—Full Paper—

Evaluation of the Estrogenic Activity of the Wild *Pueraria mirifica* by Vaginal Cornification Assay

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Abstract. The aim of this study was to evaluate the estrogenic activity of tuberous samples of phytoestrogen-rich *Pueraria mirifica* collected from 25 of 76 provinces in Thailand by vaginal cornification assay. Tuberous powders were prepared and administered to ovariectomized rats for 14 consecutive days at dosages of 10, 100 and 1,000 mg/kg BW respectively, and were compared with a daily treatment with 2 mg/kg BW 17 β -estradiol (E₂). Rats treated with 10 mg/kg BW *Pueraria mirifica* showed no vaginal cornification. Treatment with 100 mg/kg BW *Pueraria mirifica* from 13 out of 25 plant samples resulted in development of vaginal cornification. The cell count percentages of the vaginal smeared cells for the treatment with the 2 plant samples that exhibited the fastest vaginal cornification revealed large variation in their estrogenic activities. Treatment with 1,000 mg/kg BW *Pueraria mirifica* from all plant samples produced vaginal cornification with the mean value for the period (day) of first appearance of cornified cells being 4.08 days compared to 2 days with 2 mg/kg BW E₂. The overall appearance period (day) of cornified cells during the treatment and post-treatment period with 1,000 mg/kg BW per day *Pueraria mirifica* was shorter than treatment with 2 mg/kg BW E₂. The results demonstrate that the plant population shows differential estrogenic activity as evaluated by vaginal cornification assay.

Key words: Estrogenic activity, Phytoestrogen, *Pueraria mirifica*, Rat, Vaginal cornification

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P*ueraria mirifica* Airy Shaw et. Suvatabhandu [synonym: *Pueraria candollei* Wall. Ex Benth. var. *mirifica* (Airy Shaw and Suvat.) Niyomdham] is known to be rich in phytoestrogens. The plant's tuberous roots contain miroestrol [1–3], deoxymiroestrol [4], and isoflavones [5–10]. The estrogenic activity of miroestrol was first determined in ovariectomized rats. Administration of miroestrol produces a mammogenic effect in rats [11]. Using the vaginal cornification assay, miroestrol was estimated to have 0.25 times the

estrogenic activity of 17 β -estradiol [12]. Thus, various types of research have been conducted on the estrogenic effects of *P. mirifica*, especially in recent years. Crude *P. mirifica* powder can relieve the climacteric symptoms of post-menopausal women [13]. The estrogenic activities of *P. mirifica* powder and extract has been evaluated in various species of experimental animals and found to have no toxicity [14]. *P. mirifica* has biphasic effects on MCF-7 cell proliferation, both suppression and stimulation [15], and an anti-proliferation effect on HeLa cells (ER α -negative human cervical adenocarcinoma cells) [16]. Considering the effects on the reproductive organs and reproductive-related hormones, *P. mirifica* suppresses luteinizing

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hormone (LH) and follicle stimulating hormone (FSH) in male and female rats [17] and female monkeys [18–20] and reduces the levels of bone resorption hormone parathyroid hormone in aged female monkeys [21]. It also initiated the reversal of sexual skin in aged monkeys [22].

P. mirifica is widely distributed in deciduous forests throughout Thailand. The tubers of wild plants have been heavily harvested for commercial purposes recently. This could cause not only extinction of the plants in the natural habitat but also loss of plant-derived products. Cultivation of *P. mirifica* with high phytoestrogen content is urgently needed for the plant products at the industrial scale. Screening for estrogenic activity is thus a first essential step for evaluation of quality of plant tubers collected from different sources. We therefore set up bioassays for estrogen activity by means of vaginal cornification assay using a huge number of *P. mirifica* samples collected from 25 out of 76 provinces in Thailand to find and rank the plants with high estrogenic activity based on plant cultivar and collection site.

Materials and Methods

Plant materials

The tuberous roots of wild *P. mirifica*, Family Leguminosae, were surveyed and collected from 25 out of a total of 76 provinces in 5 regions of Thailand (Table 1). To minimize the seasonal variation in phytoestrogen content of *P. mirifica* [23], the plants were collected only in March and April of 2000 [24]. The plants were identified by Cherdshewasart with reference to the study of Kasemsanta *et al.* [25] and were compared with voucher specimen for *P. mirifica* (No. BCU 11045) deposited at the Department of Botany, Faculty of Science, Chulalongkorn University (Bangkok, Thailand). Powders were prepared from the tubers as described previously [15].

Animals

Adult female Wistar rats were supplied from the National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand). They were housed in stainless steel cages (5 animals/cage) with a standard animal husbandry environment of controlled lighting (lights on 0600–2000 h) and temperature (25 ± 1 °C) at the Primate Research

Unit, Department of Biology, Faculty of Science, Chulalongkorn University (Bangkok, Thailand). Rat chow and tap water were provided *ad libitum*. The animal experiments conducted in the morning between 0800 and 1000 h. The experiment protocol was approved by the Animal Ethical Committee in accordance with the University guideline for the care and use of laboratory animals.

The rats used were 60 days old and 200–250 g in weight, and each had at least 3 consecutive estrous cycles (4–5 days) before the start of this study. The 5 rats in each group were ovariectomized under ether anesthesia in the diestrous phase of the 4th estrous cycle. The day of ovariectomy was designated as the 1st day of the study period. The rats were divided into the following 3 groups; *P. mirifica*, negative control, and positive control. The treatment schedule was separated into the following 3 periods: pretreatment for 14 days, treatment for 14 days, and post-treatment for 14 days. In the pretreatment and post-treatment periods, the rats were administered 0.7 ml/kg distilled water. In the treatment period, the rats of the *P. mirifica* group were fed 10, 100 or 1,000 mg/kg BW of *P. mirifica* powder suspend in 0.7 ml/kg distilled water daily, the rats of the negative control group were fed 0.7 ml/kg distilled water daily, and the rats of the positive control group were subcutaneously injected with 2 mg/kg BW of 17 β -estradiol (Sigma, St. Louis, MO, USA) dissolved in corn oil daily, respectively.

Vaginal cornification assay

The vaginal epithelium was checked daily between 0800 and 0900 h with the aid of a small glass rod sterilized with 70% alcohol solution and soaked in 0.9% normal saline solution before use. The vaginal cells were smeared onto a slide with a drop of 0.9% normal saline solution, observed under a light microscope, and identified and then their cell types were recorded. Vaginal cells were categorized into the following 3 types: leukocyte cells (L), nucleated cells (O), and cornified cells (Co). The representative cell-type was determined by selecting the type of cells that comprised the majority of cells. The results of examination of vaginal smear cells from 5 rats in each treatment group were expressed as a mode value (the most frequently occurring cell type in 5 rats). The appearance of cornified cells (or a majority of Co-type cells) was used as an indicator of estrogenic

activity. Thus, 25 cultivars of *P. mirifica* were ranked according to their estrogenic activity. The criteria for ranking were as follows: 1) earlier stimulation of Co-type cell appearance (or the first day of cornified cell appearance) after 100 mg/kg BW of *P. mirifica* was judged as the higher estrogenic activity, and 2) if the first day of cornified cell appearance was the same between two (or more) cultivars of *P. mirifica* after judgement with the first criterion, earlier stimulation of Co-type cell appearance after 1,000 mg/kg BW of *P. mirifica* was used for subsequent judgement for the next step. Since no cornified cell occurred after treatment with 10 mg/kg BW of *P. mirifica* in all 25 cultivars, the results for this dose were not taken into consideration.

Additionally, the percentage of cornified cells was randomly calculated for the negative control (distilled water) group, positive (17 β -estradiol) control group, and the two cultivars of *P. mirifica* in the treatment groups (cultivars Kanchanaburi and Mae Hong Son) that showed the highest estrogenic activity. In brief, after evaluation of vaginal smears the 3 types of vaginal cells were counted randomly for total of 100 cells and the percentage of cornified cells were then calculated [26].

Statistical analysis

The results are expressed as means $\pm$ SEM. The differences in estrogenic activity between the regions of *P. mirifica* collection were analyzed by one-way analysis of variance. Significant differences were then confirmed using the least significant difference (LSD) test. The significance level was set at $P < 0.05$. SPSS version-10 was used for statistical analysis.

Results

Estrogenic activity of 25 cultivars of *P. mirifica*

All the rats had only L-type cells throughout the pretreatment period after ovariectomy. Administration of distilled water (negative control group) did not influence on the vaginal epithelium, and only L-type cells were found. In contrast, subcutaneous injection of 2 mg/kg BW of 17 β -estradiol induced cornification of the vaginal epithelium as early as the second day of treatment, and Co-type cells were retained until the 7th day of the post-treatment period (Fig. 1). The occurrence of

vaginal cornification in rats after *P. mirifica* treatment depended on the dose and cultivar. A higher dose of *P. mirifica* produced an earlier response and slower recovery of vaginal cornification. Treatment with 10 mg/kg BW of *P. mirifica* collected from 25 provinces produced no estrogenic response for any of the samples and no Co-type cells were observed. Treatment with 100 mg/kg BW of *P. mirifica* only induced vaginal cornification for 13 out of 25 cultivars. The appearance of cornified cells varied between the 4th and 7th day of the treatment period (Fig. 1). Rats treated with 100 mg/kg BW of *P. mirifica* collected from the Kanchanaburi and Mae Hong Son had the earliest vaginal cornification responses which occur at the same day (the 4th day). Treatment of 1,000 mg/kg BW of *P. mirifica* resulted in development of cornified cells in all rats but with differing first day of appearance, from day 2–8th (Fig. 1). Combining the results of the 100 and 1,000 mg/kg BW *P. mirifica* treatments, it was found that the first day of cornified cell appearance after *P. mirifica* treatment was related to the last day of its appearance (Fig. 2; $r^2 = 0.5446$, $P < 0.01$). That is, *P. mirifica* with the highest estrogenic activity also exhibited the longest duration of action.

Estrogenic activity of *P. mirifica* based on region

Based on the first day of Co-type cell appearance after *P. mirifica* treatment at a dose of 100 mg/kg BW and then at a dose of 1,000 mg/kg BW, *P. mirifica* was ranked into 12 levels, arbitrarily designated using numbers between 12 (the highest estrogenic activity) and 1 (the lowest estrogenic activity) (Figs. 1 and 3). The Kanchanaburi cultivar had the highest estrogenic activity, and the Uttaradith and Petchaburi cultivars had the lowest activity. Excluding the East and South regions, from which only one cultivar was collected each, comparison of the estrogenic activity of *P. mirifica* by region showed that the Central-West region had the highest estrogenic activity, but this result was not statistically significant difference ($P = 0.18$ and 0.17 , comparing between Central-West vs. North and Northeast, respectively, Table 1 and Fig. 3).

Estrogenic activity of *P. mirifica* based on the percentage of cornified cells

Judgement of estrogenic activity based on only the first day of Co-type cell appearance could be problematic if two (or more) cultivars have the

Fig. 1. The appearance of cornified cells in ovariectomized rats treated with 100 and 1,000 mg/kg BW of *P. mirifica* and 17β-estradiol. The graphs for each *P. mirifica* treatment are ranked sequentially in accordance with their estrogenic bioactivity from 12 (highest activity) to 1 (lowest activity) as shown by superscript numbers. "L" and "Co" stand for the "leukocyte" and "cornified" cells. The vertical dotted lines indicate the first and last days of Co-type cell occurrence in the 100 and 1,000 mg/kg BW of *P. mirifica* groups. The vertical line indicates the last day of the treatment period.

Fig. 2. Correlation analysis of the last day of cornified cell appearance and the first day of cornified cell appearance.

same first day of the Co-type cell appearance after treatment with 100 and 1,000 mg/kg BW of *P. mirifica*. Thus, calculation of the percentage of cornified cells should resolve this ranking problem.

The two cultivars of *P. mirifica* with the highest estrogenic activity, Kanchanaburi and Mae Hong Son, were selected and compared the percentage of cornified cells. The estrogenic activity calculated by the percentage of cornified cells resembled the results of the first day of Co-type cell appearance. That is, the percentage of cornified cells for 2 mg/kg BW of 17 β -estradiol was higher than for the Kanchanaburi, Mae Hong Son cultivars and distilled water, respectively (Table 2).

Discussion

P. mirifica phytoestrogens were found to have a dose-dependent estrogenic effect on rat vaginal cornification. A dose of 10 mg/kg BW from all plant samples did not produce a rat vaginal cornification, and only 13 of 25 *P. mirifica* samples induced a vaginal cornification at a dose of 100 mg/kg BW. The responses in the *P. mirifica*-treated rats were initiated by binding of phytoestrogens with the ER β and α type vaginal epithelial [27, 28]. Binding affinity to ER α and initiation of estrogenic response by *P. mirifica* has also been demonstrated in ER α human breast adenocarcinoma, MCF-7 [15]. The phytoestrogen concentrations in the blood

Fig. 3. A map of Thailand showing distribution of *P. mirifica* populations in 25 of the 76 provinces of Thailand. The degree of estrogenic activity is represented by the size of the circles.

circulation of the animals treated with 100 mg/kg BW *P. mirifica* samples have been too low compared with the maximum binding amount to ER, and thus the maximum estrogenic response could not be attained. This assumption was confirmed by induction of vaginal cornification occurring in all the rats after they were treated with a higher dose (1,000 mg/kg BW) of *P. mirifica*. In conclusion, the *P. mirifica* collected from different provinces exhibited great variation in estrogenic activity in terms of the vaginal cornification assay. The plants showing the highest estrogenic activity also maintained activity for a longer period. The plant sample from the Kanchanaburi cultivar exhibited the highest estrogenic activity and longest duration of action. Thus, the Kanchanaburi cultivar should be a candidate of choice for large-scale cultivation. Taken together, selection of the wild *P. mirifica* for cultivation to serve the market demand should take into consideration not only the estrogenic action of the plant but also profit calculations related to

Table 1. Regions, collection sites, estrogenic bioactivity, and first day of cornified cells appearance after daily treatment with 100 and 1,000 mg/kg BW/ of *P. mirifica* for each cultivar

Region	Collection sites (province)	Estrogenic bioactivity ^a	First day of cornified cells appearance	
			100 mg <i>P. mirifica</i>	1,000 mg <i>P. mirifica</i>
North	Mae Hong Son	11	4	4
	Chiang Rai	9	5	3
	Nan	3	–	4
	Phrae	3	–	4
	Phayao	2	–	5
	Lampang	2	–	5
	Lamphun	2	–	5
	Uttaradith	1	–	8
	N=8 provinces	4.13 ± 1.32	11.63 ± 1.56 ^b	4.75 ± 0.53
Northeast	Nakorn Ratchasima	6	6	3
	Sakon Nakhon	4	7	6
	Nong Bua Lam Phu	2	–	5
	Chaiyaphum	2	–	5
	N=4 provinces	3.50 ± 0.96	10.25 ± 2.17 ^b	4.75 ± 0.63
Central-West	Kanchanaburi	12	4	2
	Ratchaburi	10	5	2
	Nakorn Sawan	9	5	3
	Sukhothai	8	5	4
	Phitsanulok	7	6	2
	Phetchabun	7	6	2
	Kamphaeng Phet	6	6	3
	Tak	3	–	4
	Uthai Thani	3	–	4
	Lop Buri	3	–	4
	Phetchaburi	1	–	8
	N=11 provinces	6.27 ± 1.04	8.45 ± 1.34 ^b	3.45 ± 0.53
East	Phrachin Buri	5.00	6	4
South	Chumphon	9.00	5	3

Dashes indicate “no” cornified cells were observed during the treatment period.

^a Estrogenic bioactivity was arbitrarily designated as a number from 1 to 12 based on the first day of cornified cell appearance after daily treatment with 100 and 1,000 mg/kg BW of *P. mirifica* for each cultivar (a detailed explanation is included in the text).

^b Fourteen (the 14th day) was used as the first day of cornified cell appearance for calculations when no cornified cells were observed for daily cultivar treatment of 100 mg/kg BW/day.

Table 2. The percentage of rat cornified cells counted on the 4th day of the treatment period for 100 mg/kg BW *P. mirifica*, distilled water and E₂

Treatment	Percentage of cornified cells
17 β -estradiol (positive control)	100
Kanchanaburi	81.20 ± 3.12
Mae Hong Son	52.80 ± 4.09
Distilled water (negative control)	0

cultivation cost such as tuber size and the growth rate of plants.

There are at least three methods popularly used to investigate the estrogenic activity of synthetic estrogens, xenoestrogens, and phytoestrogens.

These methods include yeast estrogen screening (YES) of recombinant yeast cells [29, 30], proliferation assay with MCF-7 cells (E-assay) [30, 31] and uterotrophic assay in rodents [32]. Although the YES and E-assay methods are rapid methods, the results can not be transformed and applied to humans directly because metabolism (absorption, distribution and biotransformation) of estrogens and estrogen-like substances in humans is different from those of yeast and MCF-7 cells. Use of the uterotrophic assay in rodents seems to be the candidate of choice to resolve this problem. However, the uterotrophic assay has two disadvantages; firstly we need to kill the animals, and secondly we can not follow up changes of the

uterus weight during the treatment and post-treatment periods. Recently, we reported a sensitive, simple, and inexpensive method for evaluation of the estrogenic activity of synthetic estrogens and phytoestrogen-rich herbs [33]. This method uses the vaginal cornification assay in ovariectomized rats. We compared the estrogenic activity of those substances between the uterotrophic and vaginal cornification assays and obtained the similar results. Moreover, the vaginal cornification assay resolves the two disadvantages of the uterotrophic assay.

Screening of the estrogenic activity of *P. mirifica* by the vaginal cornification assay is sensitive, simple, and inexpensive. However, this method has the following weaknesses: 1) the results are expressed as a non-continuous number (or all-or-none); including stimulation or no stimulation of Co-type cells and 2) two (or more) plants always show the same estrogenic activity ranking. This problem can be resolved by calculation of the percentage of cornified cells.

It had been realized that Phytoestrogen Replacement Therapy (PRT) is an effective alternative for Estrogen Replacement Therapy (ERT). This study confirmed the differential estrogenic effect of wild *P. mirifica* collected from a majority of the plant population, 25 out of a total of 76 provinces in Thailand using the vaginal cornification assay. A similar study with *Pueraria Radix*, a plant with tubers and high amount of isoflavone, especially puerarin has also been conducted. In that study the estrogenic activity of *P. Radix* tubers collected from many parts of Korea were compared using uterotrophic assay with that of *P. mirifica* collected from Thailand. None of the collected samples of *P. Radix* expressed an uterotrophic effect, although the Thai *P. mirifica* did. The authors concluded that the isoflavone contents in *P. Radix* were not related to estrogenic

activity [34].

Considering the variability in the estrogenic activity of *P. mirifica* by region, the plants of the Central-West region showed high estrogenic activity in accordance with the phytoestrogen contents analyzed by HPLC [24]. Our investigation into the major isoflavonoid contents of the Thai *P. mirifica* population is intriguing because of the great diversity of puerarin, daidzin, genistin, daidzein and genistein in the plant tubers collected from the different sites. This is probably being one of the reasons why the plant tubers derived from different collection sites also varied in estrogenic activity. A possible explanation for this may be that the forest type and climate of the Central-West region may critical for plant growth [35]. This conclusion could be more precise if the differences in plant genetics are clarified because both genetics and environment, especially geographic distribution, could influence the isoflavone contents of plant materials [36]. The results of this study should benefit not only ranking of the quality of the *P. mirifica* plants based on the strength of their estrogenic activity but also individuals who need to farm the *P. mirifica* plants or process their tuber products. Based on the latest information we have obtained, it is possible that Thai *P. mirifica* populations have high potential to be introduced as an effective herbal phytoestrogen source [13, 15–22]. The plant should be considered the most potent phytoestrogen source for replacement of estrogen therapy.

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