CHAPTER III

MATERIALS AND METHODS

1. Animals

Adult ICR mice, age 50-60 days, weighing 30-35 gm for males and 25-27 gm for females, from National Laboratory Animal Center, Mahidol University were used in this study. They were housed 5 animals/cage in a room with controlled lighting (lights on 06.00 a.m. – 08.00 p.m.) 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 habituated by handling regularly for 2 weeks before the experiments were conducted. All experiments were performed between 08.00 - 11.00 am.

2. Treatments and Sample collection

Experiment 1. Effects of *P. mirifica* on hormone-related testicular functions, reproductive organs and fertility in male mice.

The mice were divided into 4 groups (10 mice/group) as below.

Group I: a negative control or DW group, mice were orally treated with 0.2 ml/day of distilled water.

Group II: 10-PM group, mice were orally treated with the powder suspension of 10 mg/kg/day of *P. mirifica* in 0.2 ml of distilled water.

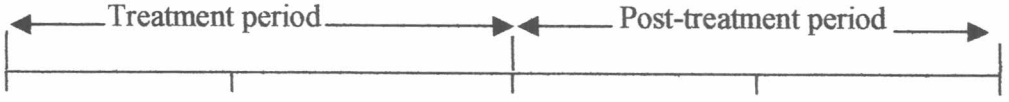
Group III: 100-PM group, mice were orally treated with the powder suspension of 100 mg/kg/day of *P. mirifica* in 0.2 ml of distilled water.

Group IV: a positive control or DES group, mice were subcutaneously injected with 200 μg/kg/day of diethylstilbestrol (DES) in 0.2 ml of corn oil.

The treatment schedule was separated into 2 periods; treatment and post-treatment. The duration in each period was 8 weeks. Mice were collected blood by cardiac puncture for LH, FSH and T assays in every 4 weeks.

After the blood collection, male mouse was paired with three virgin female mice for 4 days, night-time only, approximately 12 h/day. After mating, male mouse was returned to the treatment schedule. Half of them were decapitated at the end of treatment period (K8) and the remaining half was decapitated at the end of post-treatment period (K8P8). The testis and epididymis were dissected and weighed. The sperm concentration, motility and viability, and testicular histology were examined.

Experimental procedure 1



0 wks	4 wks	8 wks	12 wks	16 wks
C	**C,M**	**C,M,A**	**C,M**	**C,M,A**

M Mated the treated male mouse with virgin female mice for 4 days

C Collected the blood sample for LH, FSH and T assays

A Autopsied half of mice in each group (n=5), weighed the testes and epididymes, and determined the sperm concentration, sperm motility and sperm viability

Experiment 2. **Effects of *P. mirifica* on hormone-related ovarian functions, reproductive organs and fertility in female mice**

The mice were divided into 4 groups (36 mice/group) as below.

Group I: a negative control or DW group, mice were orally treated with 0.2 ml/day of distilled water.

Group II: 10-PM group, mice were orally treated with the powder suspension of 10 mg/kg/day of *P. mirifica* in 0.2 ml of distilled water.

Group III: 100-PM group, mice were orally treated with the powder suspension of 100 mg/kg/day of *P. mirifica* in 0.2 ml of distilled water.

Group IV: a positive control or DES group, mice were subcutaneously injected with 200 µg/kg/day of diethylstilbestrol (DES) in 0.2 ml of corn oil.

The treatment schedule was separated into 2 periods; treatment and post-treatment. The duration in each period was 8 weeks.

In each group, mice were further divided into 6 subgroups (6 mice/group) as below.

Subgroup I: K4 group, mice were treated with DW or *P. mirifica* or DES for 4 weeks. After that, the three proestrous female mice were paired with fertile male mouse, and then washed out from the treatment schedule.

Subgroup II: K8 group, mice were treated with DW or *P. mirifica* or DES for 8 weeks. After that, the three proestrous female mice were paired with fertile male mouse, and then washed out from the treatment schedule.

Subgroup III: K8A group, mice were treated with DW or *P. mirifica* or DES for 8 weeks. After the cessation of treatment, they were decapitated. The ovaries and uterus were dissected, weighed, and preserved in 10% buffer formalin for histological study.

Subgroup IV: K8P4 group, mice were treated with DW or *P. mirifica* or DES for 8 weeks. After that, they were kept for 4 weeks of the post-treatment period and the three proestrous female mice were paired with fertile male mouse, and then washed out from the treatment schedule.

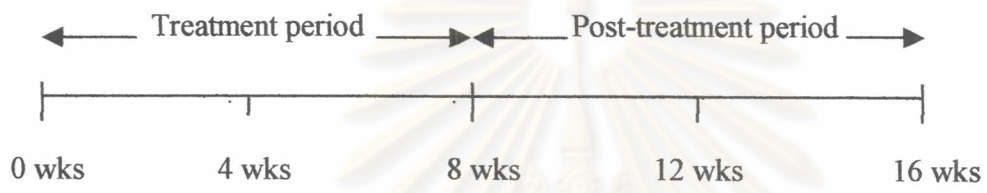
Subgroup V: K8P8 group, mice were treated with DW or *P. mirifica* or DES for 8 weeks. After that, they were kept for 8 weeks of the post-treatment period and the three proestrous female mice were paired with fertile male mouse, and then washed out from the treatment schedule.

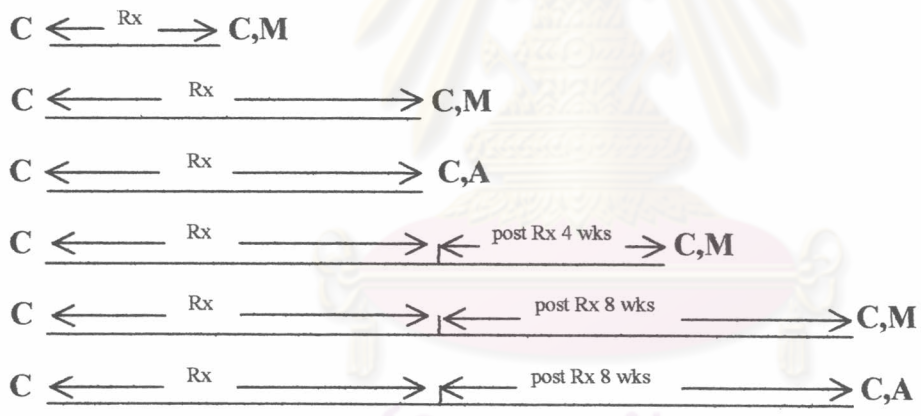
Subgroup VI: K8P8A group, mice were treated with DW or *P. mirifica* or DES for 8 weeks. After that, they were kept for 8 weeks of the post-treatment period and decapitated. The ovaries and uterus were dissected, weighed, and preserved in 10% buffer formalin for histological study.

Mice were collected blood by cardiac puncture for LH, FSH and E_2 assays in every 4 weeks. After the blood collection, the three female mice in subgroup I, II, IV, and V in the

proestrous stage was selected and paired with the fertile male mouse for 1 night. After mating, the female mice were washed out from the treatment schedule. They were observed for pregnancy and delivery.

Experimental procedure 2





M Mated the three treated female mice with fertile male mouse for 1 night

C Collected the blood sample for LH, FSH and E_2 assays

A Autopsies the female mice (n=6) at the end of treatment period for the Subgroup III and at the end of post-treatment period for the Subgroup VI, and weighed the ovaries and uterus

Rx Treatment period

Experiment 3. **Effects of *P. mirifica* on reproductive organs and malformation of litters born form *P. mirifica*-treated parents.**

After mating, all females were quantified the presence of sperm plug, pregnancy rates, duration of pregnancy, number of litters born and malformation of litters. The weights of litters were recorded at birth, 21 days of age, and every week thereafter. The pups were decapitated on 50 days of age, gonad and accessory sex organs were dissected and weighed.

3. Vaginal smear

Daily vaginal cytology was monitored using staging criteria for the phases of the estrous cycle; diestrus, proestrus, estrus, and metestrus. Vaginal smear was performed at 08.00-09.00 a.m. using a glass spatula with a physiological saline and spreaded on a glass slide. The vaginal epithelium cells observed under the microscope (x100 magnification) were classified into 3 types; leukocyte cells (L), nucleated epithelial cells (O), and cornified epithelial cells (Co). The representative cells-type was determined by choosing the majority.

4. Mating studies

Treated male mice

Male mouse was paired with three virgin female mice for 4 days (night-time only, approximately 12 h/day). Positive mating was verified by the presence of either sperm plug or the finding of sperm in the vaginal smear done between 08.00-09.00 a.m. in the morning and was considered to be the first day of pregnancy.

Treated female mice

Three female mice were paired with fertile male mouse for 1 day (night-time only, approximately 12 h/day). During experimental period, if female mice showed a regular estrous cycle, three proestrous female mice were selected and mated with male mouse, if they showed an irregular estrous cycle, three female mice were randomly picked up and mated with male mouse according to the protocol on Experiment 2 (every 4 weeks). Positive mating was verified by the presence of either sperm plug or the finding of sperm in the vaginal smear done between 08.00-09.00 a.m. and considered to be the first day of pregnancy.

5. Blood collection

The mice were anesthetized with diethyether in the dessicator jar. After anesthetization they were collected the blood for approximately 0.5 ml/time by a closure cardiac puncture (Semler, 1992) at 09.00-10.00 a.m. The 1 ml-syringe and the 26 G x 1/2

inch-needle were used for cardiac puncture. Blood samples were centrifuged at 2,000 rpm at 4°C for 30 minutes. The serum was separated and kept for analysis of FSH, LH and E_2 (in female mice) or T (in male mice).

6. Preparation of the powder suspension of *P. mirifica*

Pueraria mirifica stain Wichai-III was collected from Chiangmai province, Thailand. *P.mirifica* roots used in this study were the same lot, collected from the same locality and season. Its root was sliced and dried at 70-80 °C and pulverized in a mortar. The powder was filtered through a 100-mesh size. The powder was kept as a stock into the dark bottles at the room temperature. During treatment, the dried powder of *P.mirifica* was mixed with the distilled water into the concentrations of 10 and 100 mg/kg BW/day/0.2 ml distilled water. The suspension was force-fed to the mice during 10.00-11.00 a.m. using a gastric feeding needle size 19 x 11/2 inch with 1 ml-syringe (Semler, 1992).

7. Preparation of diethylstilbestrol solution

Diethylstilbestrol in the dose of 200 μg/kg BW/day (Sigma Chemical Company, Merck, USA) in 0.2 ml of corn oil was used as a positive control in this study. The powder of DES was weighed and dissolved in a small volume of absolute ethanol (Sigma Chemical Company, Merck, USA). After the powder was completely dissolved, the corn oil in appropriate volume was added. The solution was then standed at the room temperature to

evaporate the ethanol out. The DES solution was kept as a stock into dark bottles at the room temperature until used. The solution was subcutaneously injected to mice during 10.00-11.00 a.m. using 26 G x 1/2 inch -needle with 1 ml -syringe.

8. Semen analysis

Semen quality was determined by 3 parameters; sperm concentration, viability, and motility. They were interpreted as the spermatogenic suppression in various degrees.

8.1 Sperm collection

Mice were sacrificed and removed the cauda epididymis into 1 ml of phosphate buffer saline (PBS) (**Table 3.**). Cauda epididymis was cut to 3 pieces and incubated 37 °C in incubator maintaining at 5% CO_2 in air, for 10 min. The processes of semen analysis were done thereafter.

Table 3. Composition of phosphate buffer saline (PBS) pH 7.2 - 7.4.

Chemicals	Concentration (mM)	Concentration (g/l)
NaCl	136.89	8.00
KCl	4.77	0.36
$CaCl_2.2H_2.O$	1.70	0.25
KH_2PO_4	1.19	0.16
$Na_2\,HPO_412H_2.O$	4.29	1.54
$MgCl_2.\,6H_2.O$	0.49	0.09
$C_6H_{12}O_6.\,H_2.O$	5.05	1.00
$NaOOCCOCH_3$	0.25	0.03

8.2 Sperm concentration

Sperm concentration was analysed by standard World Health Organization (WHO) method (WHO, 1999), the heamocytometer method. Semen collected from cauda epididymis was diluted 1:20 with diluents (50g $NaHCO_3$, 10 ml of 35% formalin and distilled water for a final volume of 1000 ml). The diluted specimens were thoroughly mixed and transferred about 10 µl to the heamocytomete using a pasteur pipette. The counting chamber was left in a humid environment for 10 min to allow the spermatozoa to settle onto the counting grid before being counted under the phase-contrast microscope at 400x magnification. Spermatozoa concentration was estimated by counting the number of spermatozoa present in the four corner large squares and the center square of each of the two grids in the heamocytomete chamber **(Figure 4)**. The concentration of spermatozoa present in the

original semen sample is calculated as **the average of the total for the five squares for each of the two grids in the chamber**. The sperm concentration was expressed in term of millions/ml.

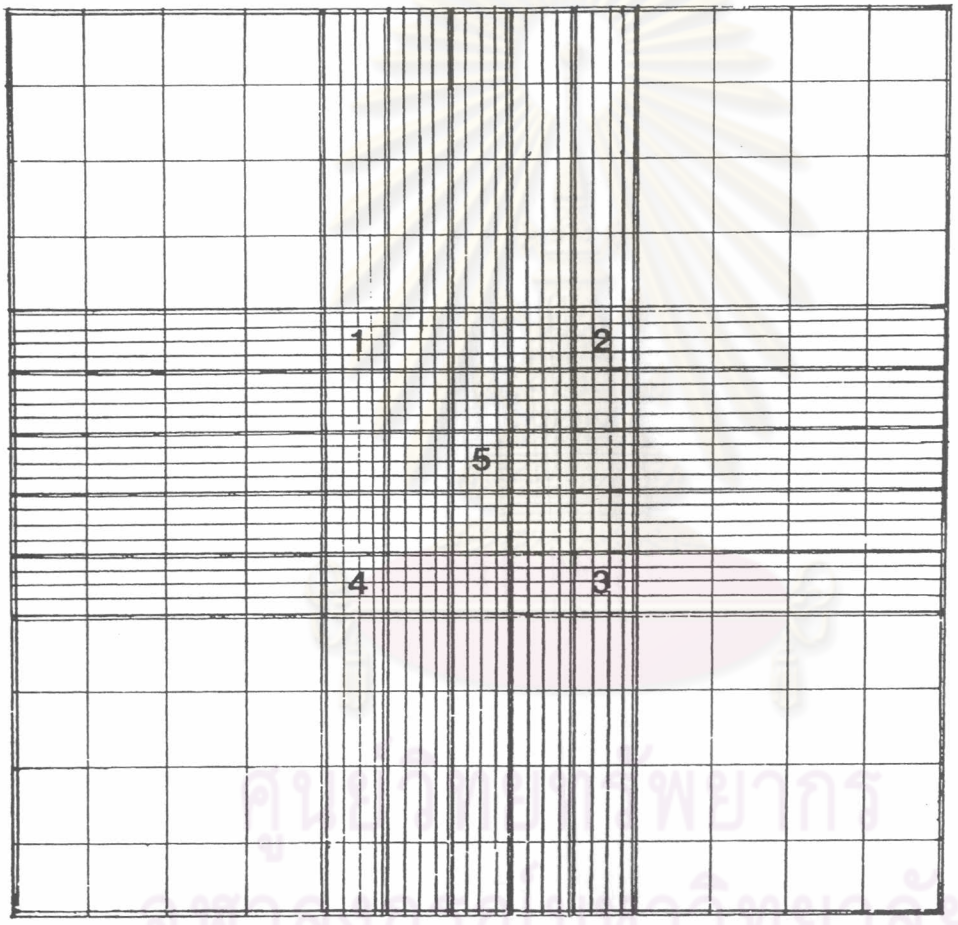


Figure 4. Chamber markings on an improved Neubauer haemocytometer

8.3 Sperm viability

Sperm viability were analysed by standard WHO methods (WHO, 1999), the eosin-nigrosin staining method. One drop of the original semen and 1% eosin y solution were placed on a clear glass slide and mixed together and left it for 30 second. After that, two drops of 10% nigrosin solution, using as a background, were mixed with a suspension of semen and smeared on the glass slide. The specimen was left in the room temperature until it was dried up. The viable sperm, which were not stained with eosin y, and dead sperm, which were stained with eosin y, were counted under the light microscope. The percent of viable sperm was calculated as **the number of viable sperm per the total number of counted sperm x 100**.

8.4 Sperm motility

Sperm motility were analysed by multiexposure photography method (Makler, 1978). A drop of semen from a well-mixed, undiluted sample was placed on a clear glass slide and covered with a small cover slip. The weight of the cover slip on semen drop spreaded the sample for optimal viewing. The slide was immediately examined in room temperature. The determination of sperm motility was done as mentioned below.

The slide was put on a stage of a phase-contrast microscope, and a 10x objective and 15x eyepiece were used. A still camera loaded with 100 ASA black and white film (Kodak T max 100 film; Px 135-36) was attached with the aid of a bellows or extension

rings to the microscope. The bellows or rings were adjusted so that the film will cover an area of approximately 0.5 x 0.35 Sq mm. With the microscope fine adjustment knob, spermatozoa that were moving within the space of cover slip, rather than nonmotile spermatozoa lying on the bottom, were brought into focus; thus all spermatozoa could be seen clearly on the film. While exposed for 1 full second, six light pulses, each of 1/200–second duration and at intervals of 1/6 second, were given. This multiple exprosure technique was achieved by a stroboscope made of a six – slotted black disc placed between the light source and the condenser of the microscope and rotating at 60 rpm (1 rps) by electric motor **(Figure 5)**. As a result during this 1 second, only about 1/30 of the light penetrates the camera, so that images of nonmotile spermatozoa were not appear overexposed, motile spermatozoa were not underexposed, and both were clearly represented on the film. Practically, images of nonmotile spermatozoa were 6 – fold brighter than images of motile spermatozoa were. The latter were seen as small six – ringed chains, were their length described the true distance that they traveled during this 1second. The percent of sperm motile was calculated as **the number of motile sperm per the total number of counted sperm**.

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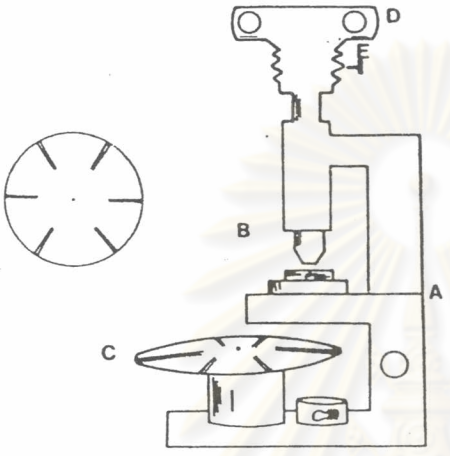

Figure 5. Assembly of the system of multiple exposure photography.

9. Hormonal determinations

Concentrations of serum FSH and LH were measured by heterologous RIA system. 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 LH-RP-3 reference standards (Niswender *et al.*, 1968).

Serum concentrations of E_2 and T were measured using the established RIA methods of WHO after the samples were extracted with ether (Sufi *et al.*, 1986).

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 2.24%, 6.34%, 10.71% and 12.64% respectively.

10. Histological analysis

After overnight fixation in formalin, ovary, uterus and testis 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, 1972). Tissue sections were photographed using a Nikon camera mounted on the microscope. Permanent slides of all experiment organs were analyzed under Olympus microscope and photograph.

11. Statistical analysis

The results were expressed as mean ± standard error (SE). The relative organ weight was calculated as the organ weight per the body weight of mice. Statistical analyses were performed using SPSS version 11 software. T – test was submitted for comparison the data between 0wks and other wks of treatment and post-treatment periods in each group. Comparisons between control and treated groups were done by one-way analyses of variance (ANOVA) for factorial or repeated measures designs with post – hoc testing by LSD test. The P values of less than 0.05 ($P < 0.05$) were considered to be statistically significant.