CHAPTER VI

DISCUSSION

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) (Chansri, 2002).

To rank the estrogenic potency of the plant samples, from this study, it can conclude that 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 totally cornified cells whereas the number of days that the vaginal epithelium becomes cornified during a sum up number of day during the 14 days of plant material treatment plus the 14 days post treatment period was a second parameter. The treatment with the dosage of 100 mg/kg BW/day from Kanchanaburi, Mae Hong Son, Sukhothai, Ratchaburi, Chiang Rai, Chumphon, Nakorn Sawan, Phetchabun, Nakorn Ratchasima, Phitsanulok, Kampaeng Phet, Phrachin Buri and Sakon Nakhon resulted in fully proliferation of the rat vaginal epithelium. It was found from the vaginal smear cell count that at Day 4th after treatment with *P. mirifica* collected from Kanchanaburi, the rats exhibited the highest percentage of cornified cells which was the fastest response as compare with the others. Thus, it may conclude that *P. mirifica* from Kanchanaburi exhibited the highest estrogenic activity in term of vaginal cornification assay, in accordance with the first parameter.

The treatment with the dosage of 1,000 mg /kg BW/day, *P. mirifica* from 25 provinces resulted in clearly expression of differential estrogenic activity. The second parameter in our analysis revealed that the plant samples from Kanchanaburi showed the maximum of estrogenic response (18.00 $\pm$ 0.00 days) from Table 4-3 as compared with the

mean value of the plant population (14.32 ± 0.48 days) or 75.37 % (in comparison with 19.20 ± 0.20 for 17β -estradiol, positive control of 100 % estrogenic response). The statistic analysis revealed that there are 6 and 7 plant samples exhibited a significant lower and higher days of cornification than the mean value of the population, respectively. The minimum estrogenic response is the sample collected from Uttraradith (8.60±0.89 days),(Figure 4-33). The difference between the least and greatest expressed is 52.64%. The data demonstrated clearly that there is a differential expression in estrogenic activity among plant materials collected from different source.

Attempts were made to analyse the estrogenic effect of the plant samples by the aid of cornification assay in relavant with the geographic distribution of the plants. The plant samples were classified as 15, 4, 5 and 1 samples from the northern part, north-eastern part, central and southern part of Thailand. Even the statistical analysis of the mean value of total cornified cells and the first appearance of cornified cells showed no significant but the results demonstrated the interesting trend. The plants collected from the cental part exhibitted the strongest estrogenic activity as evaluated with the first and second parameter, 7.40 ± 3.17 and 8.60 ± 2.22 cornified days, respectively. The samples from the northern and north-eastern part showed the similar activity from Table 4-1 and 4-3 (north; 5.26 ± 1.52 and 9.33 ± 1.16 ,northeast; 5.00 ± 3.00 and 10.25 ± 2.17). Besides the top-ranked of the samples collected from the 3 parts of Thailand, Kanchanaburi, Mae Hong Son and Nakorn Ratchasima were compared for the second parameter and the results are in the same trend that is the same rank as appear from the population analysis.This observation should benefit those who need to do a farming production of the plant tubers as areas in the central part may be the better place to establish plant materials with high estrogenic effect. This conclusion can be more precise if the difference in plant genetics is clarified because both genetics and environments, especially geographic distribution could influence the chemical content of the plant materials (Joseph *et al.*, 2000)

There were many studies on the effects of phytoestrogens on the female 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 10 mg/kg BW/day did not 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, all of the tested *P. mirifica* 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 (81.2 %, of cornified cell count of D_4 after treatment with Kanchanaburi 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.

The uterus weight in the dose 10 and 100 mg/kgBW/day *P. mirifica* treated rats was not significant difference from the negative control, but did difference from the 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 14[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*.

However, the results in our study were not related with the MCF-7 cell proliferation assay. Oral consumption of *P. mirifica* created metabolized form of chemicals by the influence of gut normal flora and liver drug metabolizing enzymes, and finally bound to ER. MCF-7 cells has low capacity of such modification, thus the chemicals bound to ERα within the cells should be somehow different with those bound to ER of the rat vaginal epithelium.

There was a similar study with *Pueraria Radix*, a plant with tubers and high amount of isoflavone, especially puerarin. The tubers were collected from many parts of Korea and submitted to estrogenic activity analysis in comparison with the Thai *P. mirifica* by means of uterotrophic assay. It was found that all collected samples expressed no uterotrophic effect but the Thai plant did. There was a conclusion that the content of isoflavone in the *Pueraria Radix* is not ralated to the estrogenic activity (Kim *et al.*, 2003). However, it is known that daiadzein and genistein of isoflavone in Luguminosae family plants can be a substitutive substance for estrogen by binding to the estrogen receptor even if the chemical affinity is weaker than estrogen (Kim *et al.*, 2002; Franke *et al.*, 1994).

Attempts have been done to correlate the cornification response with the isoflavone amount in the wild *P. mirifica* (Subtang, 2003). It was found that there is no correlation between total isoflavone or individual isoflavone including puerarin, daidzin, genistin, daidzein and genistein ($P>0.05$). It was found that puerarin from *P. mirifica* exhibited low estrogenic effect (Chansakaow *et al.*, 2000[a]). The high amount of puerarin in *P. mirifica* should also exhibit non-significant estrogenic response in our study.

It was noticed that the HPLC fingerprint analysis of the plant sample collected from Kanchanaburi had the maximum amount of total isoflavone (198.29 mg/100 g powder, Subtang, 2003). This finding is not correlated with the analysis of another samples. Therefore, it could not draw the final conclusion that the total isoflavone contents in the tuberous root are directly related with estrogenic activity.

Consider with the previous report that miroestrol initiated cornification in immature mice (Jones *et al.*, 1961) and ovariectomized rats (Benson *et al.*, 1961) it could deduce that induction of cornification by the wild *P. mirifica* in our study is mainly initiated by miroestrol. It is pity that any commercial supply for miroestrol was not possible and a standard for HPLC analysis could not set up as did success with isoflavone, so could not be correlated the cornification response degree with the tuberous miroestrol content.

This study confirmed the variation of *P. mirifica* tubers collected from various provinces in Thailand as had been previously shown in the chemical content study (Subtang., 2003) and MCF-7 proliferative study (Trisap *et al.*, 2004). This study is the first report demonstrating the differential estrogenic effect of the wild *P. mirifica* collected from a majority of plant population, 25 provinces in Thailand by ovariectomy and cornification test

approach. 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. 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 sources.

In term of product development for human consumption, an *in vivo* test in animals could benefit most as the animal system is similar to living human system. Thus even the non-correlated results had occurred among the different tests with the same group of samples, it can rely most on the animal *in vivo* test that is demonstrated in this study. To make a choice for plant material which may be derived from the wild or field-grown plants, the plants with strongest vaginal cornification effect should be the first choice. This will end up with a strong estrogenic product for human consumption. As it is realized that PRT (Phytoestrogen Replacement Therapy) is an interesting alternative with ERT (Estrogen Replacement Therapy), this study had demonstrated that the selected sample of *P. mirifica* (Kanchanaburi) could exhibit estrogenic effect up to 81.2 % in comparison with 0.2 mg/100g BW E_2 (100%). The plant is endemic species for South East Asia, especially Thailand. Thai society should take benefit from the plant by accept it as a precious phytochemical-source, especially phytoestrogen and make full afford to develop a full range of products from the plant that can be ranked from traditional medicine, cosmetics and beverage.