Chapter IV

Ovulation-Block by *Pueraria mirifica*, an Indigenous Thai Herb: A Study of Its Endocrinological Effect in Adult Female Cynomolgus Monkeys.

Abstract

Pueraria mirifica, an indigenous Thai herb, containing phytoestrogens, is suggested to act as estrogen and to influence on reproduction. Therefore, we investigated long-term effect of this plant on the menstrual cycle length and concentrations profile of reproductive hormones, using nine normal cyclic monkeys (*Macaca fascicularis*), which were separated into 3 groups. Each group (n = 3) was fed with 10, 100, and 1,000 mg/day of *P. mirifica* for 3 cycles. The first day of menstruation was designated as the day 1 of the cycle. Blood samples were collected on days 3, 9 - 14, 19, 24, 29; and then, every 10 days until the next menstruation. The result shows that the length of menstrual cycles was increased significantly during the treatment period in monkeys with 10 and 100 mg/day of *P. mirifica*. Menstruation stopped completely throughout the treatment and post-treatment periods in monkeys treated with 1,000 mg/day of *P. mirifica*. Follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and progesterone levels were lower during treatment in a dose-dependent manner. The menstrual cycle length and levels of these hormones were recovered during post-treatment in monkeys treated with 10 and 100 mg/day of *P. mirifica*. These findings demonstrated that *P. mirifica* greatly influences the menstrual cycles and suppresses the ovulation by lowering serum levels of gonadotropins.

Key words: *Pueraria mirifica*, cyclic monkey, gonadotropin, ovarian hormone, phytoestrogen

4.1 Introduction

Pueraria mirifica, called as "white kwao krua" in Thai, is an indigenous Thai herb that belongs to family Leguminoceae. This plant are of interest because its tuberous root abundantly contains many kinds of phytoestrogens having estrogenic potencies, such as miroestrol (Pope et al., 1958), puerarin (Pope et al., 1986), deoxymiroestrol, kwakhurin (Chansakaow et al., 2000a, 2000b), and others in the isoflavone and coumestrol groups (Ingham et al., 1986, 1989). In recent years, the use of *P. mirifica* as an alternative medicine has become popular in Thailand. Many products in the form of cream, tablet, and solutions were developed from *P. mirifica* roots, and are widely used in normal cycling women as an age rejuvenation drug and cosmetic products including breast enlargement creams, skin moisturizers, and eye gels. However, there were no scientific reports showing its estrogenic effect on reproduction and hormones in humans.

Estrogenic effects of this plant to alter reproductive function have been found in mice and rats (Jones and Pope, 1960; Songkaew and Smitasiri, 1985). Miroestrol, phytoestrogenic substance that found only in *P. mirifica* roots, increased uterine weight in immature female mice (Jones and Pope, 1960). Its crude extract stopped rat pregnancy when it was given during embryo transport (Songkaew and Smitasiri, 1985). In addition, phytoestrogen isoflavones from other plants were firstly reported as causing sheep infertility (Bennetts et al., 1946). Coumestrol isolated from alfalfa reduced the ovulation rate in mice (Fredricks et al., 1981). Furthermore, previous reports showed that soy isoflavones disturbed hormonal characteristics in premenopausal women, although there have been conflicting reports (Cassidy et al.,

1994, 1995; Duncan et al., 1999; Lu et al., 1996, 2000). From the epidemiological data, Japanese and Chinese women who consumed high amounts of soy diet had lowered circulating levels of estradiol during menstrual cycles (Bernstein et al., 1990; Nagata et al., 1998).

From these evidences, it is important to investigate the exact effects of *P. mirifica* containing phytoestrogens on the menstrual cycle and reproductive hormones. To avoid the limitations of the long-term study effect of *P. mirifica* in humans, a nonhuman primate, the cynomolgus monkeys (*Macaca fascicularis*) were used as the model of normal cyclic women. Female cynomolgus monkey has been proved to have similar reproductive function and hormonal pattern with those in women (Krajewski et al., 2003), and offer the advantage of allowing control diet and exposure to other environment factor that may confound the influence. Therefore, this study examined the changes in the menstrual cycle length and the concentration of reproductive hormones in adult cyclic cynomolgus monkeys treated with *P. mirifica.*

4.2 Materials and Methods

4.2.1 Animals

Adult female cynomolgus monkeys (*Macaca fascicularis,* n=9) with regular menstrual cycles for at least 4 consecutive months, 30 $\pm$ 4 days in length and weighing 4.0 – 6.5 kg before the study, were used. The first day of menstrual bleeding was designated as day 1 of the menstrual cycle. The monkeys were housed in individual cages at the Primate Research Unit, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Lighting conditions of the animal room were controlled (12 : 12 h light to dark cycle). Temperature and humidity fluctuated

slightly depending on the season. The monkeys were fed daily with monkey chow (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) in the morning (09:00 – 10:00 h) and supplemented with fresh fruits in the afternoon (14:00 – 15:00 h). The experimental protocol was approved by the ethical committee in accordance with the guide for the care and use of laboratory animals prepared by the Primate Research Unit, Chulalongkorn University.

4.2.2 Experimental Design

Nine female monkeys were divided into three groups; each group was fed with the suspension of *P. mirifica* at doses of 10, 100, and 1,000 mg/5 ml of distilled water/individual/day, respectively. The treatment schedule was composed of 3 periods: pre-treatment, treatment, and post-treatment. Occurrence of menstrual bleeding was checked daily by vaginal swabbing method. During the pre-treatment and post-treatment periods, all monkey groups were fed daily 5 ml of distilled water at 08:00 h for one and two menstrual cycles, respectively. During the treatment period, all monkey groups were fed the suspension of *P. mirifica* for 3 menstrual cycles. However, in the treatment and post-treatment periods, if monkeys had no menstrual bleeding, 90 and 60 days were allotted to treatment and post-treatment periods, respectively. Three-ml blood samples were collected from the femoral vein without anesthetization between 08:00 – 09:00 h on day 3 (the early follicular phase), days 9, 10, 11, 12, 13, and 14 (the late follicular phase); days 19, 24, and 29 (the late luteal phase) of the menstrual cycle. However, in the treatment and post-treatment periods, if there were no menstrual bleeding, blood collections were performed every 10 days until 90 and 60 days during the treatment and post-treatment periods, respectively. After that, Blood samples were centrifuged at 1,700 x g, 4 ° C, for 20 minutes, and sera were stored at –20 ° C until follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and progesterone assays.

4.2.3 Preparation of the Suspension of *P. mirifica*

The fresh tuberous roots of *P. mirifica* were sliced, dried in hot air oven at 70 ° C, and subsequently ground into powder at size of 100 Mesh. Then, the stock of its powder was kept in the dark desiccator until the preparation of its suspension. *P. mirifica* powder was suspended into 5 ml of distilled water, and then kept in a dark bottle at 4 ° C until the feeding time.

4.2.4 Hormonal Analyses

Concentrations of serum FSH and LH were measured by heterologous RIA system. Iodinated preparations were rat NIDDK-rat FSH -I-5 and rat LH-I-5. The anitisera used were anti-ovine FSH (NIDDK-H-31) and anti-ovine LH (YM#18) (Hodgen et al., 1976). The latter was kindly provided by Dr.Y.Mori (University of Tokyo, Tokyo, Japan).

Serum concentrations of progesterone were determined by a double-anti body RIA system using ^{125}I-labeled radioligands as described previously (Taya et al., 1985). Antisera against progesterone (GDN#377) were kindly provided by Dr. G. D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, U.S.A). After extraction of ether, serum level of estradiol was determined by double-antibody RIA with ^{3}H-labeled radioligands as described in the established method of World Health Organization (WHO) (Sufi et al., 1986).

4.2.5 Statistical Analysis

The lengths of menstrual cycle of each monkey group were expressed as the mean $\pm$ S.E.M. The significance of the differences between the means was evaluated by the Analysis of Variance (ANOVA). The observed significance was then confirmed using the least significant difference (LSD) test. $P < 0.05$ was considered to be statistically significant.

4.3 Results

4.3.1 Changes in Menstrual Cycle Length of Monkeys Treated with *P. mirifica*

The menstrual cycle length during the pre-treatment period in nine monkeys was 28.2 ± 0.8 days. The menstrual cycle lengths after treatment with 10, 100, and 1,000 mg/day of *P. mirifica*, as shown in Table 4.1, were increased, and they were recovered during the post-treatment period in all monkeys treated with 10 mg/day of *P. mirifica* and one of three monkeys treated with 100 mg/day of *P. mirifica*, but no mokeys treated with 1,000 mg/day. When menstrual cycle length was analyzed in each monkey, it was found that 10 mg/day of *P. mirifica* extended the menstrual cycle length to 50, 81, and 52 days in monkey no. 601, to 49 and >90 days in monkey no. 627, and completely stopped the menstruation throughout the treatment period in monkey no. 619. The extended menstrual cycles recovered in all monkeys during the post-treatment period. In monkeys treated with 100 mg/day of *P. mirifica*, the menstrual cycles became shorter during the early treatment period to 15 days in monkey no. 616 and to 21 days in monkey no. 801. Subsequently their cycles were stopped throughout the treatment period. Monkey no. 108 did not show menstruation throughout the treatment and post-treatment periods. All monkeys (nos. 624, 77, and 633) treated with 1,000 mg/day of *P. mirifica* showed complete cessation of menstruation throughout the treatment period and did not recover the cycle in the post-treatment period.

4.3.2 Changes in Serum Levels of Gonadotropins and Ovarian Hormones in Monkeys Treated with *P. mirifica*

To evaluate changes of gonadotropin and ovarian hormones in monkeys treated with *P. mirifica*, their concentration patterns during the treatment and post-treatment periods were compared with those during the pre-treatment period.

In monkeys treated with 10 mg/day of *P. mirifica*, as shown in Figure 4.1, there were no changes in pattern of gonadotropins and ovarian hormones during the treatment and post-treatment periods. All monkeys showed peak levels of FSH and LH during the follicular phase while progesterone levels remained low during the treatment periods. The increased estradiol levels appeared to coincide with the increased levels of FSH and LH. After the decline of FSH and LH levels, progesterone levels elevated slightly.

Monkeys treated with 100 mg/day of *P. mirifica* had no surges of FSH and LH during the treatment period (Figure 4.2). The basal levels of FSH decreased significantly and could be recovered during the post-treatment period; meanwhile, basal levels of LH did not decrease significantly. There was no evidence of high levels of estradiol and progesterone during treatment in monkeys nos. 801 and 108. However, high level of estradiol was observed in the first menstrual cycle of monkey no. 616. After that, serum levels of estradiol and progesterone were recovered in all monkeys.

As shown in Figure 4.3, serum levels of gonadotropins and ovarian hormones in monkeys treated with 1,000 mg/day of *P. mirifica* were obviously decreased. There were no high levels of FSH, LH, estradiol, or progesterone throughout the treatment and post-treatment periods in all monkeys. Furthermore, the basal levels of FSH and LH decreased prominently during the treatment period and were recovered during the post-treatment period.

Table 4.1 The menstrual cycle length of monkeys treated with 10, 100, and 1,000 mg/day of *P. mirifica* during the treatment and post-treatment periods.

	Menstrual cycle length (days)	
Treatment group	Treatment period	Post-treatment period
10 mg/day	no. 601: 50, 81, 52	no. 601: 29, 28
	no. 627: 49, > 90 [CC]	no. 627: 36, > 60 [NR]
	no. 619: > 90 [CC]	no. 619: 38, 30
100 mg/day	no. 616: 15, > 90 [CC]	no. 616: > 60 [NR]
	no. 801: 21, > 90 [CC]	no. 801: 38, 32
	no. 108: > 90 [CC]	no. 108: > 60 [NR]
1,000 mg/day	no. 624: > 90 [CC]	no. 624: > 60 [NR]
	no. 77: > 90 [CC]	no. 77: > 60 [NR]
	no. 633: > 90 [CC]	no. 633: > 60 [NR]

The menstrual cycle length during the pre-treatment period in nine monkeys was 28.22 $\pm$ 0.78 days. CC represents a complete cessation of menstruation during the treatment period. NR represents that the non-recovery of the menstruation during the post-treatment period.

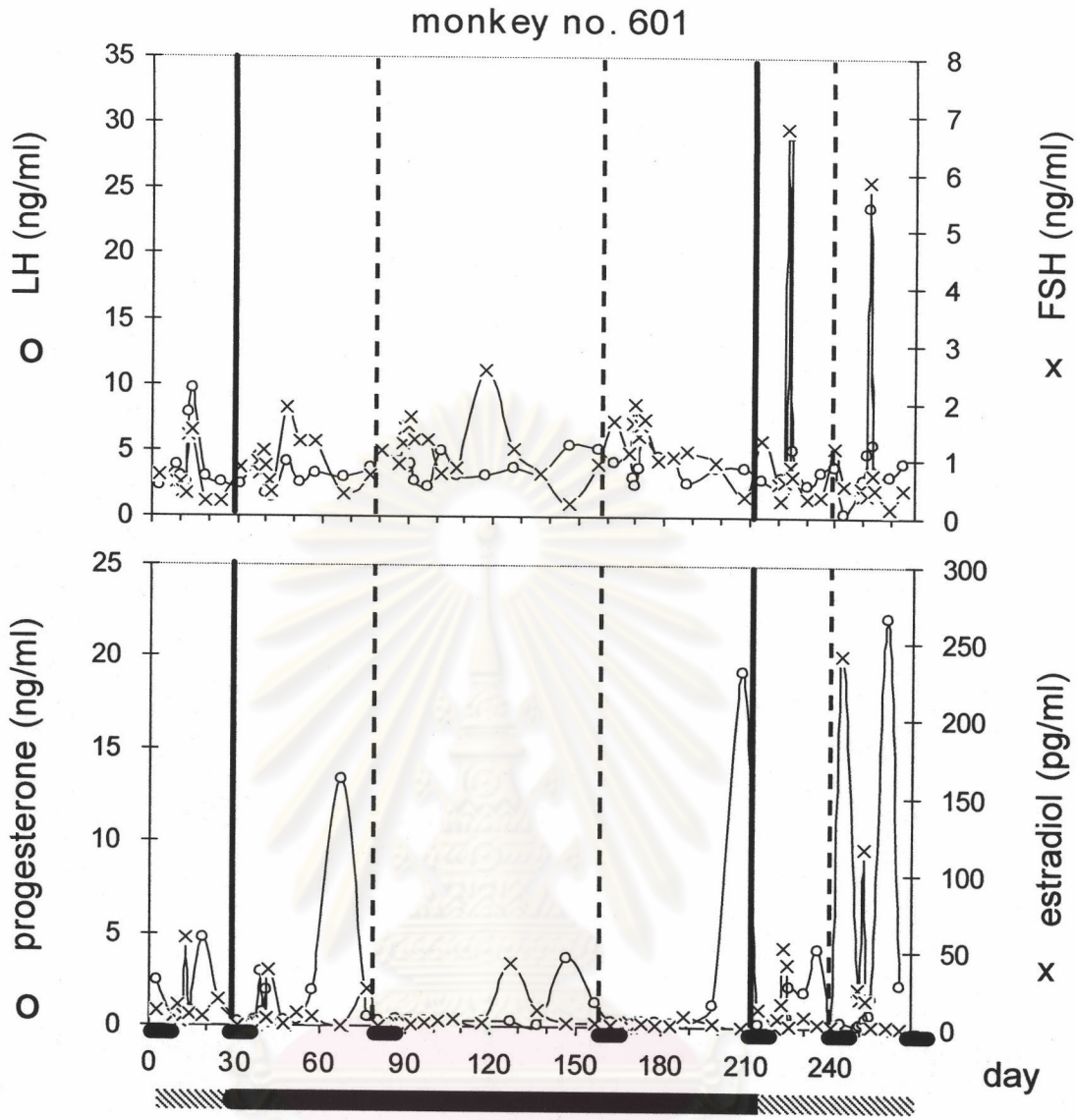


Figure 4.1 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkeys treated with 10 mg/day of *P. mirifica*. Day 1 represents the day of menses in the pre-treatment period. The short horizontal bars at the abscissa represent the day of menses. Period between solid vertical lines (thick bar bellow the abscissa) represents the treatment period. The dotted vertical lines represents each menstrual cycle.

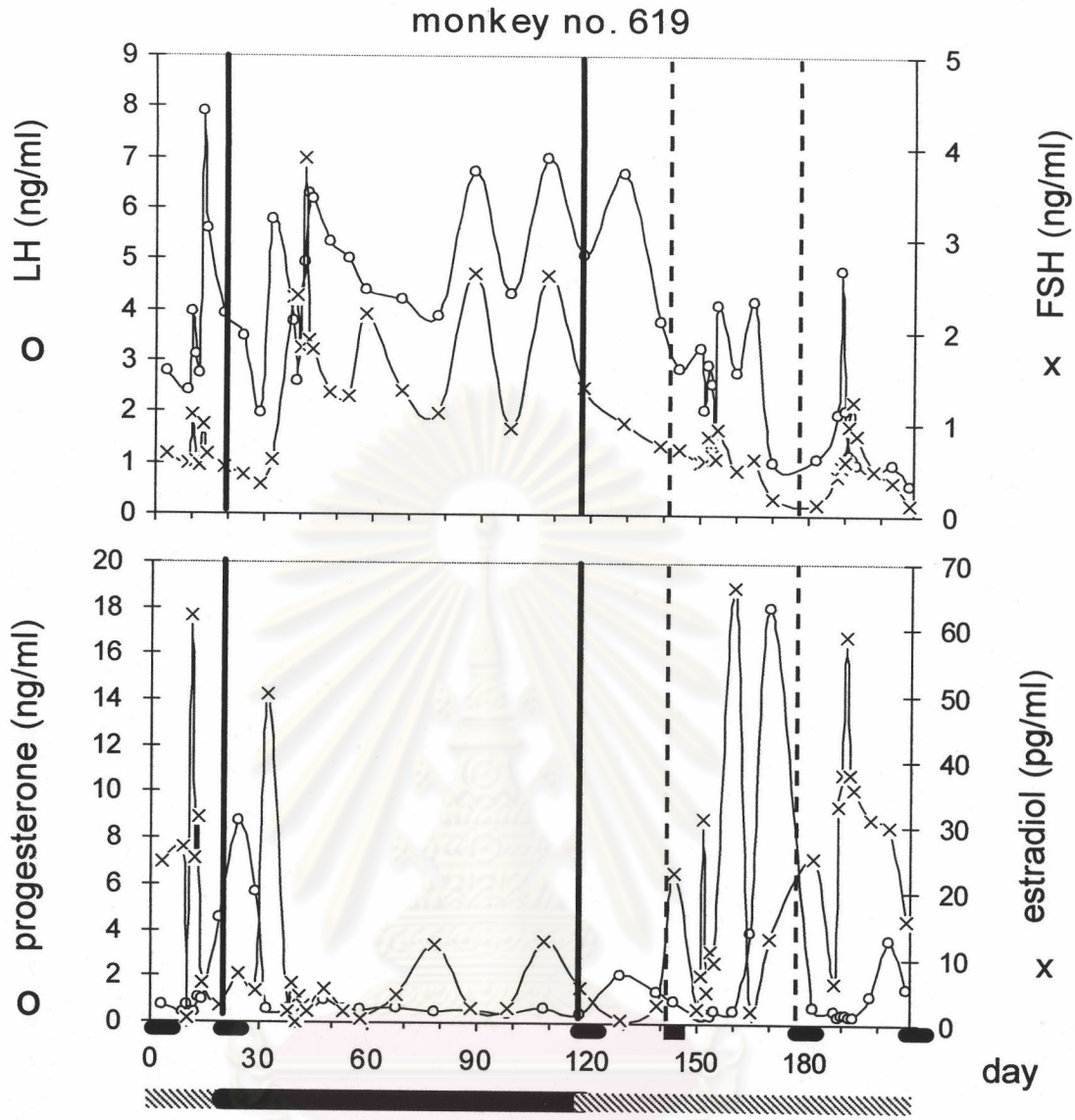


Figure 4.1 (cont.)

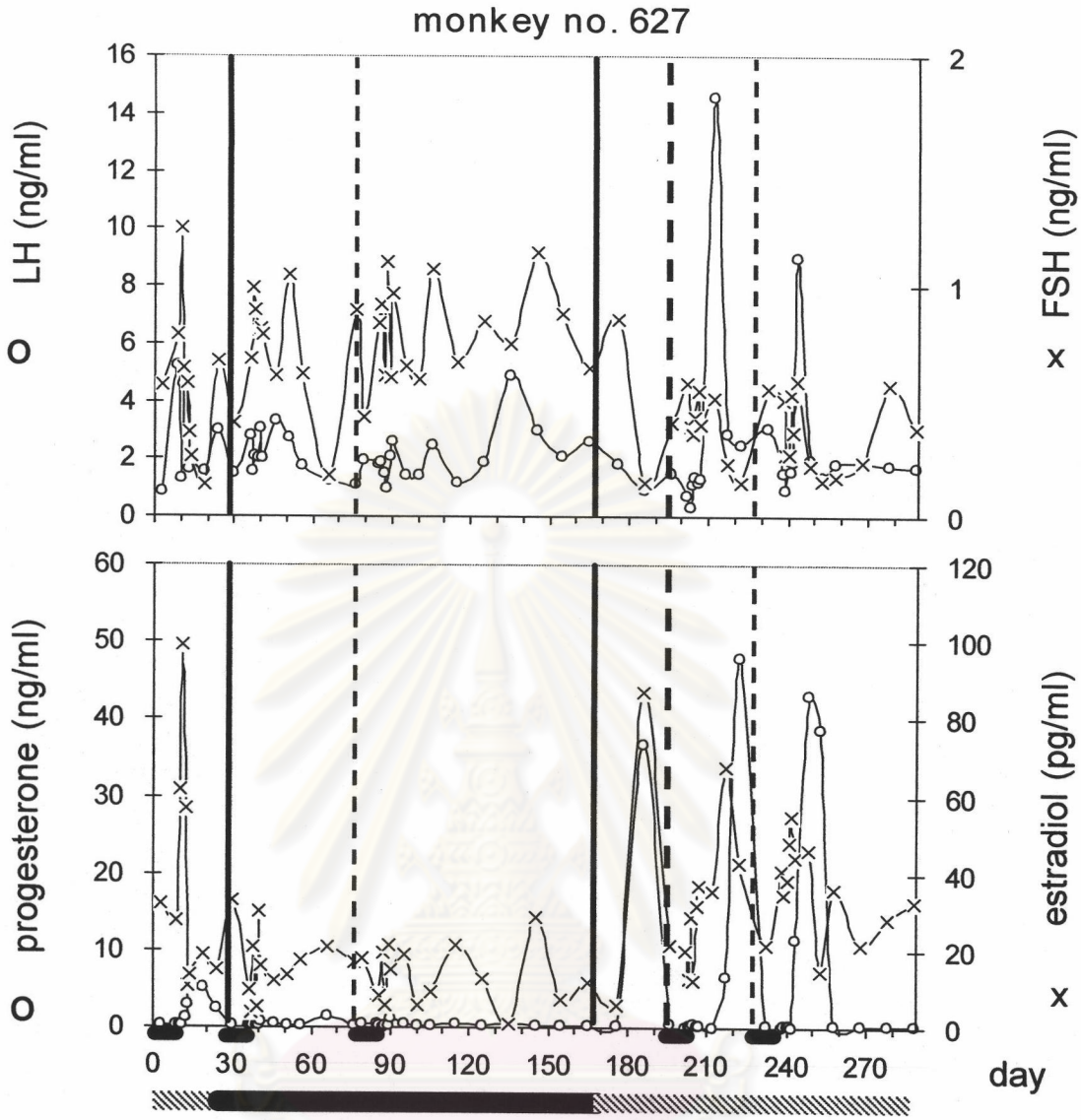


Figure 4.1 (cont.)

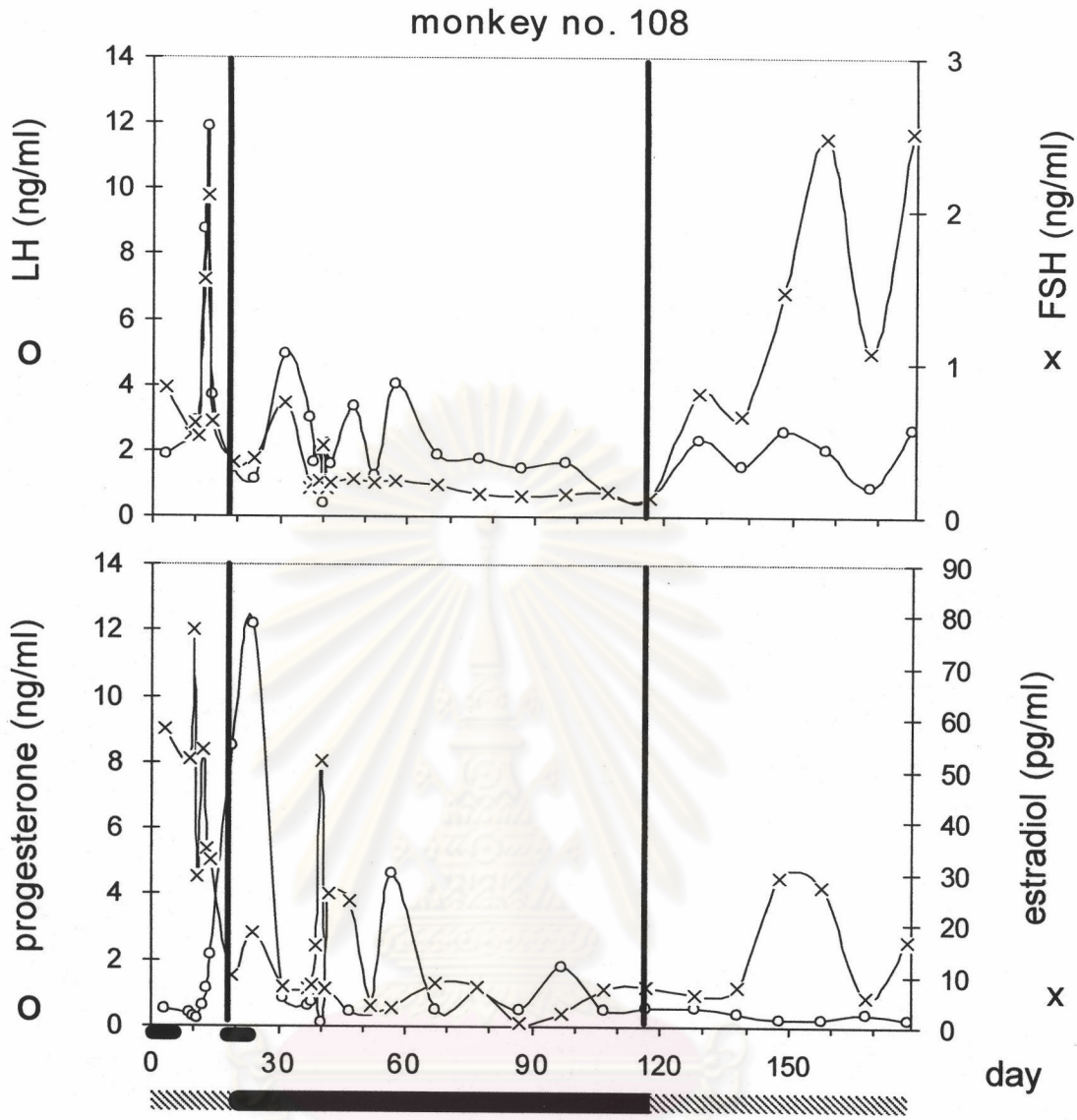
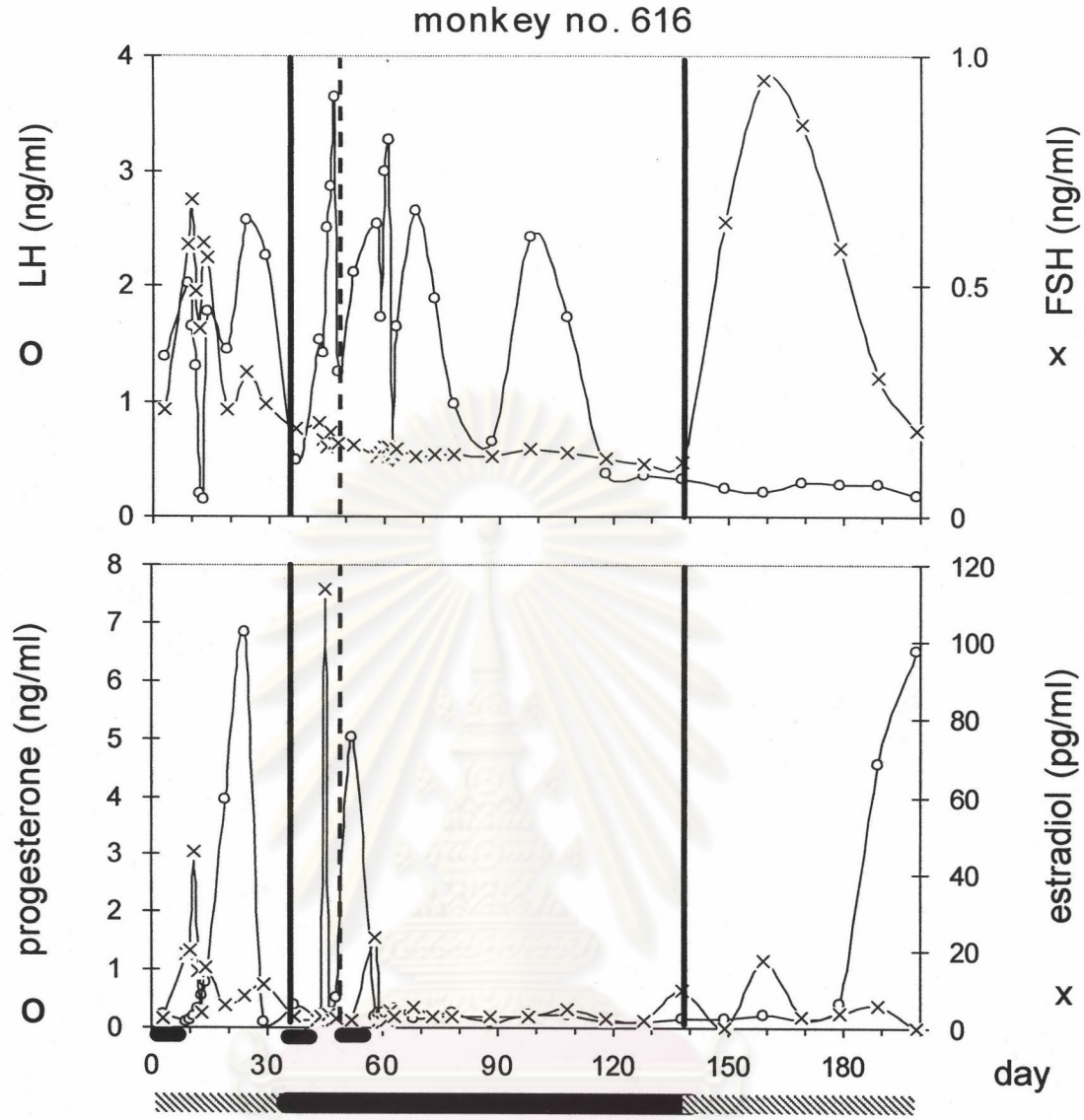


Figure 4.2 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkeys treated with 100 mg/day of *P. mirifica*. The meaning of the horizontal bars and vertical lines were the same as in Figure 4.1



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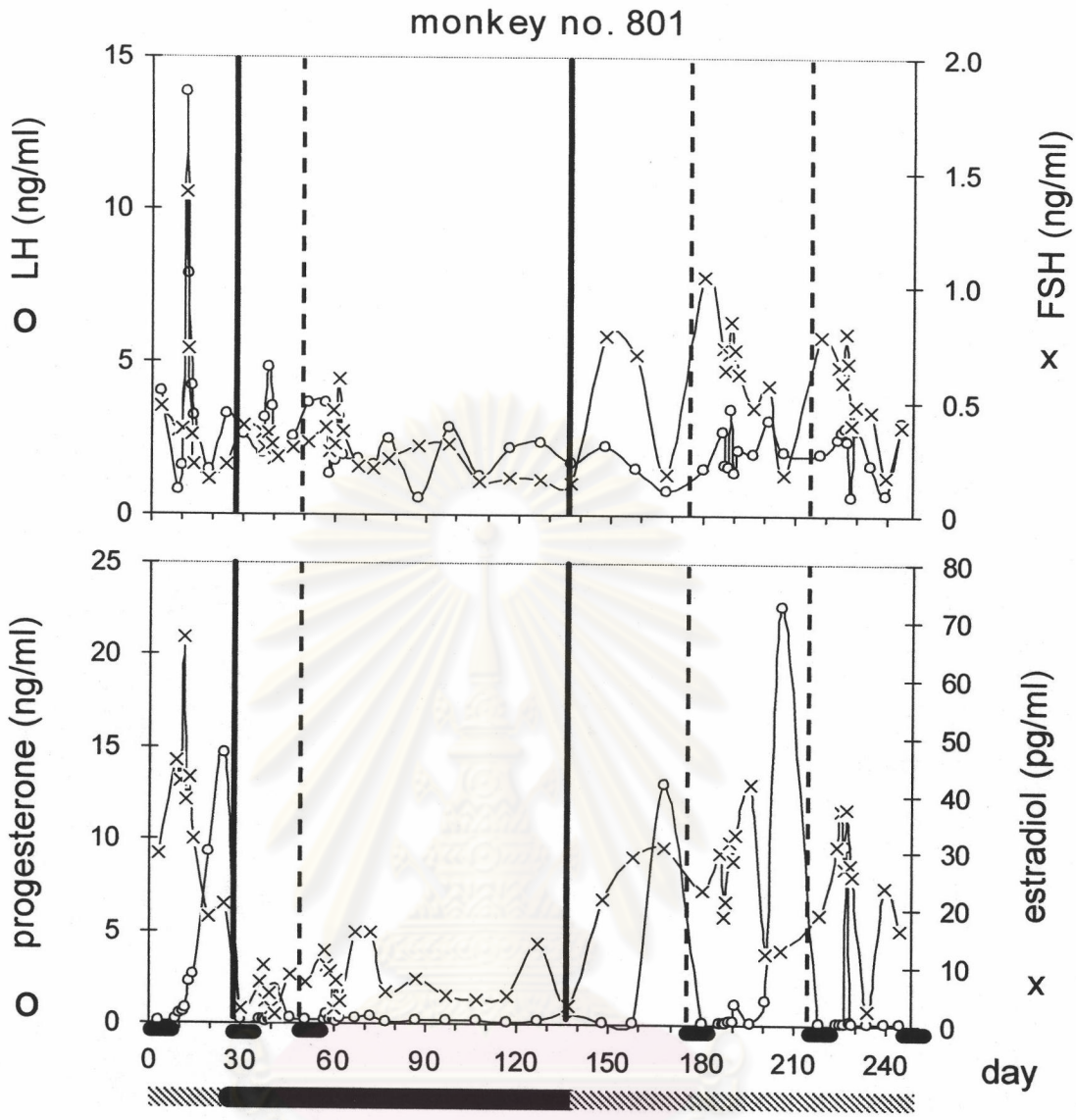

Figure 4.2 (cont.)

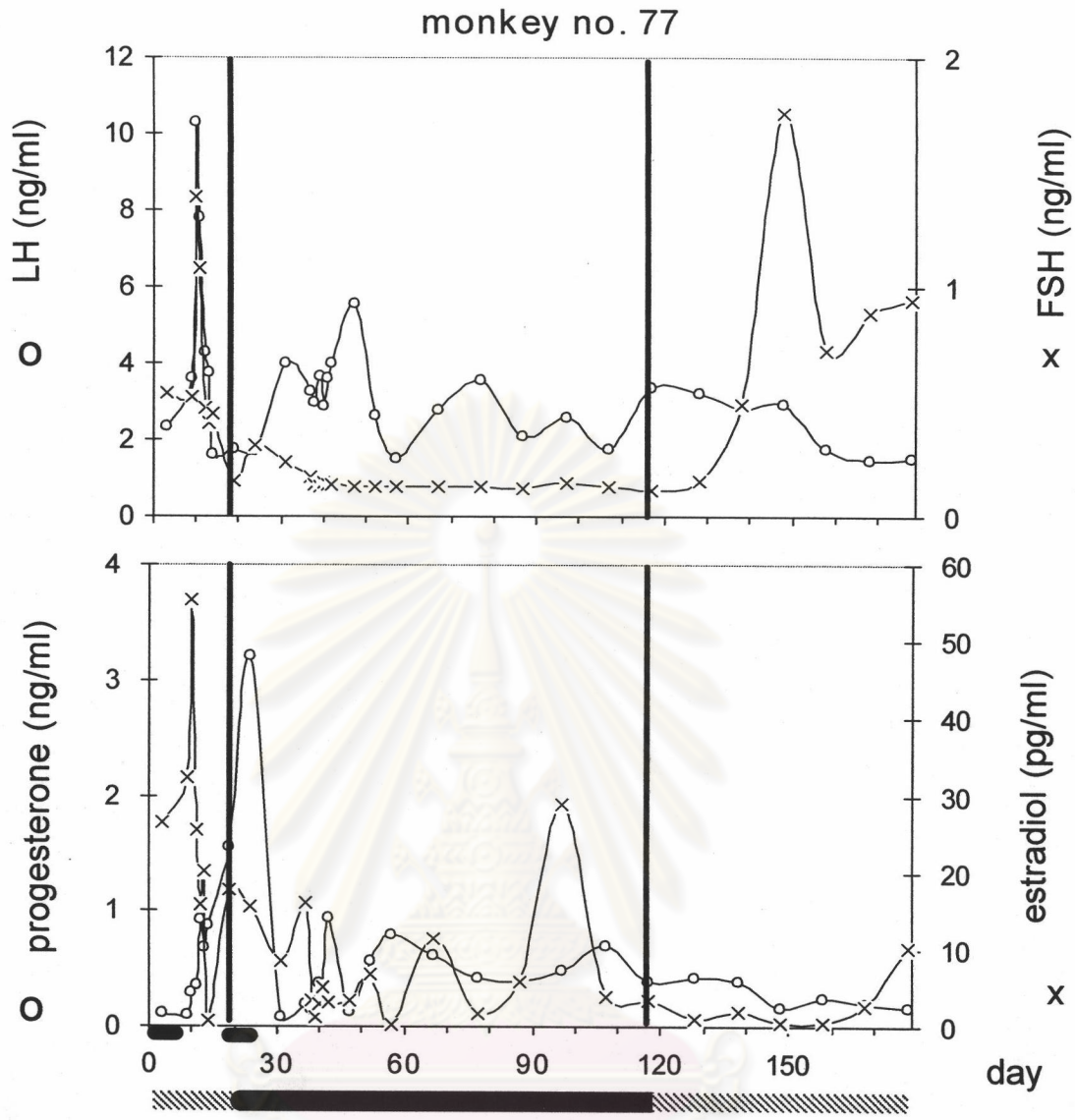

Figure 4.3 Changes in serum levels of gonadotropins (FSH and LH) and ovarian hormones (estradiol and progesterone) of monkeys treated with 1,000 mg/day of *P. mirifica*. The meaning of the horizontal bars and vertical lines were the same as in Figure 4.1

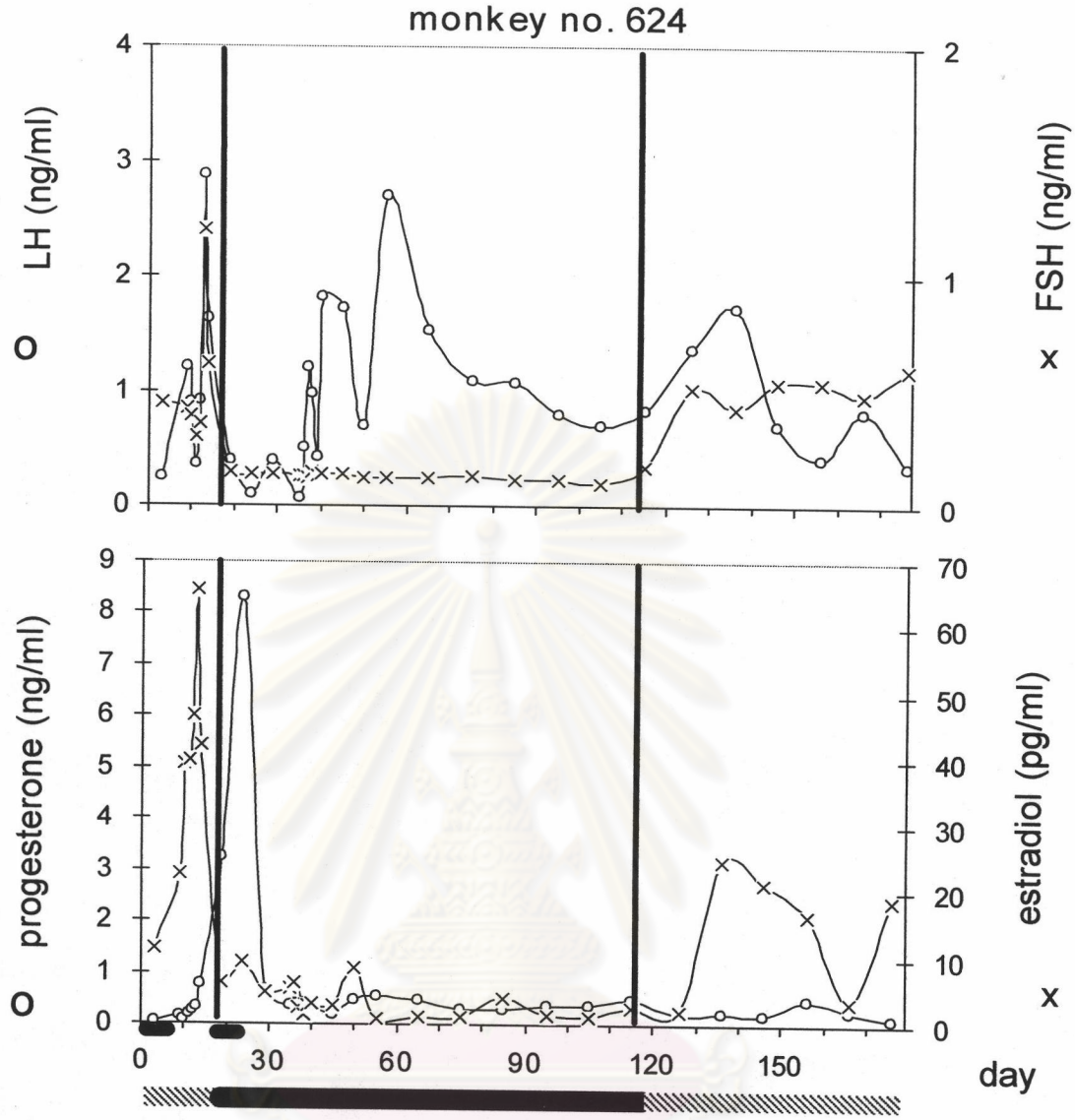

Figure 4.3 (cont.)

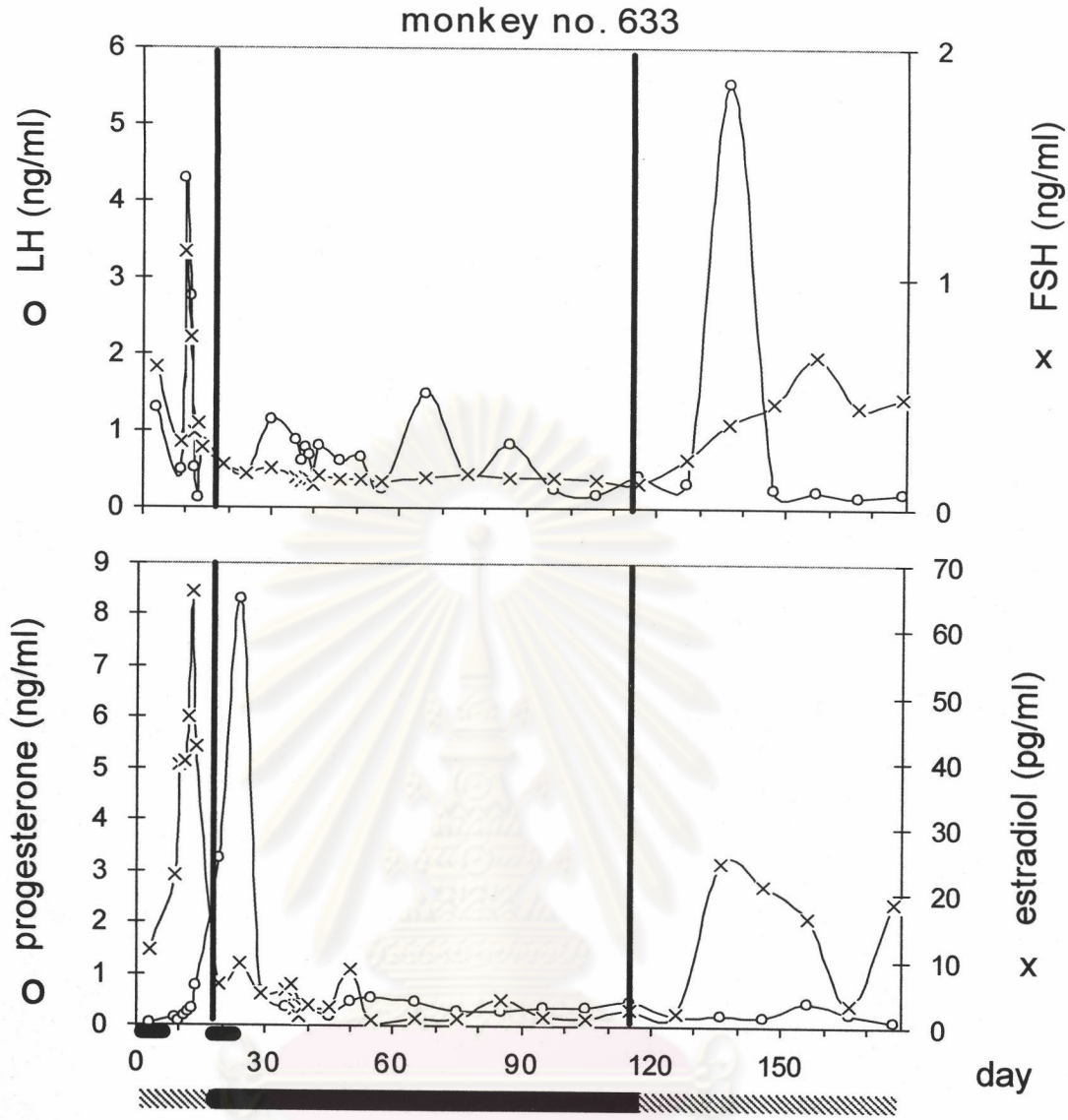

Figure 4.3 (cont.)

4.4 Discussion

This is the first report demonstrating effect of an indigenous Thai herb, *P. mirifica*, on the menstrual cycle length and reproductive hormone levels in female monkeys. The present study clearly demonstrated the estrogenic effect of *P. mirifica* on the menstrual cycle in monkeys. There was an increase in menstrual cycle length of monkeys treated with *P. mirifica* in a dose-dependent manner. The highest dose of *P. mirifica* (1,000 mg/day) showed a complete cessation of the menstruation throughout the 90-days of *P. mirifica* treatment and 60-days of post-treatment. The lower doses of *P. mirifica* (10 and 100 mg/day) showed the prolongation of menstrual cycle length during the treatment period and the recovery during the post-treatment period in some monkeys (2/3 and 1/3 of monkeys treated with the doses of 10 and 100 mg/day, respectively).

The prolongation of the menstrual cycle length was concurrent with the decrease in the serum levels of FSH and LH during *P. mirifica* treatment, which depended on the doses. In monkeys treated with the highest dose of *P. mirifica* (1,000 mg/day), serum levels of FSH and LH were completely suppressed throughout 90 days of the treatment period and 60 days of the post-treatment period. The suppression of these hormone levels seems to be very strong. The medium dose of *P. mirifica* (100 mg/day) also suppressed serum FSH and LH levels during the treatment period. At the lowest dose of *P. mirifica* (10 mg/day), there was a trend of decreasing serum levels of FSH and LH.

FSH normally stimulates the aromatization of testosterone to estradiol by the granulosa cell during the follicular development. In turn, estradiol supports follicular growth and development by increasing the number of FSH receptors, which induces stimulated estrogen production from developed granulosa cells. FSH together with estradiol regulates the expression of LH receptors on granulosa cells of large follicles

during the late follicular phase, inducing an increase in serum LH and ovulation (Tonetta and Dizerega, 1989). Accordingly, our result can be inferred that *P. mirifica* containing phytoestrogens impairs the follicular growth and development due to decreased FSH and LH secretion from the anterior pituitary gland. The impairment of the ovarian function in the monkeys is proved by the decrease in both basal and peak levels of estradiol within entire menstrual cycles.

Moreover, the levels of estradiol and progesterone, which were mainly secreted from the corpus luteum, decreased significantly during the luteal phase. Estradiol and progesterone levels are known to correlate positively with corpus luteum function. The decrease in both serum estradiol and progesterone levels during the luteal phase can be assumed as the indicator of anovulation of the menstrual cycle (Goldstein et al., 1982). Accordingly, the decrease in progesterone throughout the treatment can support the present hypothesis.

Estrogenic effects of *P. mirifica* phytoestrogens are concurrent with the previous reports investigating effect of soy phytoestrogens. Premenopausal women with regular ovulatory cycles who ingested soy protein containing 25 - 60 mg of isoflavones for 1 - 2 months showed an extension in the follicular phase length and delayed the menstruation (Cassidy et al., 1994, 1995; Lu et al., 1996). In addition, there were the suppression of FSH and LH surge at the follicular phase (Cassidy et al., 1994, 1995; Duncan et al., 1999) and decreased levels of estradiol and progesterone entire menstrual cycle (Lu et al., 1996, 2000).

The potential effect of *P. mirifica* phytoestrogens are mediated by estrogen receptor (ER) at target sites including the pituitary gland, hypothalamus, gonad, and uterus (Shughure et al., 1998). Previous studies on the chemical structures of phytoestrogens indicated that the heterocyclic phenol with a close similarity in structure to estradiol, is a prerequisite for binding to ER (Kuiper et al., 1998; Patisaul,

Whitten, and Yong, 1999). When both structures of phytoestrogens and estradiol are overlaid, the distance between the hydroxyl groups of both molecules is identical. Thus, based on its structure, phytoestrogens can bind to ER and act as an estrogen agonist (Hopert et al., 1998). The relative binding affinities of genistein compared with that of estradiol is about 36% for ER α and 5% for ER β (Patisaul et al., 1999). Miroestrol exhibits a high estrogenic potency when assayed by the immature mouse uterine-weight and rat vaginal-cornification tests (Pope et al., 1958; Jones et al., 1960). Therefore, phytoestrogens contained in *P. mirifica* may have an estrogenic effect by the direct interaction with the ER, at least at the levels of pituitary and/or ovary and result in the decreased serum levels of FSH and LH secreted from the pituitary by a negative feedback mechanism.

In recent years, tuberous root of *P. mirifica* was analyzed by the HPLC and found that it contains the high amounts of isoflavones (169.1 mg/100 g of dried weight) and the lower amounts of miroestrol, deoxymiroestrol, and the other (Muangman and Cherdshewasart, 2001). It is therefore deduced that isoflavones are the phytoestrogens in *P. mirifica* influencing on gonadotropins and ovarian hormones. However, the present study could not eradicate the potential role of other phytoestrogens.

Summary: this finding strongly indicate that as is anticipated by the fact that *P. mirifica* contains a high amount of phytoestrogens, it has effect on the menstrual cycle, suppressions of the ovulation and secretion of reproductive hormones, gonadotropins and ovarian hormones.

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