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Different Effects of *Pueraria mirifica*, a Herb Containing Phytoestrogens, on LH and FSH Secretion in Gonadectomized Female and Male Rats

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Abstract. To investigate the effect of *Pueraria mirifica* (*P. mirifica*) containing phytoestrogens on reproductive systems, both sexes of rats were gonadectomized and treated orally with 0, 10, 100, and 1,000 mg/kg BW per day of *P. mirifica* suspended in water (abbreviated as P-0, P-10, P-100, and P-1000), respectively. The treatment schedule was separated into 3 periods: pre-treatment, treatment, and post-treatment. The duration for each period was 14 days. Blood samples were taken once a week. Serum LH and FSH levels were significantly increased within 1 week after gonadectomy; and there were no changes after administration of P-0, P-10, and P-100. However, the increase of LH levels in both sexes and FSH levels in females were attenuated within 1 week after P-1,000 treatment. The attenuation of LH levels in males was smaller than that of females. The decrease of gonadotropin levels was recovered within 1 week in males and 2 weeks in females, respectively, during the post-treatment period. The increase of uterine weight and vaginal cornification were observed in female rats treated with P-100 and P-1,000, whereas only the increase of epididymis weight was found in male rats treated with P-1,000. From this study, it can be concluded that *P. mirifica* can influence the reproductive functions in both sexes of rats, but the response in females is greater than in males.

Keywords: *Pueraria mirifica*, phytoestrogen, LH, FSH, vaginal cytology

Introduction

The *Pueraria mirifica* is an indigenous herb of Thailand, known in Thai as "white *kwao krua*". It was firstly classified as *Butea superba*, and finally identified as *P. mirifica* (1). It belongs to the family *Leguminosae*, subfamily *Papilinoideae*. The plants are commonly found in the forests in the north, west, and northeast of Thailand. Its tuberous root contains at least 13 known chemicals classified as phytoestrogens and comprised of isoflavones (daidzin, daidzein, genistin, genistein, and puerarin) and others such as miroestrol and its derivatives, β-sitosterol, stigmasterol, coumestrol, puerarin, mirificoumestan, kwakhurin, and mirificin (2 – 4). Due to the constituents of phytoestrogens, *P. mirifica* has been postulated to have effects on reproductive organs. Most of the reports about the effect of *P. mirifica* on reproductive organs, however, have been done in female animals or women. Sukavattana (5) firstly reported that the alcoholic extract of *P. mirifica* stimulated the proliferation of vaginal and uterus epithelium in female rats and women. Pope et al. (2) found that *P. mirifica* contained phytoestrogens that behave as an estrogen in inducing vaginal cornification in ovariectomized rats. *P. mirifica* also inhibited the follicular growth and ovulation in female rats (6). Our previous study showed that a single dose of 10, 100, and 1000 mg/kg of *P. mirifica* feeding prolonged the menstrual cycle length in adult cyclic cynomolgus monkeys (7). Muangman and Cherdshewasart (8) showed that *P. mirifica* clearly reduced the menopausal symptoms in women.

In recent years, *P. mirifica* has been used as an alternative medicine for the estrogen effect in humans.

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Many commercial products in the form of cream, tablet, and solution developed from *P. mirifica* roots are widely used not only by women, but it has also become popular in men as an age rejuvenation drug. However, there are no published reports about whether *P. mirifica* has an effect, especially on the reproductive organs, in men as well as in women when administered in the same dosage. In this study, we have examined whether the same dosage of *P. mirifica* is capable of inducing changes of gonadotropins and accessory sex organs in male as well as in female rats.

Materials and Methods

Animals

Adult female and male Wistar rats, 100 days of age, were used in this study. They were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. They were housed 5 animals per cage in a room with controlled lighting (lights on 06.00 – 20.00 h) in which the temperature was maintained at 25 ± 1°C at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand. The animals were fed with the rat chow diet (Pokaphan Animal Feed Co., Ltd., Bangkok, Thailand) and water ad libitum. The experimental protocol was approved by the animal ethical committee in accordance with the guide for the care and use of laboratory animals prepared by Chulalongkorn University.

Experimental design

Female rats: Forty adult female rats, body weight of 230 – 270 g, with regular estrous cycles (4 – 5 days) for 3 consecutive cycles before the study period were used. When the rats showed the diestrous phase (leukocyte cells) on the fourth estrous cycle, blood was collected from the animals by cardiac puncture and then they were ovariectomized (OVX) under ether anesthesia. The day of ovariectomy was designed as day 1 of the study period. The rats were divided into 4 groups (10 rats/group) and orally treated with 0, 10, 100, and 1000 mg/kg BW per day of *P. mirifica* suspended in 0.7 ml distilled water (abbreviated as P-0, P-10, P-100, and P-1,000, hereafter), respectively. The treatment schedule was separated into 3 periods: pre-treatment, treatment, and post-treatment. The duration for each period was 14 days. At the end of the treatment period, half of rats (5 animals in each group) were killed by decapitation 24 h after the final dose, and the remaining half of the rats were killed at the end of post-treatment period. The uterus, liver, and kidney were dissected and weighed thereafter.

Vaginal smears were checked daily between 0800 – 0900 h. The vaginal epithelium cells observed under the microscope were classified into 3 types: leukocyte cells (L), nucleated cells (O), and cornified cells (Co). The representative cell-type was determined by choosing the majority of cells. The results of examined vaginal smear cells from 10 rats in each treatment group were expressed as a modal value.

Male rats: Forty adult male rats, body weight of 380 – 450 g, were used in this study. On day 1, blood was collected from the animals and then they were orchidectomized (ODX) under ether anesthesia. After orchidectomy, the rats were divided into 4 groups (10 rats/group) and treated with P-0, P-10, P-100, and P-1,000, respectively. The schedule of *P. mirifica* treatment was the same as that in female rats. Both at the end of treatment and post-treatment periods, half of the rats (5 animals in each group) were killed by decapitation, and the seminal vesicle, epididymis, liver, and kidney were dissected and weighed.

The preparation of P. mirifica suspension and feeding

To minimize the variation of phytoestrogens content in *P. mirifica* with seasons and locations, the tuberous roots of *P. mirifica* cultivar-Wichai III used in this study were obtained from the same lot (9). The constituents of phytoestrogens investigated by the HPLC technique in 100 g of dry powder are as follows: daidzin = 51 mg, daidzein = 8.1 mg, genistin = 12 mg, genistein = 20 mg, and puerarin = 96 mg. The roots were sliced, dried in a hot air oven at 70°C, and subsequently ground into powder to the size of 100 Mesh. The powder was kept in desiccators until used. The suspensions of *P. mirifica* were freshly prepared from the powder and suspended into distilled water. The rats were fed daily with the suspension of *P. mirifica* between 1000 – 1100 h by gavage.

Blood collection

One-milliliter of blood sample was collected once a week (day 1, 8, 15, 22, 29, 36, and 43) by cardiac puncture under ether anesthesia after the vaginal smear checks in female rats or at 0900 – 1000 h in male rats. The blood samples were additionally collected at 24 and 48 h after the first and last gavage administration of distilled water or *P. mirifica* at the treatment period (day 17 and day 31, respectively). Immediately after the blood clotted, blood serum was separated by centrifugation at 2,000 rpm 30 min and then kept it frozen at –20°C until assayed for LH and FSH concentrations.

Hormonal assays

Concentrations of serum FSH and LH were measured using NIDDK kits for rat FSH and LH. Iodination

preparations were rat FSH-I-5 and rat LH-I-5. The antisera used 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-2 reference standards.

To minimize the interassay variation, all samples in each group were run in a single assay of each hormones.

Statistical analyses

Paired-samples *t*-test and analysis of variance (ANOVA) was used to determine the differences of means using SPSS, a statistical analysis program. The observed significance was then confirmed using the least significant difference (LSD) test. Significance was set at $P<0.05$.

Results

Female rats

Effect of P. mirifica on LH and FSH levels: There were highly significant increases in both the serum LH and FSH levels at 1 week after the ovariectomy (Fig. 1). FSH levels showed an abrupt increase ($P<0.0005$, from 0.56 ± 0.05 ng/ml at day 1 to 5.99 ± 0.27 ng/ml at day 8) while the LH levels showed a gradual increase ($0.02 \le P \le 0.12$, from 0.46 ± 0.05 ng/ml at day 1 to 3.97 ± 0.36 ng/ml at day 8) in all 4 groups. The administration of P-10 and P-100 to OVX rats had no effects on the increase in LH and FSH levels throughout the treatment period. However, the feeding of P-1,000 attenuated the increase in both LH and FSH levels after 1 week of treatment. Even after the cessation of *P. mirifica* treatment, LH and FSH levels were kept lower at 2 weeks and 1 week, respectively, than those at day 8 and day 15. When compared to the control group, significant differences were found in the period from day 22 to day 31 in FSH and from day 22 to day 36 in LH.

Effect of P. mirifica on vaginal smears: All rats in those 4 groups had only L-type cells throughout the pre-treatment period. It was confirmed by this fact that the ovaries were completely removed and no endogenous ovarian estrogens were produced. The P-0 and P-10 treatment did not influence the vaginal epithelium, and only L-type cells were found. In contrast, P-100 and P-1,000 treatments induced a cornification of the vaginal smear. In the P-100 group, cell types changed from L to Co within 5 days after treatment and returned to L-type within 2 days after the cessation of *P. mirifica* treatment. The treatment of P-1,000 had a stronger effect on vaginal epithelium. The cell type changed from L to Co within 3 days after treatment and returned to L by 3 days after the cessation of treatment.

Effect of P. mirifica on the uterus, liver, and kidney: The uterine wet weight after treatment with *P. mirifica* was increased in a dose-dependent manner (Table 1). The uterine weight of P-10 rats did not significantly differ from that of the control. On the other hand, the uterine weight was significantly increased by the treatment of P-100 and P-1000 ($P<0.05$ and $P<0.01$, respectively) compared with the control, respectively. The increment of uterine weight by the treatment of P-1,000 was significantly higher than that of P-100 ($P<0.05$; 373 ± 103 mg and 213 ± 34 mg by P-1,000 and P-100, respectively). At the end of post-treatment, the uterine weights of rats treated with P-100 and P-1,000 were reduced from those of the end of treatment, but they were still significantly higher than those of the control rats.

No significant change has been observed in the liver and kidney weights from the end of treatment to the end of post-treatment in any *P. mirifica* treatment groups. There were also no significant differences in the same period of treatment among those 4 groups of rats.

Male rats

Effect of P. mirifica on LH and FSH levels: There were highly significant and abrupt increases in both of serum LH and FSH levels ($P<0.0005$; from 1.21 ± 0.12 ng/ml at day 1 to 6.40 ± 0.33 ng/ml at day 8 for LH, and from 1.13 ± 0.05 ng/ml at day 1 to 4.44 ± 0.18 ng/ml at day 8 for FSH) after 1 week of orchidectomy (Fig. 2). The increase of FSH levels at day 8 in ODX male rats was smaller than in OVX female rats (increase 291.28% in males *vs* 971.07% in females). However, the increase of serum LH levels in male rats was comparable to that of female rats after the gonadectomy for 1 week (increase 430.48% in males *vs* 769.05% in females). The serum LH and FSH levels compared to day 1 were significantly and consistently higher throughout the study period. When compared to the control group, treatments of P-10 and P-100 had no influences on LH and FSH levels. In contrast to female rats, the P-1,000 treatment attenuated ($P<0.05$) only the increase of serum LH levels. The attenuation was maintained for only 1 week (day 22 to day 29) and the level was immediately recovered to the pre-treatment levels (day 8 and day 15), 1 week after the cessation of treatment. The levels of FSH showed a slight decrease during the treatment period, and the decrease was not significant.

Effect of P. mirifica on the seminal vesicle, epididymis, liver, and kidney: There were no significant differences on the organ weights in each group of rats when compared between at the end of treatment and at the end of the post-treatment periods. The treatments of P-10 and P-100 did not influence the weights of any of the

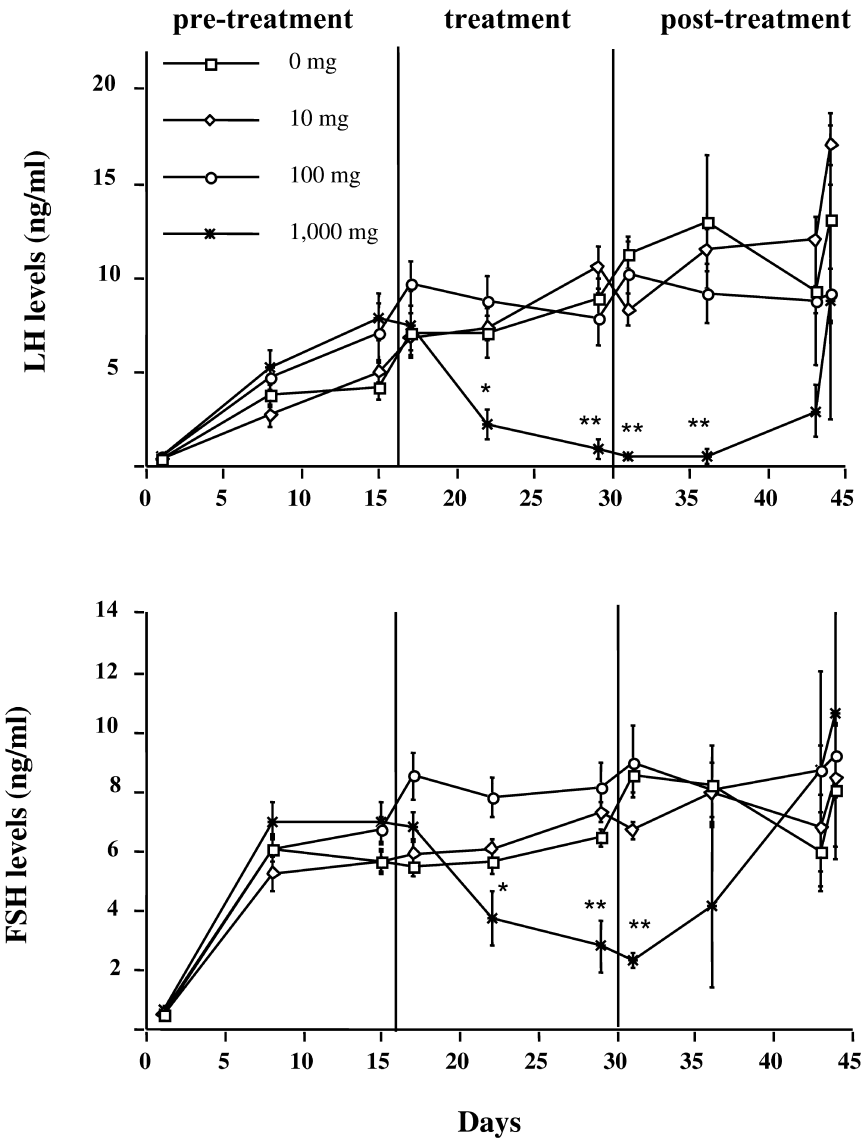

Fig. 1. Serum LH and FSH levels in ovariectomized rats treated with 0, 10, 100, and 1,000 mg/kg BW per day of *Pueraria mirifica*. Each value is expressed as the mean ± S.E.M. of ten (pre- and treatment periods) or five animals (post-treatment period). **P*<0.05 and ***P*<0.01, compared to 0 mg/kg BW of *P. mirifica*.

Table 1. Weights of uterus, kidney, liver, and body in ovariectomized rats at the end of treatment and post-treatment periods of *Pueraria mirifica* treatment

Dose of *P. mirifica* (mg/kg BW)	Uterus (mg)		Kidney (g)		Liver (g)		Body weight at day 1 (g)
	Treatment	Post-treatment	Treatment	Post-treatment	Treatment	Post-treatment	
0	140.75 ± 58.11	118.33 ± 53.78	1.30 ± 0.14	1.38 ± 0.13	6.73 ± 0.89	7.47 ± 1.09	245.50 ± 5.90
10	126.71 ± 15.79	97.50 ± 7.77	1.45 ± 0.15	1.44 ± 0.06	8.28 ± 0.99	7.99 ± 2.14	249.14 ± 5.03
100	213.40 ± 34.98*	173.80 ± 52.85*	1.36 ± 0.08	1.35 ± 0.04	6.66 ± 0.34	6.10 ± 0.33	253.20 ± 4.36
1,000	373.66 ± 103.61**	239.80 ± 105.64*	1.25 ± 0.07	1.30 ± 0.20	8.05 ± 1.61	7.15 ± 1.13	239.50 ± 5.47

Each value is expressed as the mean ± S.E.M. of five animals. **P*<0.05 and ***P*<0.01, compared to 0 mg/kg BW of *P. mirifica*.

organs studied (Table 2). The treatment of P-1,000 induced the significant increase of weight only in the epididymis after 2-week treatment, and the increment was recovered within 2 weeks after the cessation of the treatment.

Discussion

This is the first report demonstrating the effect of *P. mirifica* on secretion of gonadotropins in rats and the sex differences in the effect. Rats were gonadectomized

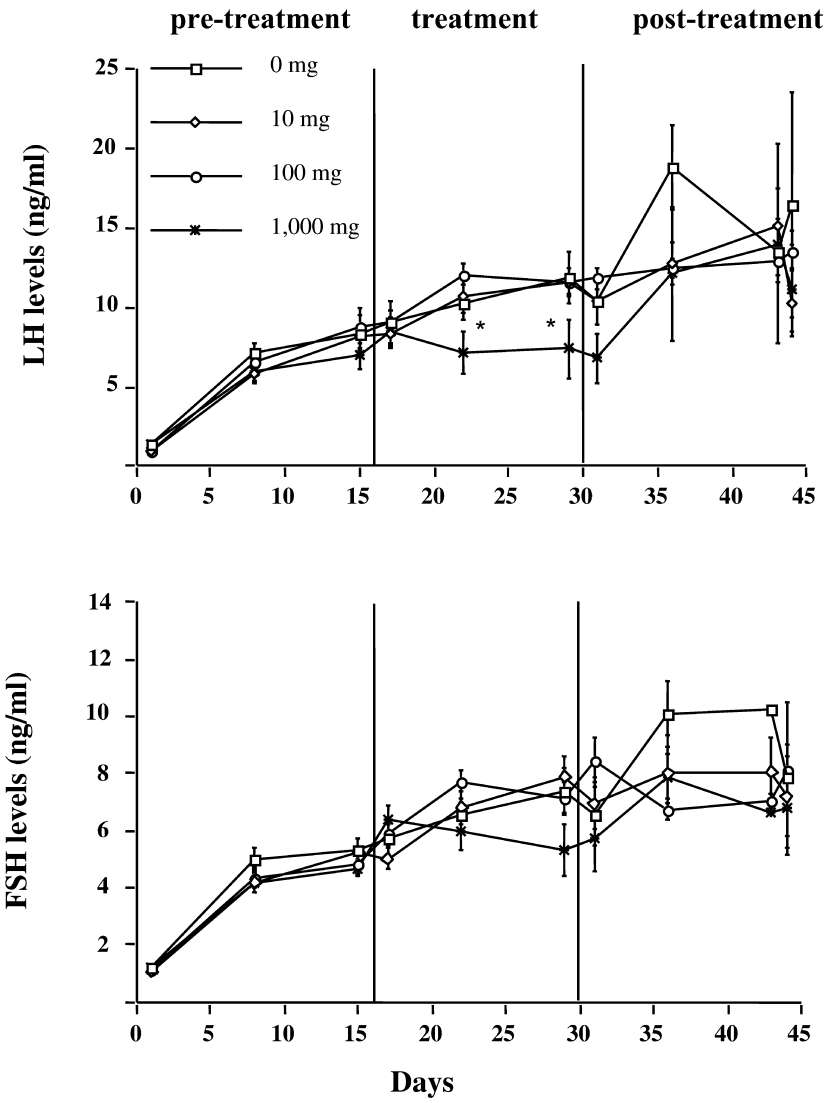

Fig. 2. Serum LH and FSH levels in orchidectomized rats treated with 0, 10, 100, and 1,000 mg/kg BW per day of *Pueraria mirifica*. Each value is expressed as the mean ± S.E.M. of ten (pre- and treatment periods) or five animals (post-treatment period). **P*<0.05 compared to 0 mg /kg BW of *P. mirifica*.

Table 2. Weights of epididymis, seminal vesicle, liver, kidney, and body in orchidectomized rats at the end of treatment and post-treatment periods of *Pueraria mirifica* treatment

Dose of *P. mirifica* (mg/kg BW)	Epididymis (g)		Seminal vesicle (g)		Liver (g)		Kidney (g)		Body weight at day 1 (g)
	Treatment	Post-treatment	Treatment	Post-treatment	Treatment	Post-treatment	Treatment	Post-treatment	
0	0.25 ± 0.08	0.23 ± 0.05	0.19 ± 0.07	0.15 ± 0.01	9.71 ± 1.17	12.07 ± 0.15	1.98 ± 0.17	2.16 ± 0.24	426.42 ± 9.96
10	0.19 ± 0.04	0.23 ± 0.04	0.15 ± 0.02	0.18 ± 0.01	11.33 ± 3.37	12.33 ± 0.81	2.18 ± 0.30	2.22 ± 0.23	435.10 ± 7.64
100	0.22 ± 0.10	0.24 ± 0.05	0.18 ± 0.07	0.16 ± 0.02	9.52 ± 0.52	11.87 ± 1.41	1.99 ± 0.12	2.16 ± 0.10	428.66 ± 7.19
1,000	0.55 ± 0.20*	0.23 ± 0.04	0.22 ± 0.09	0.15 ± 0.02	10.49 ± 1.63	14.04 ± 0.17	2.12 ± 0.17	2.44 ± 0.17	425.40 ± 6.36

Each value is expressed as the mean ± S.E.M. of five animals. **P*<0.05 compared to 0 mg/kg BW of *P. mirifica*.

before the onset of study to prevent the ambiguous effect of the endogenous sex steroid hormones on changes of gonadotropins and accessory sex organs by *P. mirifica*. Furthermore, the removal of feedback inibition of gonadotropin-releasing hormone (GnRH) secretion by sex steroid hormones is predominantly responsible for the rise in gonadotropins (10), and the alteration can be observed clearly after *P. mirifica* treatment. From this study, we can clearly conclude that *P. mirifica* has an effect on accessory sex organs and gonadotropin levels in both sexes of rats similar to estrogen (11 – 14). Thus *P. mirifica* functions on the accessory sex organs through the two pathways, stimulating directly and suppressing through the hypothalamic-pituitary-gonadal

axis. The results agreed with those previously published reports for other phytoestrogens. Genistein and miroestrol produced a persistent or prolonged estrus and increased uterus weight in female rats (11, 15). Coumestrol inhibited a pulsatile LH secretion in female rats (16). Genistein reduced pituitary LH-contents and prostate weight in male mice (17). Trisomboon et al. (18) found that the *P. mirifica* induced decreasing of LH and FSH by the long-term treatment for 90 days in aged menopausal cynomolgus monkeys. The effect was dose-dependent; that is, the higher dose induced an earlier and greater decrease in gonadotropin levels.

Comparing the effect of *P. mirifica* in both sexes of rats, our study showed that the response of rats to *P. mirifica* was greater and more sensitive in females than males. Considering the effect on the accessory sex organs (vaginal cornification and uterus weight in females and seminal vesicle and epididymis weights in males), the increase of uterine wet weight and vaginal cornification in females started to be observed from the treatment of 100 mg/kg BW of *P. mirifica*, whereas only the increase of epididymis weight was found in male rats and only at the dose of 1,000 mg/kg BW. On gonadotropin levels, *P. mirifica* could significantly attenuate the increase both of LH and FSH levels at the dose of 1,000 mg/kg BW in female rats, but only LH levels in male rats. In addition to this, the reduction of LH level was smaller in male rats. The differences in reduction of LH and FSH after *P. mirifica* treatment in relation to sex are presently unknown. One possibility is that the percentage of gonadotropes in the rat pituitary gland storing only LH or FSH or both LH/FSH is different between female and male rats. From quantitative immunocytochemical analysis on rat pituitary, the percentage of multihormonal LH/FSH cells in female rats is 37 – 40%, as compared with 70% in male rats (19).

The ovariectomy in female rats induced an abrupt increase in FSH level but a gradual increase in LH level. These increases are largely attributable to the removal of estradiol and inhibin, negative feedback regulators. Inhibin and estradiol are main regulators to suppress the secretion of the FSH in rats and only the later is for LH suppression (20, 21). The inhibins are secreted by the gonads, and the feedback effects of inhibin appear to be exerted at the level of the gonadotropes. Inhibin reduces FSH synthesis and cell content, as well as basal and GnRH-stimulated FSH release in dispersal pituitary cells (22 – 24). Recent evidence suggests that ovarian steroids, particularly estradiol, may act directly on the GnRH-producing neurons (10). The rise in LHβ mRNA can be completely blocked by administration of estradiol at the time of OVX. In contrast to LHβ, the rapid increase in FSHβ mRNA is only partially suppressed by estradiol at the time of OVX, suggesting that the loss of inhibin is casually related to the rapid increase in FSHβ mRNA expression (25, 26). Consequently, the administration of *P. mirifica* phytoestrogens decreased LH levels more pronouncedly than the FSH levels.

In male rats, both the FSH and LH levels showed a rapid increase after orchidectomy. The present data suggest that the relative importance of inhibin in normal physiology may differ between the sexes. During sexual maturation in female rats, plasma inhibin levels rise steadily and are inversely related to those of FSH, as they are during cyclic variations during the estrous cycle (27, 28). In contrast, circulating inhibin levels are at their highest level in immature males but fall to low or immeasurable values by the time of maturity (29). The levels in testicular lymph, rete testis fluid, and semen are some 100-fold lower than the level in follicular fluid (10). After administration of an antiinhibin antiserum, serum FSH increased in immature male rats, but this effect was absent in adult male animals (30). In contrast, serum FSH rose in both immature and adult female rats (27, 30, 31). Taken these data together, inhibin maintains a selective inhibition on FSH secretion in rats and this effect appears to be more prominent in adult female than adult male rats, and thereby the FSH levels in females (5.99 ± 0.27 ng/ml) are higher than in males (4.44 ± 0.18 ng/ml) after gonadectomy in our study. However, the suppression of estradiol on LH levels in males may be comparable to that in females, as represented by the similar levels of LH in both sexes after gonadectomy. In normal male rats, testosterone inhibits LH synthesis through the aromatization to estradiol (10).

When we follow the various changes in reproductive function in each sex of rats, we found that in females, cornification in the vaginal smear was observed at a lower dose of *P. mirifica* than the reduction of gonadotropin levels and the response was also earlier. All of the OVX rats treated with 100 and 1,000 mg/kg BW of *P. mirifica* showed a persistent cornification of the vaginal smear, while the attenuation of increase in LH and FSH levels was induced only by the administration of the highest dose at 1,000 mg/kg BW. At the highest dosage of *P. mirifica*, fully cornified cells were observed within 3 days while the decrease of LH and FSH levels was started at approximately 1 week after treatment and the maximal decrease occurred at 2 – 3 weeks. In addition, the recovery of vaginal proliferation could occur within 3 days; that is, the vaginal smear returned to being composed of leukocytes, while the recovery of LH and FSH levels took 7 or 14 days. Phytoestrogens

have a higher binding affinity to the ERβ than the ERα (32). ERβ is expressed in both the vagina (33) and pituitary gland (34, 35). Kuiper et al. (36) reported a low expression of ERβ mRNA in the adult rat pituitary by RT-PCR and the distribution was also restricted (34). From this study, we may conclude that the expression of ERβ in vagina of adult rats is higher than in the pituitary. Thus, the response of vaginal epithelium is greater and more sensitive to *P. mirifica* phytoestrogens than the hypothalamic-pituitary axis. Nowadays, the uterotropic assay is a classical in vivo tool for the prediction of estrogenicity of a given substance, that is, determining the stimulatory activity by weighing the uterus in OVX or juvenile mice or rats (37). The present study, however, showed that a vaginal cytology could be an alternative useful tool to assess the estrogenic effects. The advantages of vaginal cytology assay as an intervention with estrogenic effects are 1) we do not need to kill the animals, and therefore, 2) we can follow the recovery after the cessation of treatment.

Since no changes of liver and kidney weights after *P. mirifica* treatment were found in our study, even if the dosage is as high as 1,000 mg/kg BW, it may suggest that *P. mirifica* has considerably safety for human use. Chivapat et al. (38) reported that *P. mirifica* produced no signs and symptoms of acute toxicity in mice, and the LD_{50} value was greater than 16 g/kg BW. That value is rather higher than the ordinary dose consumed by the native Thai people, 200 mg/day or 4 mg/kg BW (8). It is, however, important to note that when using *P. mirifica* in different sexes or for different results, one should be aware of the dosage. Thus, for example, in the application of *P. mirifica* to andropausal men for estrogenic effect, higher dosage is needed than that for the therapeutic application in menopausal women. It is also important to note that there is a considerable variation in phytoestrogen content in *P. mirifica*. Thus we used *P. mirifica* powder prepared from the same lot of tuberous roots throughout this study. Sukavattana (5) found that ovariectomized rats treated with 1 mg of alcoholic extract of *P. mirifica* collected in the different seasons showed a different response of vaginal cornification. It was also known that the phytoestrogen content in soy can vary considerably (39, 40). Thus, in two batches of one rat diet (Altromin A and B) produced mainly from soy, an approximately twofold difference in daidzein and genistein content was found (41). In addition, the isoflavones content in 100 g of *P. mirifica* is higher than that in soy (42, 43).

From this study, it can be concluded that *P. mirifica* phytoestrogens can influence the reproductive functions in both sexes of rats. However, the response in female rats was higher than in male rats and the recovery was slower. Thus, if the application of *P. mirifica* is considered for andropausal men, the dosage used should be higher than that for the therapeutic dosage in menopausal women.

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