# CHAPTER V
# DISCUSSIONS

There are at least 3 approaches to investigate estrogenic effect of any substances, yeast estrogen screening (YES) (Lee *et al.*, 2002), proliferation assay with MCF-7 or E-assay (Strobl and Lippman,1979) and uterotrophic assay in ovariectomized rats (Benson *et al.*,1961). It had previously demonstrated that, apart from uterotrophic assay, vaginal epithelium cornification assay was also a reliable tool (Jones and Popes, 1960). The later method was recently applied to demonstrate the difference of estrogenic effect among *P. mirifica* samples collected from 3 provinces in Thailand; Prachuabkirikhan, Saraburi and Chiang Mai (or cultivar Wichai-III, Malaivijitnond et al., 2006).

PM-III and PM-IV is selected as experimental plants in this study because the 2 plant clones exhibited significant different in PM-III : leaf morphometry of 6 out of 9parameters, in tuber morphometry of 3 out of 3 parameters. PM-IV: leaf morphometry of 5 out of 9 parameters, in tuber morphometry of 2 out of 3 parameters and in pod morphometry found that PM-III have the length of pods longer than PM-IV and the width of pods less than PM-IV,respectively.

To rank the estrogenic potency of the plant samples, from this study 2 parameters can represent the response of the rat vaginal epithelium submitted to the treatment of *P. mirifica*. The first parameter was the first day during the 14 days of *P. mirifica* treatment that exhibited mostly cornified cells whereas the number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post-treatment period was a second parameter. The 2 parameters are quantitative analysis.

The treatment with the dosage of 100 mg/kg BW/day of PM-III samples showed significant in mean of the first day of appearance among rainy season (day 12.17$^{th}$), winter (day 6$^{th}$) and summer (day 6.67$^{th}$). The samples collected in July, August, September and October exhibited no cornified cells while the other plant samples exhibited cornified cells with the first day of appearance of cornified cells of 6-9 days (Table 4.1). The rainy season in the field trial site was May to October. PM-III and PM-IV exhibited the lowest estrogenic activity in October considered from the

first parameter which was one day delayed in the treatment with the dose of 1,000 mg/kg BW, and % cornified cells at the first day of appearance which was lower than the other months. The nonestrogenic expression of the collected tubers was in the middle and late period of rainy season. There must be an influence of water obtained during rainy season with the estrogenic activity of the tubers. During the first 2 months of rainy season; May and June, the amount of obtained water from rain may not enough to initiate change in phytoestrogen synthesis/storage in the plant. That certain amount left of phytoestrogen can thus initiate vaginal cornification response. Nevertheless the partial influence of rain was exist as the number of first day of cornified cell appearance in May is day $9^{th}$ and June is $8^{th}$ in PM-III which was late than the left, day $6^{th}$-$7^{th}$ in PM-III and day $5^{th}$-$6^{th}$ in PM-IV. When the sharp drop of rain amount happened in November, the plant samples could regain express estrogenic activity. The data confirmed our hypothesis that amount of obtain water from rain had highly influence on estrogenic activity. The results in PM-III was similar to PM-IV with most likely more expression of estrogenic activity in PM-IV consider from less mean value of first day of cornified cell appearance in winter (day $5.33^{th}$ /day $6.00^{th}$) and summer (day $6.00^{th}$ /day $6.67^{th}$). Nevertheless in the treatment of 1,000 mg/kg BW, PM-IV exhibited stronger estrogenic effects evaluated from the less mean value of first day of cornified cell appearance in rainy season (day $2.33^{th}$ in PM-IV / $3.50^{th}$ in PM-III), winter (day $2.00^{th}$ in PM-IV / $2.33^{th}$ in PM-III) and summer(day $2.00^{th}$ in PM-IV / $3.00^{th}$ in PM-III)

Evaluation by the second parameter, the number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post treatment period, showed the similar results. At the dose of 100 mg/kg BW/day, ovariectomized rats treated with plant samples collected in rainy season showed the least number (2.33 days in PM-III and 2.50 days in PM-IV) as compared with winter (11.00 days in PM-III and 11.67 days in PM-IV) and summer (10.00 days in PM-III and 10.67 days in PM-IV, Table 4.3). Nevertheless in the treatment of 1,000 mg/kg BW, PM-IV exhibited stronger estrogenic effects evaluated from the number of days that the vaginal epithelium became cornified during a sum up number of day during the 14 days of plant material treatment plus the 7 days post treatment period in rainy season (15.50 days in PM-IV / 13.50 days in PM-III), winter (17.67 days in PM-IV / 15.33 days in PM-III) and summer(17.33 days in PM-IV / 15.33 days in PM-III). The data confirmed that PM-IV expressed stronger estrogenic activity than PM-III.

The first day of appearance of leucocyte cells during the post-treatment period is an additional parameter to mark for the end of expression of estrogenic response period in rat vaginal epithelium. PM-III and PM-IV treatment at the dose of 100 mg/kgBw/day showed less value of the first day of appearance of leucocyte cells in rainy season (day 3.33$^{th}$ un PM-III and 3.33$^{th}$ in PM-IV) than winter (day 3.33$^{th}$ in PM-III and day 4.67$^{th}$ in PM-IV) and summer (day 4.00$^{th}$ in PM-III and day 4.33$^{th}$ in PM-IV). The stronger estrogenic activity of the plant extracts leaded to prolong of appearance of leucocyte cells during the post-treatment period as seen in the treatment with 1,000 mg/kgBw/day, in rainy season (day 4.67$^{th}$ in PM-III and 5.50$^{th}$ in PM-IV) showed less value of the first day of appearance of leucocyte cells than winter (day 4.67$^{th}$ in PM-III and day 5.67$^{th}$ in PM-IV) and summer (day 5.33$^{th}$ in PM-III and day 6.00$^{th}$ in PM-IV). The treatment with the dosage of 1,000 mg/kgBw/day, *P. mirifica* collected in 12 months resulted in clearly expression of differential estrogenic activity. The second parameter in our analysis revealed that the PM-IV samples collected in January, April and November showed the maximum of estrogenic response (18.00 days) from Table 4-2 as compared with the mean value of the 3 seasons or 90.00% in comparison with 20.00 ± 0.00 days for 17β-estradiol, positive control of 100 % estrogenic response. The minimum estrogenic response is the PM-III sample collected in October (12.00 days) or 60.00% (in comparison with 20.00 ± 0.00 days for 17β-estradiol, positive control of 100 % estrogenic response), (Table 4-2). The difference between the least and greatest expressed is 30.00%. The data demonstrated clearly that there is a differential expression in estrogenic activity among plant materials collected in different months and different cultivars (PM-III and PM-IV).

Qualitative analysis of the vaginal cornification is also an additional parameter to evaluate for the different in estrogenic activity of the 2 plants. The percentage of cornified cells at the first day of appearance is choosen to demonstrate this analysis. The results revealed that PM-III and PM-IV exhibited different % cornified cell at the first day of appearance of cornified cells in the dose of 1000 mg/kg BW in some months. Besides, the expression period was delayed from the first day in E2 treatment to the second to third day of PM treatment in some observed months. The results suggest that estrogenic activity of PM was lower than E2 but it can reach the maximum response as did by E2 once it worked.

From the two parameters plus an additional one from the first day of appearance of leucocyte cells during the post-treatment period, Seasonal and genetic influence on estrogenic activity of the plant tubers is clearly demonstrated.

Using uterine weight as parameter, the results are in the same direction as previously described. At the concentration of 100 mg/kg BW/day, plant tubers colleted during rainy season initiated less uterine weight (170.15 mg in PM-III and 191.58 mg in PM-IV) than in winter (204.80 mg in PM-III and 199.80 mg in PM-IV) and summer (207.27 mg in PM-III and 230.67 mg in PM-IV). Seasonal has definitely influence on uterine weight. Besides, the influence of genetics is still observed as the uterine weights in PM-IV were more than PM-III during rainy season and summer (Table 4-4). With application of the same parameter, the influence of season was not significant in PM-III but significant in PM-IV. The uterine weight at the end of the treatment period from samples collected during winter gained the highest uterine weight (573.90 mg) in comparison with rainy season (478.72 mg) and summer (559.53 mg).

The uterine weight in the dose 100 and 1,000 mg/kg BW/day *P. mirifica* treated rats was significant difference from the negative control and positive control. There were previous studies that miroestrol (Jones and Pope, 1960) and powder of *P. mirifica* could exhibit uterotrophic effect (Malaivijitnond *et al.*, 2004). Our data was recorded at day $7^{th}$ after abolish treatment with *P. mirifica*. The uterotrophic response by the plant treatment at 100 and 1,000 mg/kg BW should happened but was later diminished according to the rapid secretion of the phytochemicals from the rat bodies (Malaivijitnond *et al.*, 2004). Besides, the increment of uterine weight at the end of the post- treatment period should be agreed with the changes of vaginal epithelium cells that were recovered to a stage before treatment with *P. mirifica*.

The plant samples with stronger estrogenic activity could be distinguished at the end of the treatment period and also could maintain more uterotrophic effect, as seen at the end of the post-treatment period. It was found that the uterine weight of PM-IV at the end of treatment period in winter and summer seasons are higher than PM-III while there was no different in rainy season. (Table 4.5). The results were in the same direction as in the analysis of uterine weight at the end of the post-treatment period.

Qualitative analysis is submitted to analyze the cross section area of uterine tissue is expressed into 3 parts of the uterus, including myometrium, endometrium and lumen. In PM-III there is no difference in cross section area of myometrium. It means that estrogenic effects is not much influence to myometrium. Endometrium treated with plant samples collected in winter is thicker than in rainy season and summer. Estrogenic effect to uterus is thus dominated at endometrium. Lumen treated with plant samples collected in rainy season is larger than the other 2 seasons. There is thus a correlation change of endometrium and lumen. In PM-IV, myometrium shows estrogenic response. It is thicker in winter than summer and rainy season. It means that PM-IV expresses stronger estrogenic effect to myometrium than PM-III. Endometrium is thicker in winter than summer and rainy season. This is additional evidence that PM-IV has stronger estrogenic activity than PM-III. Lumen is larger in rainy season than winter and summer. It confirms that the enlargement of lumen should result from the reduction in thickness of endometrium in rainy season.

The further analysis is the qualitative analysis of the estrogenic activity. Uterus was submitted to histology preparation and analysis. The key used parameter is uterine gland number. There was significant different of the mean value of uterine gland number in rainy season of the 2 plants that were lower than in winter and summer. This finding confirms the strong seasonal influence on plant estrogenic activity.

In comparison of estrogenic effects initiated by E2 versus *P. mirifica* in OVX rats, the two treatment exhibited an increase in uterus wet weight in different degree. E2 initiated more uterus wet weight than *P. mirifica*. In the analysis of endometrial tissue, it was found that uterus in the treatment with *P. mirifica* showed higher degree of glandular proliferation as well as endometrial area while the lumen was smaller. It is a first time demonstration that phytoestrogen treatment could initiate a better quality differentiation of uterine endometrium. Besides, the estrogenic activity found in the differentiation of the uterine tissue is more clearer than the result of the cornification analysis. The result also confirmed that phytoestrogen from this plant is not only effective as alteration of estrogen replacement therapy but also more effective. Consider the safety of *P. mirifica* from the toxicity analysis of the plant powder and extract in animals and human volunteers (Cherdshewasart, 2003), it can conclude that *P. mirifica* is the most interesting natural product to be used as phytoestrogen replacement therapy.

There were many studies on the effects of phytoestrogens on the female human genital tract and female animals. It was found that miroestrol, an isolated

active chemical from *P. mirifica*, induced cornification of the vaginal epithelium in the immature female mice (Jones and Pope,1960). Dietary supplementation with phytoestrogens could also increased vaginal cytological maturation in women (Wilcox *et al.*,1990). Six month soy-rich diet treatment to the asymptomatic post-menopausal women increased cornification of vaginal epithelium identical to those found in the hormonal replacement women (Chiechi *et al.*, 2003). Even our study showed a differential estrogenic response among plant samples, all plant samples could exhibit certain level of cornification. It should conclude that *P. mirifica* exhibit a strong potential to be introduced as an effective herbal phytoestrogen source. This response is stronger than the reports from soy origin. The clinical trial of the treatment of the Thai menopausal women with 100 mg/day crude powder of *P. mirifica* cultivar Wichai-III also confirmed the certain level of estrogenic response (Muangman and Cherdshewasart, 2001).

The present study provides the first evidence that *P. mirifica* phytoestrogens has profound, dose dependent effect on the vaginal epithelium. The dose of 1000 mg/kg BW/day exhibits higher estrogenic effect than the dose of 100 mg/kg BW/day and can effectively stimulate the proliferation of rat vaginal epithelium. Such a plant concentration might be too far from the physiological dose to create a certain amount of binding to ERα and/or ERβ at the vaginal tissue and could not subsequently stimulate the vaginal cornification. At the dosage of 100 mg/kg BW/day, most of the tested *P. mirifica* samples initiate a significant cornification of the vaginal epithelium as compare with the negative control. The level of response is far less than the initiation of $E_2$ in the positive control. This response should mostly be initiated by ERβ because the binding affinity is still far from the maximum loading. At the dosage of 1,000 mg/ kgBW/day, 2 cultivars tested samples initiated a greater significant cornification of the vaginal epithelium as compare with the negative control. But this elevated estrogenic response is not in proportion with the added amount of the plant material that is increased in a log scale as compare with the previous dosage. Miroestrol, a key chemical in *P. mirifica* showed equal estrogenic activity to 17β-estradiol in the mouse uterine and to have one quarter of the potency of 17β-estradiol in the rat vaginal cornification test (Cain, 1961). It should thus imply that phytoestrogen from *P. mirifica* at this dosage could fully bind to ERβ and there is some certain amount that is still present in the circulation can bind to ERα. The affinity to ERα and initiated estrogenic response by *P. mirifica* has been already demonstrated in MCF-7, human malignant cell comprising ERα (Cherdshewasart *et*

*al.*, 2004[b]). It is noticed that the cornification response in the combination between ERα and ERβ binding at this dosage is still less than that initiated by $E_2$ (88.60 %, of cornified cell count of $D_5$ after treatment with the dose of 100 mg/kgBw/day of PM-VI sample collected in November vs. 100% for $E_2$). It should imply that ERα plays a greater role on cornification of the rat vaginal epithelium than ERβ.

It was found that the cornification response in vaginal smear to *P. mirifica* treatment was a dose dependent. Regarding ER binding affinity, $E_2$ exhibits higher binding affinity to ERα than ERβ whereas phytoestrogen exhibits higher binding affinity to ERβ than ERα (Nikov, 2000). This can be applied to the interpretation of our results. The results were agreed with the previously published reports for other phytoestrogens. Genistein and miroestrol produced a persistent or prolonged estrus and increased uterus weight in female rats (Kouki *et al.*, 2003; Jones and Popes, 1960). Coumestrol inhibited LH secretion in female rats (McGarvey *et al.*, 2001). Genistein reduced pituitary LH-contents and prostate weight in male mice (Strauss *et al.*, 1998). The long term treatment of *P. mirifica* at the dose of 1,000 mg for 90 days in aged menopausal cynomolgus monkeys could disturb ovarian function and menstrual cycle (Trisomboon *et al.*, 2004[b]). *P. mirifica* has effect on accessory sex organs in vaginal cornification and uterus weight in females and seminal vesicle and epididymis in males (Malaivijitnond *et al.*, 2003[b]). These effects were also dose dependent.

This study is the first report demonstrating the differential estrogenic effect of the cultivated *P. mirifica* yearly collected in cornification test, uterotrofic assay, uterine gland number assay and cross section area of uterus tissue assay. The results should benefit in ranking the quality of the tuber-derived materials based on the strength of estrogenic activity in rat vaginal epithelium model, rat uterotrofic response and the changing of uterine morphology. The results demonstrate clearly that there is a differential estrogenic response occurs at the rat vaginal epithelium after treatment with *P. mirifica* derived from different period of tuber collection.