available *ad libitum.* The animals were handled regularly for 2 weeks before the onset of the study. All experiments were performed between 08.00-10.00 h.

2. Experimental procedures

Adult female and male rats were divided into 4 main groups (50 rats/group) for 4 experiments as follows:

Experiment 1. The effect of *B. superba* on sex hormone levels and reproductive organs in adult cyclic female rats.

Experiment 2. The effect of *B. superba* on sex hormone levels and reproductive organs in adult ovariectomized rats.

Experiment 3. The effect of *B. superba* on sex hormone levels and reproductive organs in adult normal male rats.

Experiment 4. The effect of *B. superba* on sex hormone levels and reproductive organs in adult orchidectomized rats.

In each experiment, the rats were subdivided into 5 groups (10 rats/group) as follows;

Group 1: negative control or DW group, rats were administered with 0.7 ml of distilled water

Group 2: 10-BS group, rats were administered with suspension of 10 mg/kg.BW/day of *B. superba* in 0.7 ml distilled water

Group 3: 50-BS group, rats were administered with suspension of 50 mg/kg.BW/day of *B. superba* in 0.7 ml distilled water

Group 4: 250-BS group, rats were administered with suspension of 250 mg/kg.BW/day of *B. superba* in 0.7 ml distilled water

Group 5: positive control or TP group, rats were subcutaneously injected with 600μg/100g. BW/day of testosterone propionate in 0.2 ml of sesame oil

Experiment 1: The effect of *B. superba* on sex hormone levels and reproductive organs in adult cyclic female rats.

Fifty adult female Wistar rats with regular estrous cycle (4-5 days) for at least 3 consecutive cycles were subjected for this study. They were divided into 5 groups (10 rats/ group). The treatment schedule was separated into 3 periods: pre – treatment, treatment and post – treatment.

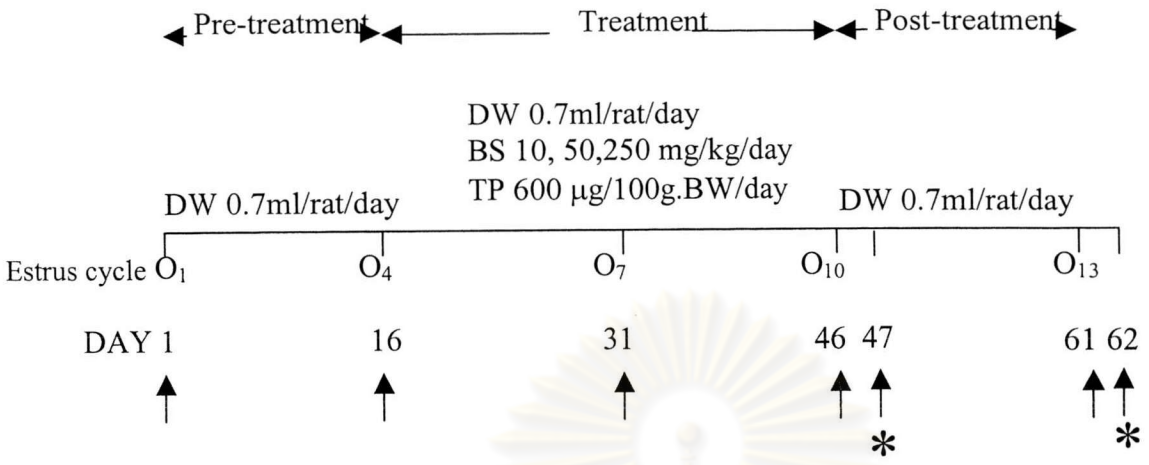
In pre - treatment period, when the rats showed a proestrous phase on the next estrous cycle, they were collected the blood by cardiac puncture between 09.00-10.00 h. This day was designed as day 1 (or O1) of the study period. The duration for this period was 15 days (or 3 consecutive estrous cycles). The rats were fed daily with distilled water between 08.00-09.00 h by gavage.

In treatment period, when the rats showed a proestrous phase on the fourth estrous cycle, they were fed with distilled water (Gr1) or *B. superba* (Gr2-4) or subcutaneously injected with testosterone propionate (Gr5) for 30 days (or 6 consecutive estrous cycles). They were collected the blood on the first day and the next 15 days (or 3 estrous cycles), that is, day 16, 31 and 46 or O4, 7 and 10, respectively. After the cessation of *B. superba* feeding or testosterone propionate injection (at the end of treatment period), half of rats (5 animals in each group) were fasted for 16 h. They were collected the blood and decapitated on the next day (day 47), and the remaining half of the rats were subjected to the next period of post-treatment. The ovary and uterus were dissected and weighed, thereafter.

In post-treatment period, when the remaing half of rats showed a proestrous phase on the tenth estrous cycle, they were fed with distilled water for 15 days (or 3 consecutive estrous cycles). They were collected the blood on the last days of this period (day 61or O13) and then they were fasted for 16 h. The rats were collected the blood and decapitated on the next day (day 62). The ovary and uterus was dissected and weighed, thereafter.

The ovary and uterus collected at the end of treatment and post-treatment period were manipulated for the histological study. The vaginal smears were checked daily in all 3 periods of experiment.

Experiment 1



DW = distilled water

BS = *Butea superba*

TP = testosterone propionate

O = Proestrous phase of the estrous cycle

↑ = blood collection

* = decapitation

Experiment 2 : The effect of *B.superba* on sex hormone levels and reproductive organs in adult ovariectomized rats.

Fifty adult female rats with regular estrous cycle (4-5 days) for at least 3 consecutive cycles were selected for this study. When the rats showed a diestrous phase on the next estrous cycle, they were collected the blood (day -14) and ovariectomized (OVX) under ether anesthesia. The rats were kept for 14 days and divided into 5 groups (10 rats/group). They were subjected for the treatment schedule, thereafter. The treatment schedule was separated into 3 periods: pre-treatment, treatment and post- treatment.

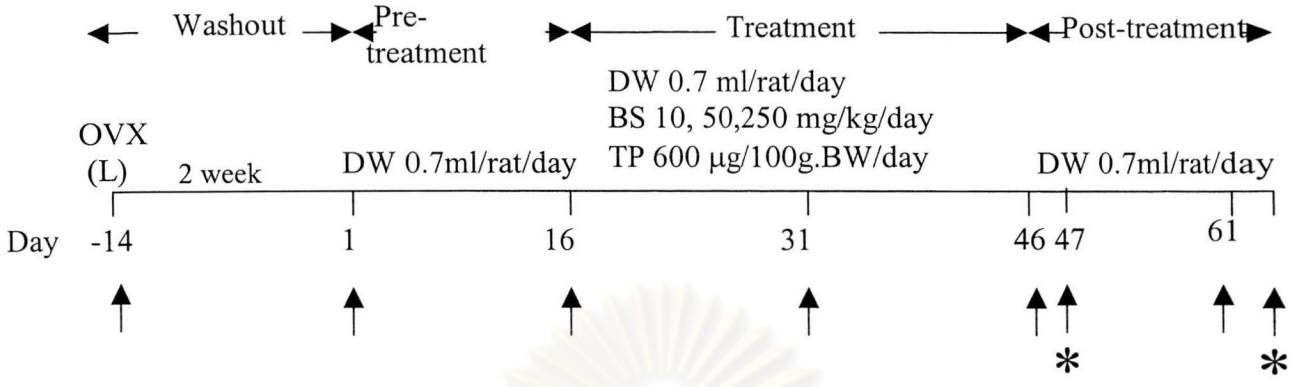
In pre-treatment period, the rats were administed with 0.7 ml of distilled water for 15 days between 08.00-09.00 h. The first day of the period designed as day 1. The rats were collected the blood by cardiac puncture on day 1 between 09.00 – 10.00 h.

In treatment period, the rats were fed with distilled water (Gr1) or *B. superba* (Gr2-4) or subcutaneously injected with testosterone propionate (Gr5) for 30 days. They were collected the blood on the first day and the next 15 days that is, day 16, 31 and 46. After the cessation of *B. superba* feeding or testosterone propionate injection (at the end of treatment period), half of rats (5 animals in each group) were fasted for 16 h. They were collected the blood and decapitated on the next day (day 47), and the remaining half of rats were subjected to the next period of post-treatment. The uterus was dissected and weighed, thereafter.

In post-treatment period, rats were fed with distilled water for 15 days. They were collected the blood on the last days of period (day 61). After that, the rats were fasted for 16 h. They were collected the blood and decapitated on the next day (day 62), and the uterus was dissected and weighed, thereafter.

The uterus collected at the end of treatment and post-treatment period was also manipulated for the histological study. The vaginal smears were checked daily in 3 periods of experiment.

Experiment 2



DW = distriled water

BS = *Butea superba*

TP = testosterone propionate

OVX = ovariectomy

↑ = blood collection

* = decapitation

Experiment 3: The effect of *B. superba* on sex hormone levels and reproductive organs in adult normal male rats.

Fifty adult male Wistar rats were randomly selected and subjected for this study. They were divided into 5 groups (10 rats/ group). The treatment schedule was separated into 3 periods: pre – treatment, treatment and post – treatment.

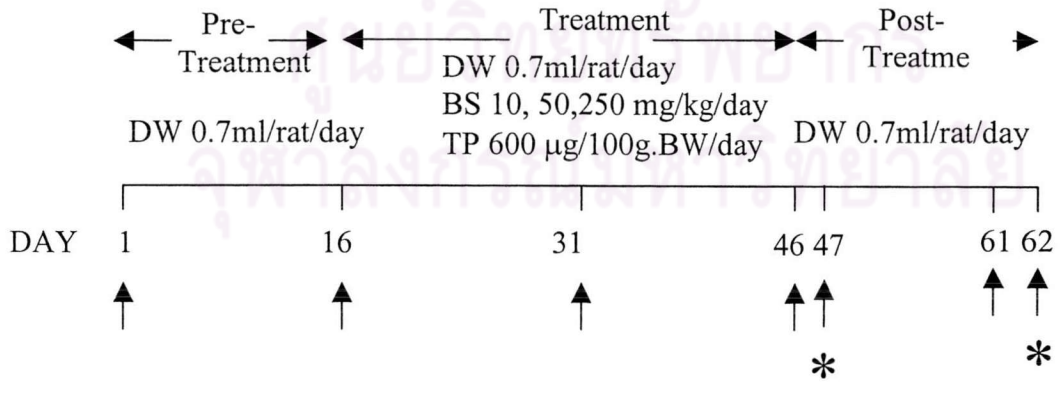
In pre – treatment period, the rats were fed daily with distilled water between 08.00 – 09.00 h by gavage. They were collected the blood by cardiac puncture between 09.00 – 10.00 at the first day of this period (designed as day 1 of the study period). The duration for this period was 15 days.

In treatment period, they were fed with distilled water (Gr1) or *B .superba* (Gr2-4) or subcutaneously injected with testosterone propionate (Gr5) for 30 days. They were collected the blood on the first day and the next 15 days of treatment period (day 16, 31 and 46). After the cessation of *B. superba* feeding or testosterone propionate injection (at the end of treatment period), half of rats (5 animals in each group) were fasted for 16 h. They were collected the blood and decapitated on the next day (day 47), and the remaining half of the rats were subjected to the next period of post-treatment. The testis, epididymis and seminal vesicle were dissected and weighed, thereafter.

In post-treatment period, the rats were fed with distilled water for 15 days. They were collected the blood on the last days of this period (day61). After that, the rats were fasted for 16 h. They were collected the blood and decapitated on the next day (day62), and the testis, epididymis and seminal vesicle were dissected and weighed, thereafter.

The testis, epididymis and seminal vesicle collected at the end of treatment and post-treatment periods were manipulated for histological study.

Experiment 3.



DW = distilled water

BS = *Butea superba*

TP = testosterone propionate

↑ = blood collection

* = decapitation

Experiment 4: The effect of *B. superba* on sex hormone levels and reproductive organs in adult orchidectomized rats.

Fifty adult male rats were randomly selected and subjected for this study. They were collected the blood and orchidectomized (ODX) under ether anesthesia, this day was designed as day -14 of the study period. The rats were kept for 14 days and divided into 5 groups (10rats/group). They were subjected for the treatment schedule, thereafter. The treatment schedule was separated into 3 periods: pre-treatment, treatment and post-treatment.

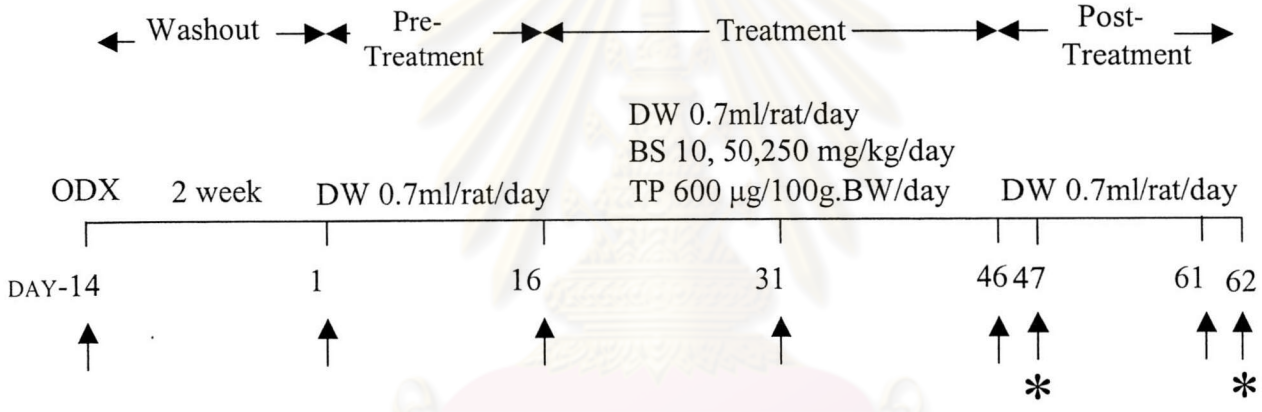
In pre-treatment period, the rats were administed with 0.7 ml of distilled water between 08.00 – 09.00 h for 15 days. The first day of this period designed as day 1 of the study period. The rats were collected the blood by cardiac puncture between 09.00-10.00 h.

In treatment period, the rats were fed with distilled water (Gr1) or *B. superba* (Gr2-4) or subcutaneously injected with testosterone propionate (Gr5) for 30 days. They were collected the blood on the first day and the next 15 days of treatment period. After the cessation of *B. superba* feeding or testosterone propionate injection (at the end of treatment period), half of rats (5 animals in each group) were fasted for 16 h. They were collected the blood and decapitated on the next day (day-47), and the remaining half of the rats were subjected to the next period of post-treatment. The epididymis and seminal vesicle were dissected and weighed, thereafter

In post-treatment period, they were fed with distilled water for 15 days. They were collected the blood on the last days of this period (day 61). After that, rats were fasted for 16 h. They were collected the blood and decapitated on the next day (day 62), and the epididymis and seminal vesicle was dissected and weighed, thereafter.

The epididymis and seminal vesicle collected at the end of treatment and post-treatment period were also manipulated for histological study.

Experiment 4



DW = distilled water

BS = *Butea superba*

TP = testosterone propionate

ODX = orchidectomy

↑ = blood collection

* = decapitation

3. Vaginal smear

Vaginal cytology was monitored daily between 08.00-09.00 h. Vaginal smear was performed using a small glass spatula that cleaned with 70% alcohol solution and soaked into 0.9% normal saline solution (NSS). The spatula was inserted into the vagina and gently touched against the vaginal wall. The vaginal cells were smeared into a drop of 0.9% NSS placed on a glass slide. The vaginal epithelium cells observed under the microscope were classified into 3 types; leukocyte (L), nucleated cells (O) and cornified cells (Co). The representative cell-type was determined by choosing the majority.

O represented the nucleated cells in proestrous period.

Co represented the cornified cells in estrous period.

L represented the leukocyte cells in both metestrous and diestrous periods.

4. Blood collection

One-milliliter of blood sample was collected by closure cardiac puncture after the ether anesthesia, between 09.00-10.00 h. The 2.5-ml of syringe and 25G X 1" of needle were used. Immediately after the blood clotted, blood serum was separated by centrifugation at 2,000 rpm, 4°C, 30 minutes and then kept it frozen at -20°C until assayed for LH, FSH and sex steroid hormones (E_2 in females and T in males)

5. Preparation of the powder suspension of *B. superba*

The tuberous roots of *B. superba* (cultivar Wichai 201) were collected in March, 2001 from Maephaluang district, Chiangrai province, Thailand. The tuberous

roots used in this study were obtained from the same lot. Its root was sliced and dried at 70-80 °C and pulverized in a mortar. The powder was filtered through a 100-size mesh. The powder was kept in the dark bottles as a stock at the room temperature. During treatment, the dried powder of *B. superba* was mixed with distilled water into the concentration of 10, 50, 250 mg/kg.BW/day. The suspension was administered to the rats during 08.00-09.00 h using a gastric feeding needle, size 18 G x 2 ½ inch and 1-ml syringe (Semler, 1992).

6. Preparation of testosterone propionate

Testosterone propionate (TP) in the dosage of 600 μg/100g.BW/day in 0.2 ml of sesame oil was used as a positive control in this study. This dose was chosen to ensure an adequate influence of testosterone propionate on GnRH-Gn-sex steroid hormones axis or hypothalamic-pituitary-gonadal axis in rat model (Borg *et al.,* 1995; Gay and Bogdanove, 1969; Moulton and Leonard, 1969; Ramirez and McCann, 1965; Swerdloff and Walsh, 1973; Wierman *et al.,* 1990). The powder of testosterone propionate at the designed dose was weighed and dissolved in a small volume of absolute ethanol. After the powder was completely dissolved, the sesame oil in appropriate volume was added. The solution was then standed at the room temperature to evaporate the ethanol out. The TP solution was kept in the dark bottles as a stock at the room temperature until used. The solution was subcutaneously injected to the rats between 08.00-09.00 h using of 23G x 1 inch of needle and 1 ml of syringe.

7. Hormonal analysis

Concentrations of serum FSH and LH were measured using reagents obtained from the National Hormone and Pituitary Program. Iodination preparations were rat NIDDK-rat FSH-I-5 and rat LH-I-5. The antisera were anti-rat FSH-S11 and anti-rat LH-S11. The results obtained are expressed in terms of the rat FSH-Rp-2 and rat LH-RP-3 reference standards (Niswender et al., 1968).

Serum concentrations of estradiol and testosterone were measured using the established RIA methods of World Health Organization (WHO) after the samples were extracted with ether (Sufi et al., 1980).

To minimize the interassay variation all samples in each group were run in a single assay of each hormones. The intra-assay coefficients of variation of LH, FSH, E_2 and T were 3.10%, 7.27%, 11.94% and 16.62% respectively.

8. Statistical analysis

The result was expressed as mean $\pm$ standard error (SE). T-test was submitted for comparison of the reproductive organs weights (testis, epididymis, seminal vesicle, ovary and uterus) collected at the end of treatment and post-treatment period. Difference of serum hormone levels among the pre-treatment, treatment and post-treatment period in each group and among the negative control group, *B. superba* treated group and TP treated group in each experiment were analyzed by one way analysis of variance with post-hoc test by LSD test. Significance was set at $P<0.05$.

9. Histological analysis

After overnight fixation in formalin, ovary, uterus, testis, seminal vesicle and epididymis were dehydrated in a series of ethanol gradient and clearing in xylene. Tissues were then embedded and blocked in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin (Humason, 1979). Permanent slides of all experiment organs were examined under Olympus microscope and photograph.