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 gm, used in this study were supplied from National Laboratory Animal Centre, Mahidol University. They were housed as 5 animals/cage. Rat chow and tap water was provided *ad libitum*. A standard animal husbandry environment of controlled lighting (lights on 06.00-20.00 h) and temperature (25 ± 1°C) at Primate Reseach Unit, Department of Biology, Faculty of Science, Chulalongkorn University was maintained. All experiments were performed between 08.00 -10.00 h.

2. Plant materials

The tuberous roots of wild *P. mirifica* were collected from 25 provinces of Thailand (from the northernmost to upper peninsula parts); Chiang Rai, Mae Hong Son, Phayoa, Nan, Lampang, Phrae, Lamphun, Uttharadith, Sukhothai, Tak, Phitsanulok, Phetchabun, Kamphaeng Phet, Nakorn Sawan, Uthai Thani, Sakon Nakhon, Nong Bua Lam Phu, Chaiyaphum, Nakorn Ratchasima, Lop buri, Kanchanaburi, Phrachin Buri, Ratchaburi, Phetchaburi and Chumphon. (Cherdshewasart, 2003[a]) (Figure 3-1). To minimize the seasonal variation of phytoestrogens content in *P. mirifica*, the tuberous roots used in this study were collected in summer period (From March to April).

The collected tubers were cleaned, sliced, dried in a hot air oven at 70°C until nearly completely dried and subsequently ground into powder at a size of 100 Mesh (Cherdshewasart, 2004[a]).

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Figure 3-1. Sources of collected plant materials(* Reported by Chansri, 2002).

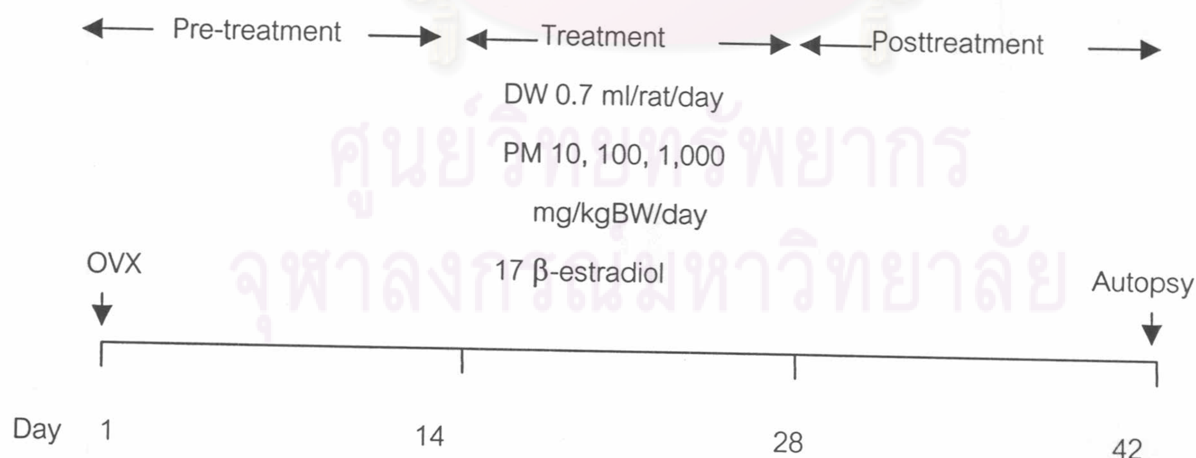
3. Experimental design

When the rats showed a diestrous phase (leucocyte cells) on the forth estrous cycle, they were ovariectomized (OVX) under ether anesthesia. The day of ovariectomy was designed as day 1 of the study period. Rat wer devided in 2 major groups; *P. mirifica* treatment group and control group. For each cultivar of *P. mirifica*, rats were divided in to 3 groups (5 rats per group); 10, 100, 1,000 mg/kgBW/day of *P. mirifica*. The rats were fed with distilled water and subcutaneously injected with 17β- estradiol during treatment period for negative and positive control groups, respectively. The treatment schedule was separated into 3 periods: pre-treatment, treatment and post-treatment, in each group as follow;

In the pre-treatment period, the rats were administered with 0.7 ml distilled water for 14 days.

In the treatment period, the rats were fed with 10, 100 and1,000 mg/kg BW/day/0.7ml DW of *P. mirifica* suspension for 14 days.

In the post-treatment period, the rats were fed with distilled water for 14 days, they were decapitated on the next day (day 42) under ether anesthesia, and the uteri were dissected and weighed. The uteri collected at the end of the post-treament period were manipulated for histological study.



4. Vaginal cytology assay

The vaginal smears were checked daily during experiment between 08.00-09.00 h. A small glass rod was sterilized with 70% alcohol solution and soaked into 0.9% normal saline solution (NSS) before use. The glass rod 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 the light microscope (100X) and recorded the cell type. The cell-type was classified as follows (Figure 3-2);

O = the nucleated cells found in the proestrous period.

Co = the cornified cells found in the estrous period.

L = the leucocyte cells found in both metestrous and diestrous periods.

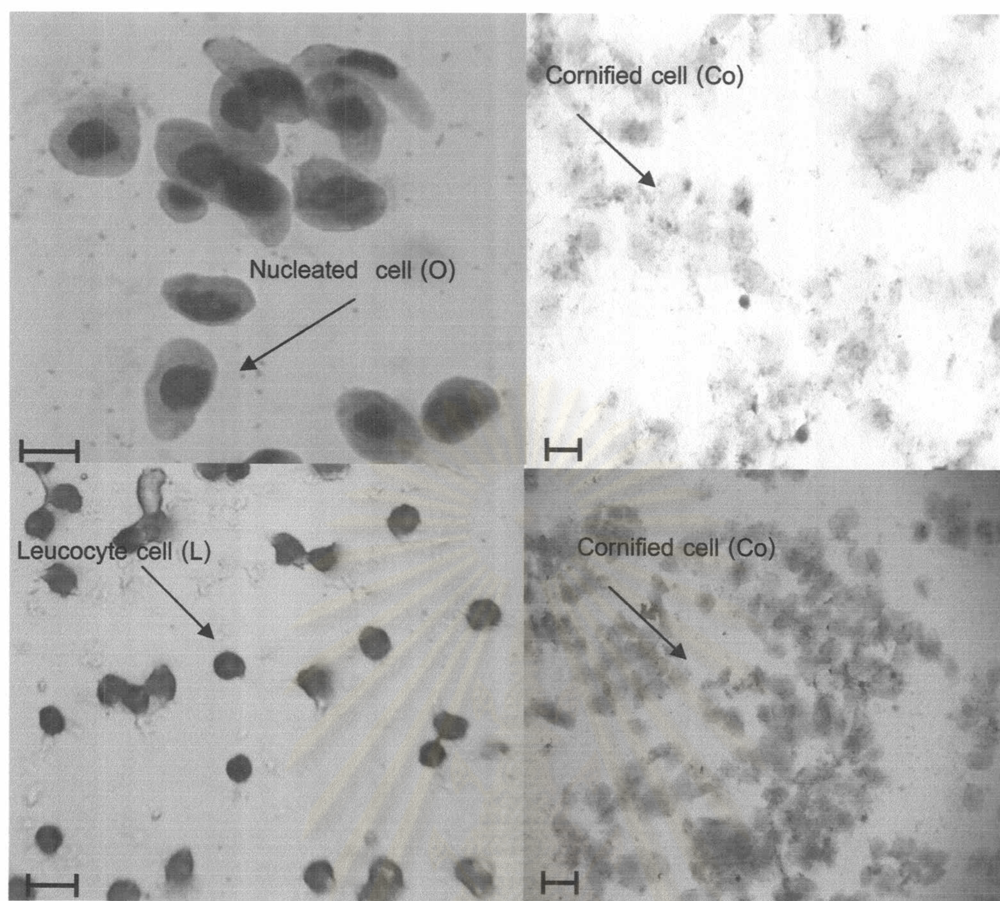


Figure 3-2. Vaginal epithelium cells found in the different phases of estrous cycle in rats. L-type cells, O-type cells, Co- type cells indicate leucocyte, nucleated and cornified cells, respectively) (The scale bar represented 10 μm).

The representative cell-type was chosen from the majority. Vaginal smear cells counts were performed randomly on 100 cells. The percentage of cornified cells was calculated according to Terenius (1971) as follows;

$$\text{Percentage of cornified cells} = \frac{\text{Number of cornified cells}}{\text{Number of leucocytes + cornified cells + nucleated cells}} \times 100$$

5. Uterotrophic assay and histological study

The uterus was removed, trimmed the fat tissue, and cut just above the junction between the cervix and the uterine horns. The uterus was weighed and fixed in 10% buffer formalin at least 24 hours and processed according to the standard histological techniques (Humanson,1979). Histological analysis were send to Physiological Laboratory of Chulalongkorn University. It was performed under a light microscope.

6. Statistical analysis

The result from vaginal cytology were compared between dosage and region in Thailand to seek for the high estrogenic activity that have the earliest and longest response.

Uterus weight and body weight were analyzed by one way analysis of variance using a PC-based version of the Statistical Program for the Social Sciences (SPSS) program version 10.0.

The correlation between in estrogenic activity determined by vaginal cytology assay and MCF- 7 proliferation assay, or between the estrogenic activity determined by vaginal cytology assay and the isoflavone content are also analyzed by SPSS program, version 10.00. The significant level was taken at $P \leq 0.05$.