CHAPTER III

MATERIALS AND METHODS

I. Animals

Wistar rats that obtained from National Laboratory Animal Center, Mahidol University were used in this study. They were 5-6 rats/cage housed in Animal Laboratory's House, Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University. The cage was made of stainless steel. The size of each cage was 24.5 cm in width, 46 cm in length and 14 cm in height. The size of each net that used to close each cage was 0.85 cm x 0.85 cm. The temperature was controlled at 25±1 °C and the photoperiod regimen was 14 hours of light and 10 hours of dark, the lights were automatically turned on at 6.00 h and turned off at 8.00 p.m. The rats were fed with rat chow diet (Pokapan Animal Food Center, Thailand) and tap water *ad libitum*.

II. Experimental protocol

Experiment I: Effects of *Mucuna collettii* on hormone-related ovarian functions and reproductive organs in cyclic female rats.

Fifty-five normal female rats, 60 to 65 days of age, 197 to 265 g of body weight, with regular vaginal estrous cycles (4-5 days) for 3 consecutive cycles before the study period were selected. When the rats showed a proestrous stage on the next

cycle, designed as D_1 or O_1 of the study period, they were randomly divided into 5 groups (10-13 rats/group) as follows;

DW group; rats (n=11) were orally treated with 0.7 ml of di-distilled water (DW) per day.

TP group; rats (n=11) were subcutaneously injected with testosterone propionate (TP) at the dosage of 600 μg/100gBW/day dissolved in 0.2 ml of sesame oil.

Mc-1 group; rats (n=13) were orally treated with the powder suspension of *M. collettii* (Mc) at the dosage of 1 mg/kgBW/day in 0.7 ml of DW.

Mc-10 group; rats (n=10) were orally treated with the powder suspension of Mc at the dosage of 10 mg/kgBW/day in 0.7 ml of DW.

Mc-100 group; rats (n=10) were orally treated with the powder suspension of Mc at the dosage of 100 mg/kgBW/day in 0.7 ml of DW.

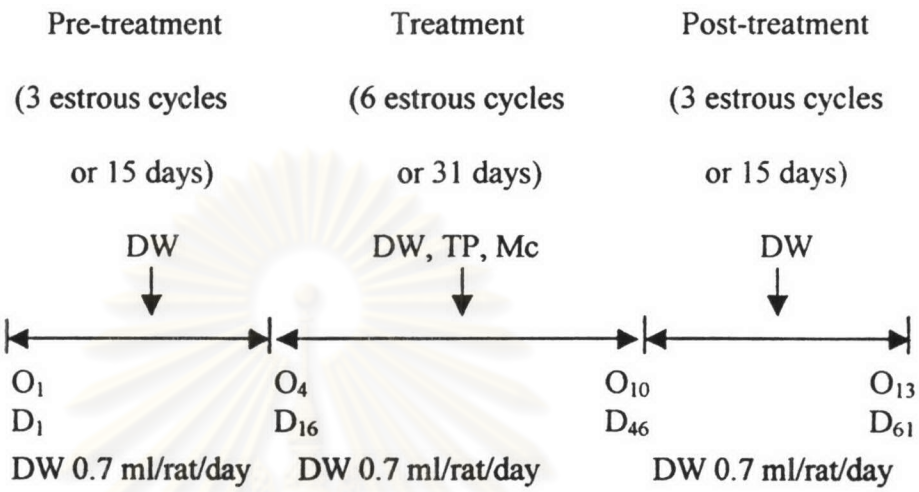
The treatment schedule was divided into 3 periods: pre-treatment, treatment and post-treatment. During pre-treatment and post-treatment periods, the rats were orally administered with 0.7 ml of DW everyday for 3 estrous cycles or 15 days. During treatment period, the suspension of Mc at the dosages of 1, 10 and 100 mg/kgBW/day were orally treated and the solution of TP were subcutaneously injected for 6 estrous cycles or 31 days as shown in the diagram I.

During the study period the rats were determined the following parameters.

1. Body weight, vaginal smear and general appearance of the rats were recorded everyday.
2. Food intake of each rat was determined every week.

3. Blood samples were collected approximately 1 ml/time by closure cardiac puncture every 3 estrous cycles or every 15 days. Blood collections were performed at the proestrous stage when the rats showed the regular estrous cycle (O_1, O_4, O_7, O_{10} and O_{13}). When the rats showed an irregular or absent estrous cycle, blood collections were performed every 15 days (D_1, D_{16}, D_{31}, D_{46} and D_{61}). Blood samples were centrifuged and serum was analysis of FSH, LH and E_2 levels by radioimmunoassay (RIA) method.
4. After, the cessation of Mc feeding or at the end of treatment period (D_{46} and O_{10}), half of the rats were killed by anesthetization with ether after the final dose, and the remaining half of the rats were killed at the end of post-treatment period (D_{61} or O_{13}). The rats were fasted for 18-24 hours before sacrifice. The blood samples were collected before sacrifice, and the ovaries and uteri were dissected and weighed, thereafter.

Diagram I: Experimental protocol in normal female rats treated with distilled water, *Mucuna collettii* and testosterone propionate.



Blood collections

- normal rats;	O_1	O_4	O_7	O_{10}*	O_{13}*
-abnormal rats;	D_1	D_{16}	D_{31}	D_{46}*	D_{61}*

Body weight; Everyday

Vaginal smear; Everyday

General appearance; Everyday

Food intake; Every week

* = the rats were sacrifice.

Experiment II; Effects of *Mucuna collettii* on hormone-related ovarian functions and reproductive organs in bilateral ovariectomized (OVX) female rats.

Sixty-three normal female rats, 60 to 65 days of age, 167 to 248 g of body weight, with regular vaginal estrous cycles (4-5 days) for 3 consecutive cycles before the study period were selected. When the rats showed a diestrous stage on the next cycle, designed as D_{-14} of the study period, they were operated for bilateral ovariectomy. After OVX for 14 days and daily vaginal smear showed no estrous cycles in the rats, designed as D_1 of the study period and the rats were randomly divided into 5 groups (10-15 rats/group) as follows;

DW group; OVX (n=11) rats were orally treated with 0.7 ml of DW per day.

TP group; OVX (n=12) rats were subcutaneously injected with TP at the dosage of 600 μg/100gBW/day dissolved in 0.2 ml of sesame oil.

Mc-1 group; OVX (n=15) rats were orally treated with the powder suspension of Mc at the dosage of 1 mg/kgBW/day in 0.7 ml of DW.

Mc-10 group; OVX (n=12) rats were orally treated with the powder suspension of Mc at the dosage of 10 mg/kgBW/day in 0.7 ml of DW.

Mc-100 group; OVX (n=13) rats were orally treated with the powder suspension of Mc at the dosage of 100 mg/kgBW/day in 0.7 ml of DW.

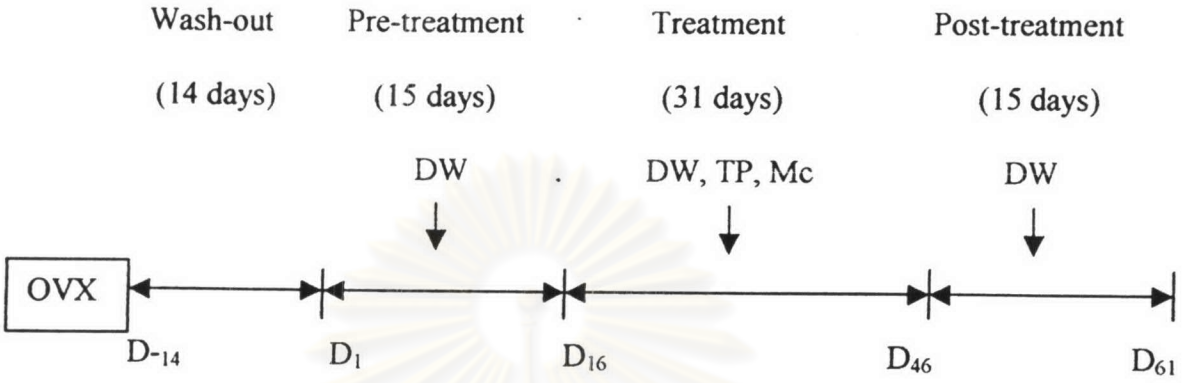
The treatment schedule was divided into 3 periods: pre-treatment, treatment and post-treatment. During pre-treatment and post-treatment periods, the rats were orally administered with 0.7 ml of DW everyday for 15 days. During treatment period, the suspension of Mc at the dosages of 1, 10 and 100 mg/kgBW/day were

orally treated and the solution of TP were subcutaneously injected for 31 days as shown in the diagram II.

During the study period the rats were determined the following parameters.

1. Body weight, vaginal smear and general appearance of the rats were recorded everyday.
2. Food intake of each rat was determined every week.
3. Blood samples were collected approximately 1 ml/time by closure cardiac puncture every 15 days (D_{-14}, D_1, D_{16}, D_{31}, D_{46} and D_{61}). Blood collection at D_{-14} served as the normal hormones levels of the rats in this study and daily vaginal smear after 14 days of OVX showed no estrous cycles in this time, it confirms that the ovaries were completely removed and sex steroid hormones levels of the rats were low, then the rats were used in this experiment because it is easy to observe the effects of Mc on hormonal levels after administrations. Blood samples were centrifuged and serum was analysis of FSH, LH and E_2 levels by RIA method.
4. After, the cessation of Mc feeding or at the end of treatment period (D_{46}), half of the rats were killed by anesthetization with ether after the final dose, and the remaining half of the rats were killed at the end of post-treatment period (D_{61}). The rats were fasted for 18-24 hours before sacrifice. The blood samples were collected before sacrifice, and the uteri were dissected and weighed, thereafter.

Diagram II: Experimental protocol in bilateral ovariectomized female rats treated with distilled water, *Mucuna collettii* and testosterone propionate.



Blood collections;	D_{-14}	D_1	D_{16}	D_{31}	D_{46}*	D_{61}*
Body weight;	Everyday					
Vaginal smear;	Everyday					
General appearance;	Everyday					
Food intake;	Every week					

Experiment III: Effects of *M. collettii* on hormone-related ovarian functions and reproductive organs in normal male rats.

Sixty-two normal male rats, 60 to 65 days of age, 265 to 333 g of body weight, were used in this study. On D_1 the rats were randomly divided into 5 groups (10-15 rats/group) as follows;

DW group; rats (n=15) were orally treated with 0.7 ml of distilled water per day.

TP group; rats (n=11) were subcutaneously injected with testosterone propionate at the dosage of 600 μg/100gBW/day dissolved in 0.2 ml of sesame oil.

Mc-1 group; rats (n=13) were orally treated with the powder suspension of Mc at the dosage of 1 mg/kgBW/day in 0.7 ml of DW.

Mc-10 group; rats (n=12) were orally treated with the powder suspension of Mc at the dosage of 10 mg/kgBW/day in 0.7 ml of DW.

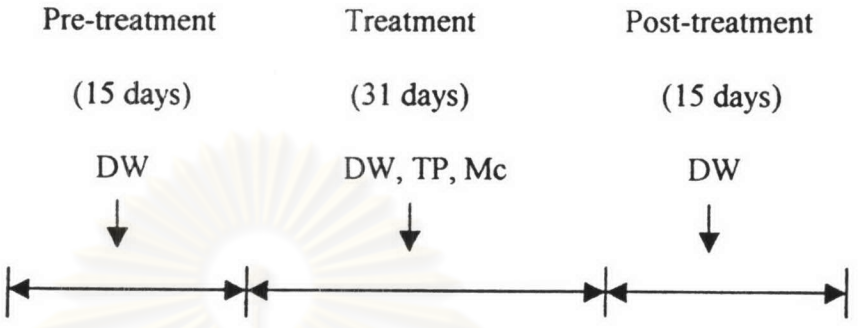
Mc-100 group; rats (n=11) were orally treated with the powder suspension of Mc at the dosage of 100 mg/kgBW/day in 0.7 ml of DW.

The treatment schedule was divided into 3 periods: pre-treatment, treatment and post-treatment. During pre-treatment and post-treatment periods, the rats were orally administered with 0.7 ml of DW everyday for 15 days. During treatment period, the suspension of Mc at the dosages of 1, 10 and 100 mg/kgBW/day were orally treated and the solution of TP were subcutaneously injected for 31 days as shown in the diagram III.

During the study period the rats were determined the following parameters.

1. Body weight and general appearance of the rats were recorded everyday.
2. Food intake of each rat was determined every week.
3. Blood samples were collected approximately 1 ml/time by closure cardiac puncture every 15 days (D_1, D_{16}, D_{31}, D_{46} and D_{61}). Blood samples were centrifuged and serum was analysis of FSH, LH and T levels by RIA method.
4. After, the cessation of Mc feeding or at the end of treatment period (D_{46}), half of the rats were killed by anesthetization with ether after the final dose, and the remaining half of the rats were killed at the end of post-treatment period (D_{61}). The rats were fasted for 18-24 hours before sacrifice. The blood samples were collected before sacrifice, and the testes, epididymis and seminal vesicle were dissected and weighed, thereafter.

Diagram of III: Experimental protocol in normal male rats treated with distilled water, *Mucuna collettii* and testosterone propionate.



Blood collections; D_1 D_{16} D_{31} D_{46}* D_{61}*

Body weight; Everyday

General appearance; Everyday

Food intake; Every week

Experiment IV; Effects of *Mucuna collettii* on hormone-related ovarian functions and reproductive organs in bilateral orchidectomized (ODX) female rats.

Sixty-one normal male rats, 60 to 65 days, 261 to 348 g of body weight, were operated for bilateral orchidectomy. This day is designed as D_{-14} of this study. After ODX for 14 days, the rats were randomly divided into 5 groups (10-13 rats/group) as follows;

DW group; ODX (n=13) rats were orally treated with 0.7 ml of DW per day.

TP group; ODX (n=11) rats were subcutaneously injected with TP at the dosage of 600 μg/100gBW/day dissolved in 0.2 ml of sesame oil.

Mc-1 group; ODX (n=13) rats were orally treated with the powder suspension of Mc at the dosage of 1 mg/kgBW/day in 0.7 ml of DW.

Mc-10 group; ODX (n=11) rats were orally treated with the powder suspension of Mc at the dosage of 10 mg/kgBW/day in 0.7 ml of DW.

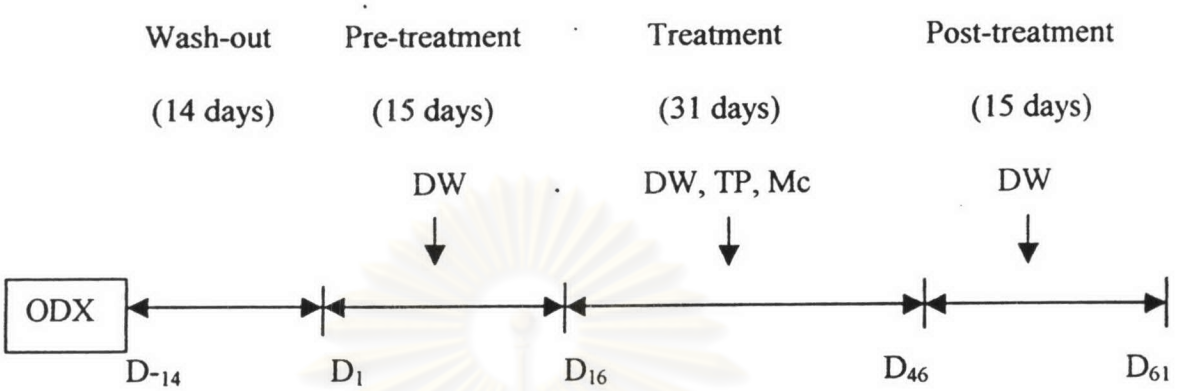
Mc-100 group; ODX (n=13) rats were orally treated with the powder suspension of Mc at the dosage of 100 mg/kgBW/day in 0.7 ml of DW.

The treatment schedule was divided into 3 periods: pre-treatment, treatment and post-treatment. During pre-treatment and post-treatment periods, the rats were orally administered with 0.7 ml of DW everyday for 15 days. During treatment period, the suspension of Mc at the dosages of 1, 10 and 100 mg/kgBW/day were orally treated and the solution of TP were subcutaneously injected for 31 days as shown in the diagram IV.

During the study period the rats were determined the following parameters.

1. Body weight and general appearance of the rats were recorded everyday.
2. Food intake of each rat was determined every week.
3. Blood samples were collected approximately 1 ml/time by closure cardiac puncture every 15 days (D_{-14}, D_1, D_{16}, D_{31}, D_{46} and D_{61}). Blood collection at D_{-14} served as the normal hormones levels of the rats. After ODX for 14 days, the rats were assumed that the testes were completely removed and sex steroid hormones levels of the rats were low, then the rats were used in this experiment because it is easy to observe the effects of Mc on hormonal levels after administrations. Blood samples were centrifuged and serum was analysis of FSH, LH and T levels by RIA method.
4. After, the cessation of Mc feeding or at the end of treatment period (D_{46}), half of the rats were killed by anesthetization with ether after the final dose, and the remaining half of the rats were killed at the end of post-treatment period (D_{61}). The rats were fasted for 18-24 hours before sacrifice. The blood samples were collected before sacrifice, and the testes, epididymis and seminal vesicle were dissected and weighed, thereafter.

Diagram IV: Experimental protocol in bilateral orchidectomized male rats treated with distilled water, *Mucuna collettii* and testosterone propionate.



Blood collections; D_{-14} D_1 D_{16} D_{31} D_{46}* D_{61}*

Body weight; Everyday

General appearance; Everyday

Food intake; Every week

II. Vaginal smear

Vaginal smear was performed during 08.00-09.00 h. A small glass rod was cleaned with 70% alcohol solution and soaked into 0.9% normal saline solution (NSS) before use. The spatula was inserted into the vagina against the vaginal wall, then smear the vaginal cells into a drop of 0.9% NSS on a slide. The vaginal cells were observed under light microscope and recorded. The cell-type was classified as follows;

O = the nucleated cells in proestrus period.

Co = the cornified cells in estrus period.

L = the leukocyte cells in both metestrus and diestrus periods.

The representative cell-type was chosen from the majority.

III. Blood collection

The rats were anesthetized with diethy-ether and collected the blood approximately 1 ml/time by closure cardiac puncture (Semler, 1992), during 08.00-09.00 h. The equipments of cardiac puncture were 2.5 cc syringe and 25 x 1 inch needle. Blood samples were centrifuged at 2,000 rpm at 4°C for 30 minutes. The serum was separated and collected for analysis of levels; FSH, LH, E_2 (in female rats) and T (in male rats).

IV. Preparation of the powder suspension of *Mucuna collettii*

Mucuna collettii (cultivar Wichai 201) used in this study was the same batch, it was collected in March, 2001 from Maephaluang district, Chiangrai province, Thailand. Its stem was sliced and dried at 70-80 °C and pulverized in a mortar. The powder was filtered through a mesh (100- mesh size). The powder was kept into the dark bottles at the room temperature. During usage, the dried powder of *M. collettii* was mixed with the distilled water into the dosages of 1, 10, 100 mg/kgBW/day. The suspension was force-fed to the rat during 08.00-09.00 h by a gastric feeding needle, size 18 x 2 ½ inch, and 1 ml syringe (Semler, 1992).

V. Preparation of testosterone propionate

Testosterone propionate in 0.2 ml of sesame oil was used as a positive control of this study. The dosage was chosen to ensure an adequate influence of testosterone propionate on hypothalamus-pituitary-gonad axis or GnRH-Gn-sex steroid hormones 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 (Sigma Chemical Company, Merck, USA). After the powder was completely dissolved, the sesame oil in appropiate volume was added. The solution was then standed at the room temperature to evaporate the ethanol out. The TP solution was kept as a stock into the dark bottles at the room temperature until used. The solution was subcutaneously injected to the rats during 08.00-09.00 h by 23 x 1 inch needle and 1 ml syringe.

VI. Hormonal analysis

Serum was collected to analyze the levels of FSH, LH, E_2 and T by RIA method. Analysis of serum LH and FSH levels followed the method of Watanabe *et al.* (1990) and analysis of serum E_2 and T levels followed the method of World Health Organization (WHO) Programme, then the samples were extracted with ether (Sufi *et al.*, 1986).

Concentrations of serum LH and FSH were measured using the reagents obtained from the National Hormone and Pituitary Programme. Iodination preparation were rat LH-I-5 and rat NIDDK-rat FSH-I-5. The antisera were anti-rat LH-S11 and anti-rat FSH-S11. The results obtained were expressed in terms of the rat LH-RP-3 and FSH-RP-2 reference standards. The percent of inter-assay coeffiency of LH and FSH were 3.10 and 7.27, respectively. The percent of intra-assay coeffiency of LH and FSH were 4.33 and 8.66, respectively.

Concentrations of serum E_2 and T were measured using the tracer (2,4,6,7-^{3}H) E_2 and (1,2,6,7-^{3}H) T. The antisera were E_2 antisera (Cloned 2F9) and T antisera (batch number K888510). The results obtained were expressed in terms of the E_2 and T reference standards. The percent of inter-assay coeffiency of E_2 and T were 0 and 15.08, respectively. The percent of intra-assay coeffiency of E_2 and T were 0 and 20.40, respectively.

VII. Histopathological study

This study used the method of haematoxylin and eosin staining to study the structural feathers of the sections of the reproductive organs for confirmed the effects of *M. collettii* on the reproductive organs.

Testes, epididymis and seminal vesicle of male rats and ovaries and uterus of female rats were fixed in 10% buffer formalin at least 24 hrs and then were processed according to the standard histological techniques (Humanson, 1979) using haematoxylin and eosin stainning (Ehrlich, 1886). All tissue blocks were sectioned at 5 μm. Histological study were performed by light microscope following two aspects; basic histology and the histological alterations.

Routine paraffin method for the certain reproductive organs were as follows;

Step 1	90% ethanol	-	1 time	-	1 hour/time.
Step 2	95% ethanol	-	2 times	-	6 hours/time.
Step 3	N-butyl alcohol	-	1 time	-	1 hour/time.
Step 4	Xylene	-	1 time	-	1 hour/time.
Step 5	Xylene + molten wax (1:1)	-	1 time	-	1/2 hour/time.
Step 6	Wax I	-	1 time	-	1/2 hour/time.
Step 7	Wax II	-	1 time	-	1 hour/time.

Step 8 Embeded and orientated in filtered wax .

VIII. Statistical analysis

The results of all parameters were expressed as the mean $\pm$ SEM. Analysis of Variance (ANOVA) was used to determine the differences of means using the Statistical Packages for Social Science (SPSS) in all of the parameters. The observed significance was then confirmed using the least significance difference (LSD) test. Statistical significance was defined as $p<0.05$. Except for comparison within each group of reproductive organ weights and estrous cycle length, unpaired Student' s t-test was used to determine the differences of means in which the statistical significance was defined as $p<0.05$.