—Research Note—

Determination of the Estrogenic Activity of Wild Phytoestrogen-rich *Pueraria mirifica* by MCF-7 Proliferation Assay

Wichai CHERDSHEWASART¹⁾, Virasinee TRAISUP²⁾ and Porntipa PICHA³⁾

¹⁾Department of Biology, ²⁾Biotechnology Program, Faculty of Science, Chulalongkorn University, Bangkok 10330 and

³⁾Section of Experimental Oncotherapy, Research Division, National Cancer Institute, Ministry of Public Health, Bangkok 10400, Thailand

Abstract. The aim of this study was to evaluate the estrogenic activity of tuberous samples of wild, phytoestrogen-rich *Pueraria mirifica* collected from 28 out of 76 provinces of Thailand by MCF-7 proliferation assay. The plant extracts were administered to MCF-7, ER α positive human mammary adenocarcinoma cell cultures, for 3 days at dosages of 0.1, 1, 10, 100 and 1,000 μ g/ml and were compared with 17 β -estradiol at concentrations of 10⁻¹²–10⁻⁶ M. The mean *P. mirifica* population at 1 μ g/ml exhibited significant proliferation. Two plant samples exhibited levels of proliferation in MCF-7 that were similar to 17 β -estradiol. The mean *P. mirifica* populations at 100 and 1,000 μ g/ml exhibited significant cytotoxicity in MCF-7. Analysis of the estrogenic activity of puerarin, representative of major isoflavonoids in *P. mirifica* tubers, revealed proliferation in MCF-7 only at the highest dose (10⁻⁶ M) that was 10²–10⁵ times less active than 17 β -estradiol. Puerarin and 17 β -estradiol at concentration of 10⁻¹²–10⁻⁶ M exhibited no cytotoxicity in MCF-7.

Key words: Isoflavonoid, MCF-7 proliferation assay, Phytoestrogen, *Pueraria mirifica*, Puerarin

(J. Reprod. Dev. 54: 63–67, 2008)

The plants in genus *Pueraria* are largely distributed in Asia and have a long history of ethnopharmacological applications [1]. *Pueraria mirifica* Airy Shaw et Suvatabhandu, family Leguminosae, is an indigenous Thai herb. The potent estrogenic activity of the plant has been proven in a 1-year human clinical trial of 4 menopausal volunteers at Ramathibodi Hospital, Faculty of Medicine, Mahidol University, Bangkok, Thailand. An average dose of 100 mg of tuberous powder per day was effective for long-term treatment of menopausal symptoms [2], and toxicological tests showed that it did not have significant adverse effects in animals and human volunteers. An acute toxicity test of *P. mirifica* treatment at a dose of 16 g/kg BW using rats did not reveal any adverse effects [3]. However, *P. mirifica* did initiate adverse effects such as anemia, during subchronic treatment for 3 months at doses of 100 and 1,000 mg/kg BW [4]. Haematological adverse effects were confirmed in a 6-month chronic toxicity test at a dose of 250 mg/kg BW [5]. Treatment with the plant extract at high doses 600 and 800 mg/kg BW, for 30 days induces micronucleus formation in male rats [6]. However, pretreatment of weanling rats with *P. mirifica* powder at a dose of 10, 100 and 1,000 mg/kg BW for 28 days resulted in a dose-dependent prevention of mammary tumors induced by 7, 12-dimethylbenz (a) anthracene (7, 12-DMBA) [7].

The phytoestrogens of *P. mirifica* exhibit the following responses: dose-dependent estrogenic effects on the reproductive system, initiated vaginal cornification, increased uterine weight with decreased follicle stimulating hormone (FSH) and luteinizing hormone (LH) serum levels in ovariectomized rats [8], increased length of the follicular phase and total menstruation cycle [9], decreased estrogen, progesterone, FSH, and LH concentrations at

the dose of 1,000 mg/kg BW [10], and ovulation blockage in female monkeys [11]. In aged menopausal monkeys, consumption of crude powder from the plant decreases the concentrations of FSH and LH [12] and parathyroid hormone (PTH) [13] and induces reappearance of sexual skin [14]. Consumption of a high amount of *P. mirifica* caused disruption of male sex organs, including the epididymis and seminal vesicle, and affects sperm motility and viability [15] but leads to protection against osteoporosis in orchidectomized rats [16].

Vaginal cornification can be used to rapidly evaluate estrogenic activity on the vaginal epithelium of ovariectomized rats [17]. This method has been adapted for evaluation of the Thai *P. mirifica* population and is capable of ranking plants based on their estrogenic activity in relation to vaginal epithelium differentiation [18]. These findings confirmed that the Thai *P. mirifica* population could be used as an alternative to estrogen.

The MCF-7 proliferation assay which is based on proliferation of ER α positive human mammary adenocarcinoma cells, is a well accepted method for evaluation of estrogenic effects initiated by phytochemicals [19], animal products [20] and plants [21]. This system has also been used in evaluating the anti-estrogenic activity of chemicals [22]. HPLC analysis of *P. mirifica* distributed in 28 provinces of Thailand revealed that isoflavonoids are major chemical components of the tubers and also that they exhibit great variation within the plant population. The results of the study lead not only to the conclusion that the plant population exhibited chemovariety but also lead to the question of whether or not the tubers collected from different sites, with different isoflavonoid contents, exhibited the same level of estrogenic activity [23]. We therefore applied the MCF-7 proliferation assay to evaluation of the estrogenic activity of the plant population. Puerarin, one of the major isoflavonoids in this plant, was also analyzed in parallel with 17 β -estradiol to aid in elucidating estrogenic mechanisms, and this

allows for this data to contribute to a better understanding of the roles of puerarin in estrogenic activity in relation to this plant. The results of this study could be beneficial not only as a guide for ranking plants from different sites but also as a guide for tuber collectors by providing information on estrogenic activity.

Materials and Methods

Plant material

The tuberous roots of wild *P. mirifica* were collected from 28 out of 76 provinces in Thailand: these plants had previously been analyzed concerning the major isoflavonoid contents of puerarin, daidzin, genistin, daidzein and genistein [23]. The plants were identified by the authors by referring to a previous study [24] and were compared with the voucher herbarium specimen, No. BCU 11045, which was collected from Chiang Mai Province, Thailand. Preparation of ethanol (EtOH) extracts from the plants was also performed as described previously [25].

Cell culture

MCF-7, estrogen receptor α (ER α) positive human mammary adenocarcinoma cells, were obtained from the Section of Experimental Oncotherapy, Research Division, National Cancer Institute, Ministry of Public Health, Thailand. The cells were grown as a monolayer in Eagle's Minimal Essential Medium (EMEM) with phenol red supplemented with 10% heat-inactivated newborn calf serum (NCS; BioWhittaker, Walkersville, MD, USA), 10^4 unit/l Penicillin G and 1 mg/l Streptomycin sulfate (antibiotics; BioWhittaker) and incubated at 37 C in a 5% CO₂ incubator. The medium was changed once every 3–4 days to maintain the optimum conditions for exponential growth.

Estrogenic activity analysis by proliferation assays

The EMEM medium was removed, and the cells were rinsed with 3–4 ml phosphate buffer saline (PBS). The cells were trypsinized with 4 ml of 0.25% trypsin. Fresh Dulbecco's Modified Eagle's Medium (DMEM), without phenol red was added and aspirated gently. The cells were counted using 0.4% trypan blue dye solution and a hemocytometer. One hundred μ l of diluted MCF-7 cell suspension (at approximately 5×10^4 cells/ml) were inoculated onto 24-well plates in DMEM medium supplemented with 10% heat-inactivated NCS penicillin and streptomycin.

Stock solutions (10^{-5} M) of puerarin and 17 β -estradiol were freshly prepared in dimethyl sulfoxide (DMSO) diluted with control solution [(DMEM culture medium with 2% DMSO (v/v))]. Stock solutions of the plant extracts were freshly prepared (10^4 μ g/ml) in DMSO, diluted with cell culture medium. The concentration of DMSO following dilution was 2% of the total volume. The assays were prepared by incubating the cells with puerarin and 17 β -estradiol (at 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} and 10^{-12} M with 3 wells per concentration), and with neither puerarin nor 17 β -estradiol [control D3, MCF-7 cells in 2% DMSO (v/v)]. The multi-well plates were incubated at 37 C in a 5% CO₂ incubator for 3 days. The assays for the plant extracts were prepared by incubating the cells with the plant extracts (at 0.1, 1, 10, 100 and 1,000 μ g/ml, with 3 wells per concentration) and without the plant extracts (con-

trol D3). The inhibitory concentration (IC₅₀) for D3 analysis of the plant extracts was based on the concentration interval of the plant extracts causing 50% cell death, in comparison with DMSO (control D3).

MTT assay

The culture flasks were medium drained by inversion. The adherent cell layer was rinsed once with 1 ml DMEM and subsequently fed into micro-well plates with 900 μ l fresh DMEM. One hundred microliters of 3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution was added to the wells and incubated for 3 h in the dark at 37 C. The medium and MTT were removed from the wells. The remaining MTT-formazan crystals were dissolved by addition of 900 μ l DMSO together with 125 μ l glycine buffer per well. The absorbance of the solution was analyzed by spectrophotometer at a wavelength of 570 nm with 900 μ l DMSO and 125 μ l glycine buffer as the blank [26].

Calculation of cell growth

The absorbance value of the control D3 was taken as 100% cell growth. The absorbance values of the other samples were converted to percentage values compared with the control D3. The concentration range at which 50% of growth inhibition occurred was considered to be the IC₅₀.

Statistical analysis

The results are shown as means $\pm$ SEM of three replicated experiments. One-way Anova was performed to determine significance differences within groups and between groups (LSD test). All statistical analysis was performed using SPSS version 10.0 (SPSS Chicago, IL, USA). A P value of less than 0.05 was considered to indicate statistical significance.

Results

Puerarin at concentration of 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} and 10^{-12} M were compared with 17 β -estradiol at the same concentrations in the incubation tests with MCF-7. MCF-7 in DMSO was used as a negative control. Puerarin at a concentration of 10^{-6} M and 17 β estradiol at concentrations of 10^{-11} – 10^{-8} M produced significant proliferation in MCF-7 ($P < 0.05$). The two chemicals did not show cytotoxicity at the tested concentrations (Table 1).

Sixteen and 1 of the 28 samples produced significant proliferation ($P < 0.05$) in MCF-7 at concentrations of 1 and 10 μ g/ml, respectively. Seven, 17 and 28 of the 28 samples exhibited cytotoxicity ($P < 0.05$) for MCF-7 at concentrations of 10, 100 and 1,000 μ g/ml, respectively. The IC₅₀ values of the plant extracts against proliferation of MCF-7 were also calculated. None of the plant samples exhibited an IC₅₀ of less than the value of 1,000 μ g/ml. At 1 μ g/ml (Table 2), the mean *P. mirifica* population exhibited significant proliferation ($P < 0.05$). At 100 and 1,000 μ g/ml (Table 2), the mean plant population exhibited significant cytotoxicity ($P < 0.05$).

Discussion

P. mirifica tuberous extracts collected from 28 out of the 76

provinces of Thailand produced proliferation in MCF-7 at a low concentration, 1 $\mu\text{g/ml}$, and cytotoxicity at high concentrations, 100–1,000 $\mu\text{g/ml}$. These results are similar to those from a previous investigation with only one sample of *P. mirifica* collected from Chiang Mai Province [25]. The results of these tests are also the same as those found in studies with genistein or daidzein, the key phytoestrogens in soya [27–29]. The plant crude extracts collected from Phitsanulok Province (exhibiting cell growth of 145.70

$\pm 8.93\%$) and Nakhon Sawan Province (exhibiting cell growth of $142.99 \pm 11.84\%$) at 1 $\mu\text{g/ml}$ showed the same level of proliferative effect as 17β -estradiol at 10^{-10} M (exhibiting cell growth of $146.00 \pm 5.81\%$). This means that phytoestrogens from the two crude extracts of *P. mirifica* at 1 $\mu\text{g/ml}$ could exhibit strong binding with the ER α of MCF-7 cells. This is strong evidence that some of the Thai *P. mirifica* samples could be effectively used as a replacement for estrogen.

In the present study, 17β -estradiol at the concentrations of 10^{-12} – 10^{-6} M exhibited no cytotoxic effects on MCF-7 cells. On the other hand, the *P. mirifica* population exhibited cytotoxic effects at concentrations of 100 and 1,000 $\mu\text{g/ml}$. We previously demonstrated in a test with the same cell line that a high concentration of this plant's extract at 1,000 $\mu\text{g/ml}$ leads to strong competitive binding with estrogen and a dramatic increase in cytotoxicity or produces a potent anti-estrogenic effect [25]. Nevertheless, the estrogenic activity of the plant tubers is considered high as only a crude EtOH extract was used, not a purified form as in a previous study [30]. It should be noted that in the estrogenic activity analysis using the vaginal cornification assay with the same plant samples, the first two plant samples that exhibited the strongest estrogenic effect were samples collected from Kanchanaburi and Chumphon Prov-

Table 1. The proliferation effect of puerarin and 17β -estradiol on MCF-7

Concentration	17β -estradiol	Puerarin
10^{-6} M	104.12 ± 3.67	$130.25 \pm 11.10^*$
10^{-7} M	126.62 ± 17.74	124.18 ± 16.04
10^{-8} M	$137.56 \pm 13.72^*$	124.58 ± 12.77
10^{-9} M	$138.22 \pm 12.54^*$	118.44 ± 19.40
10^{-10} M	$146.45 \pm 5.81^*$	122.76 ± 8.19
10^{-11} M	$142.34 \pm 16.10^*$	112.31 ± 4.06
10^{-12} M	130.16 ± 15.25	106.72 ± 14.25

Each value is the mean $\pm$ SEM (n=3). * Proliferation of growth of MCF-7 is significantly greater than for DMSO ($P < 0.05$)

Table 2. The growth response percentage of *P. mirifica* extract on MCF-7 in comparison with DMSO

Provinces	0.1 $\mu\text{g/ml}$	1 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	1,000 $\mu\text{g/ml}$	IC ₅₀ ($\mu\text{g/ml}$)
DMSO	100.00 ± 3.19	100.00 ± 3.19	100.00 ± 3.19	100.00 ± 3.19	100.00 ± 3.19	—
Phitsanulok	113.71 ± 1.70	$145.70 \pm 8.93^*$	$73.99 \pm 3.64^{**}$	$55.50 \pm 8.27^{**}$	$50.30 \pm 4.22^{**}$	>1,000
Nakhon Sawan	113.77 ± 10.35	$142.99 \pm 11.84^*$	91.65 ± 7.39	$75.76 \pm 8.53^{**}$	$61.48 \pm 10.40^{**}$	>1,000
Phetchabun	116.51 ± 12.81	$135.67 \pm 2.11^*$	$73.45 \pm 6.18^{**}$	$68.03 \pm 8.26^{**}$	$49.11 \pm 4.55^{**}$	>1,000
Sukhothai	105.95 ± 9.97	$134.73 \pm 11.90^*$	80.65 ± 3.93	$74.99 \pm 10.42^{**}$	$61.46 \pm 2.62^{**}$	>1,000
Nan	105.78 ± 4.92	$128.53 \pm 3.26^*$	83.13 ± 2.48	$59.41 \pm 2.00^{**}$	$54.55 \pm 5.56^{**}$	>1,000
Chiang Rai	113.74 ± 12.33	$128.43 \pm 5.89^*$	81.69 ± 5.13	81.45 ± 15.91	$70.01 \pm 8.80^{**}$	>1,000
Chiang Mai	100.49 ± 1.19	$128.43 \pm 8.13^*$	92.46 ± 5.04	$80.02 \pm 0.41^{**}$	$73.66 \pm 5.34^{**}$	>1,000
Prachuap Khiri Khan	111.65 ± 5.29	$127.71 \pm 8.66^*$	94.79 ± 5.43	$74.46 \pm 1.34^{**}$	$71.22 \pm 5.80^{**}$	>1,000
Ratchaburi	116.8 ± 2.17	$127.12 \pm 4.56^*$	106.06 ± 6.06	91.14 ± 1.13	$55.71 \pm 3.22^{**}$	>1,000
Mae Hong Son	102.91 ± 10.02	$127.04 \pm 8.81^*$	97.00 ± 7.39	87.32 ± 3.60	$57.37 \pm 1.77^{**}$	>1,000
Lamphun	105.73 ± 2.86	$125.83 \pm 3.26^*$	$90.35 \pm 1.88^{**}$	85.78 ± 3.81	$74.10 \pm 3.54^{**}$	>1,000
Chumphon	102.751 ± 3.33	$123.69 \pm 7.05^*$	106.38 ± 14.27	100.69 ± 8.06	$82.44 \pm 5.36^{**}$	>1,000
Kanchanaburi	101.37 ± 1.37	123.67 ± 12.49	82.27 ± 5.66	$77.27 \pm 3.38^{**}$	$69.74 \pm 5.02^{**}$	>1,000
Phrae	108.44 ± 4.41	$122.97 \pm 4.37^*$	$73.08 \pm 3.01^{**}$	$60.30 \pm 7.79^{**}$	$55.26 \pm 3.02^{**}$	>1,000
Chaiyaphum	105.92 ± 3.06	$120.62 \pm 2.74^*$	96.60 ± 5.92	87.14 ± 2.33	$75.78 \pm 2.15^{**}$	>1,000
Lop Buri	102.86 ± 0.14	$120.07 \pm 7.16^*$	83.76 ± 5.82	$79.54 \pm 6.19^{**}$	$72.14 \pm 4.07^{**}$	>1,000
Phrachin Buri	114.13 ± 3.88	$119.69 \pm 10.27^*$	$107.19 \pm 4.13^*$	93.35 ± 6.65	$78.25 \pm 5.17^{**}$	>1,000
Nong Bua Lam Phu	109.90 ± 15.06	118.46 ± 7.25	94.20 ± 7.37	80.46 ± 10.39	$56.34 \pm 6.83^{**}$	>1,000
Phayao	114.63 ± 6.35	117.55 ± 5.38	91.55 ± 4.81	92.87 ± 2.68	$72.44 \pm 5.30^{**}$	>1,000
Tak	99.82 ± 5.02	115.13 ± 6.51	$74.54 \pm 4.79^{**}$	$60.31 \pm 14.8^{**}$	$55.85 \pm 7.80^{**}$	>1,000
Sakon Nakhon	111.31 ± 2.68	114.31 ± 12.12	84.49 ± 5.87	$73.86 \pm 2.56^{**}$	$64.33 \pm 7.46^{**}$	>1,000
Uttaradit	100.25 ± 2.80	112.92 ± 3.77	85.61 ± 14.22	$63.84 \pm 7.04^{**}$	$62.88 \pm 0.89^{**}$	>1,000
Nakhon Ratchasima	100.79 ± 4.31	112.68 ± 4.87	$73.28 \pm 13.04^{**}$	$65.00 \pm 14.63^{**}$	$46.77 \pm 9.85^{**}$	>1,000
Kamphaeng Phet	104.74 ± 4.38	111.89 ± 4.05	96.39 ± 7.38	91.75 ± 6.03	$75.35 \pm 3.64^{**}$	>1,000
Lampang	105.11 ± 2.31	111.61 ± 5.45	93.51 ± 3.03	$80.50 \pm 4.77^{**}$	$66.93 \pm 6.95^{**}$	>1,000
Phetchaburi	100.03 ± 4.8	105.92 ± 4.99	81.07 ± 6.12	$68.02 \pm 6.02^{**}$	$51.34 \pm 6.99^{**}$	>1,000
Saraburi	100.29 ± 9.02	105.40 ± 4.07	$74.74 \pm 9.69^{**}$	$70.86 \pm 7.40^{**}$	$50.19 \pm 2.73^{**}$	>1,000
Uthai Thani	99.26 ± 1.46	102.35 ± 7.00	92.12 ± 5.52	78.71 ± 6.98	$76.06 \pm 3.62^{**}$	>1,000
Means $\pm$ SEM	105.66 ± 1.14	$121.07 \pm 1.56^*$	86.62 ± 1.49	$76.95 \pm 1.65^{**}$	$64.36 \pm 1.49^{**}$	>1,000

Each value is the mean $\pm$ SEM (n=3). * Proliferation of growth of MCF-7 is significantly greater than for DMSO ($P < 0.05$). ** Antiproliferation to the growth of MCF-7 is significantly greater than for DMSO ($P < 0.05$). DMSO is the negative vehicle control (without plant extract).

inces [18], which was not the case in present study. MCF-7 contains only ER α but both ER α and ER β are present in the vaginal epithelium. This may be one of the reasons why the estrogenic response of MCF-7 cells did not correlate with the estrogenic response of the vaginal epithelium. The previous estrogenic testing was done using rats, while the present study was done using a human mammary adenocarcinoma cell culture. This implies that the two estrogenic activity analysis methods share different estrogenic mechanisms and might also be initiated by different amounts or even different types of ER.

Puerarin, a selected representative of major isoflavonoids in the *P. mirifica* population (23.01 ± 1.80 mg/100g powder [23]), led to proliferation of MCF-7 only at a high concentration (10^{-6} M), while 17β -estradiol showed the same effect at concentrations of 10^{-11} – 10^{-8} M. It is clear that puerarin exhibited a proliferative effect that was 10^2 – 10^5 times lower than 17β -estradiol. *In vitro* puerarin may play a strong role in proliferation, but the plant's tuberous puerarin content may not be high enough to induce this proliferative effect. Concentrations of 10^{-12} – 10^{-6} M puerarin have no cytotoxic effect on MCF-7, so the cytotoxicity of all the plant samples at high doses, 100–1,000 μ g/ml, in this study is probably derived from other phytoestrogens. Genistin is the isoflavonoid with the highest concentration in the *P. mirifica* population (35.39 ± 2.09 mg/100 g powder [23]). The results of this study may thus be related to the genistin content in the plant tubers. Even though genistein manifested an cytotoxic action on MCF-7 by alternation of estrogen receptor dependent pathways [31] and exhibited strong competitive binding to the ER α of MCF-7 with estrogen [32], genistein and daidzein are present in small amounts in the plant population, 1.19 ± 0.10 and 6.12 ± 0.40 mg/100 g powder, respectively [23], and thus had no cytotoxic role. Tests using MCF-7 with deoxymiroestrol and miroestrol isolated from *P. mirifica* have also been conducted [33, 34]. These two chemicals were found to exhibit more estrogenic potency than genistein and daidzein. It is obvious that they also played a great role in the MCF-7 assay of the plant population.

In summary, in comparison with the vaginal cornification assay proposed for evaluation of estrogenic activity in phytoestrogen-rich herbs, testing with MCF-7 is quicker and more convenient. Furthermore, an estrogenic response and cytotoxicity can be observed within the same test, but with different concentrations of the tested materials. The MCF-7 proliferation assay is also convenient for analysis of large samples, such as the population of *P. mirifica* in this study. The only disadvantage of this method is that the results of the test may not represent the estrogenic response in animals or humans due to the absence of a metabolic activation system and differences in ER types and contents. Furthermore, the results clearly demonstrated that evaluation of the estrogenic activity of the phytoestrogen-rich plants definitely varied depending on the analytical methods used.

Acknowledgements

The authors wish to thank the Graduate School, Chulalongkorn University, and the National Center for Genetic Engineering and Biotechnology, Ministry of Science and Technology, Thailand, for

research grants and Svend Jensen for English proof reading.

References

1. van der Maesen LJG. Pueraria: botanical characteristics. In: Keung WM (ed.), Pueraria, The genus Pueraria. London and New York: Taylor & Francis; 2002: 1–28.
2. Muangman V, Cherdshewasart W. Clinical trial of the phytoestrogen-rich herb, *Pueraria mirifica* as a crude drug in the treatment of symptoms in menopausal women. *Siriraj Hosp Gaz* 2001; 53: 300–309.
3. Cherdshewasart W. Toxicity tests of a phytoestrogen-rich herb; *Pueraria mirifica*. *J Sci Res Chulalongkorn Univ* 2003; 28: 1–12.
4. Chiwapat S, Chavalittumrong P, Rattanajarasroj S, Chuthaputti A, Panyamung S. Toxicity study of *Pueraria mirifica* Airy Shaw et Suvatabhandhu. *Bull Med Sci* 2000; 42: 202–223 (in Thai with English abstract).
5. Chiwapat S, Chavalittumrong P, Rattanajarasroj S, Panyamung S. Chronic toxicity of *Pueraria mirifica* in rats. *Thai J Pharm* 2005; 27: 76–90.
6. Saenphet K, Kantarop S, Saenphet S, Aritajat S. Mutagenicity of *Pueraria mirifica* Airy Shaw & Suvatabhandhu and antimutagenicity of *Thunbergia laurifolia* Linn. *Southeast Asian J Trop Med Public Health* 2005; 36: 238–241.
7. Cherdshewasart W, Panriansaen R, Picha P. Pretreatment with phytoestrogen-rich plant decreases breast tumor incidence and exhibits lower profile of ER α and ER β . *Maturitas* 2007; 58: 174–181.
8. Malaivijitnond S, Kiatthaipipat P, Cherdshewasart W, Watanabe G, Taya K. Different effects of *Pueraria mirifica*, a herb containing phytoestrogens, on LH and FSH secretion in gonadectomized female and male rats. *J Pharmacol Sci* 2004; 96: 428–435.
9. Trisomboon H, Malaivijitnond S, Watanabe G, Taya K. Estrogenic effects of *Pueraria mirifica* on the menstrual cycle and hormone-related ovarian function in cyclic female cynomolgus monkeys. *J Pharmacol Sci* 2004; 94: 51–59.
10. Trisomboon H, Malaivijitnond S, Cherdshewasart W, Watanabe G, Taya K. Assessment of urinary gonadotropin and steroid hormone profiles of female cynomolgus monkeys after treatment with *Pueraria mirifica* herb. *J Reprod Dev* 2007; 53: 395–403.
11. Trisomboon H, Malaivijitnond S, Watanabe G, Taya K. Ovulation block by *Pueraria mirifica*—a study of its endocrinological effect in female monkeys. *Endocrine* 2005; 26: 33–39.
12. Trisomboon H, Malaivijitnond S, Watanabe G, Cherdshewasart W, Taya K. The estrogenic effect of *Pueraria mirifica* on gonadotropin levels in aged monkeys. *Endocrine* 2006; 29: 129–134.
13. Trisomboon H, Malaivijitnond S, Suzuki J, Hamada Y, Watanabe G, Taya K. Long-term treatment effects of *Pueraria mirifica* phytoestrogens on parathyroid hormone and calcium levels in aged menopausal cynomolgus monkeys. *J Reprod Dev* 2004; 50: 639–645.
14. Trisomboon H, Malaivijitnond S, Cherdshewasart W, Watanabe G, Taya K. Effect of *Pueraria mirifica* on the sexual skin coloration of aged menopausal cynomolgus monkeys. *J Reprod Dev* 2006; 52: 537–542.
15. Jaroenporn S, Malaivijitnond S, Wattanasirmit K, Trisomboon H, Watanabe G, Taya K, Cherdshewasart W. Effects of *Pueraria mirifica*, an herb containing phytoestrogens, on reproductive organs and fertility of adult male mice. *Endocrine* 2006; 30: 93–101.
16. Urasopon N, Hamada Y, Asaoka K, Cherdshewasart W, Malaivijitnond S. *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats. *Maturitas* 2007; 56: 322–331.
17. Malaivijitnond S, Chansri K, Kijkuokul P, Urasopon N, Cherdshewasart W. Using vaginal cytology to assess the estrogenic activity of phytoestrogen-rich herb. *J Ethnopharmacol* 2006; 107: 354–360.
18. Cherdshewasart W, Kitsamai Y, Malaivijitnond S. Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. *J Reprod Dev* 2007; 53: 385–393.
19. Ketcha Wanda GJM, Njamen D, Yankep E, Tagatsing Fotsing M, Taneef Fomum Z, Wober J, Starcke S, Zierau O, Vollmer G. Estrogenic properties of isoflavones derived from *Milletia griffoniana*. *Phytomedicine* 2006; 13: 139–145.
20. Mishima S, Suzuki K-M, Isohama Y, Kuratsu N, Araki Y, Inoue M, Miyata T. Royal jelly has estrogenic effects *in vitro* and *in vivo*. *J Ethnopharmacol* 2005; 101: 215–220.
21. Innocenti G, Vegeto E, Dall'Acqua S, Ciana P, Giorgetti M, Agradi E, Sozzi A, Fico G, Tomè F. *In vitro* estrogenic activity of *Achillea millefolium* L. *Phytomedicine* 2007; 14: 147–152.
22. Jung J, Ishida K, Nishihara T. Anti-estrogenic activity of fifty chemicals evaluated by *in vitro* assays. *Life Sci* 2004; 74: 3065–3074.
23. Cherdshewasart W, Subtang S, Dahlan W. Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison with *Pueraria lobata*. *J Pharm Biomed Anal* 2007; 43: 428–434.
24. Kashemsanta MLC, Suvatabhandu K, Airy SHK. A new species of *Pueraria* (Legumi-

- nosae) from Thailand, yielding an oestrogenic principle. *Kew Bull* 1952; 7: 263–266.
25. **Cherdshewasart W, Cheewasopit W, Picha P.** The differential anti-proliferation effect of white (*Pueraria mirifica*), red (*Butea superba*), and black (*Mucuna collettii*) Kwao Krua plants on the growth of MCF-7 cells. *J Ethnopharmacol* 2004; 93: 255–260.
 26. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival; Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–63.
 27. **Wang CF, Kurzer MS.** Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. *Nutr Cancer* 1997; 28: 236–247.
 28. **Zava DT, Duwe G.** Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. *Nutr Cancer* 1997; 27: 31–40.
 29. **Constantinou AI, Krygier AE, Mehta RR.** Genistein induces maturation of cultured human breast cancer cells and prevents tumor growth in nude mice. *Am J Clin Nutr* 1998; 68: 1426s–1430s.
 30. **Ye HY, Lou YJ.** Estrogenic effects of two derivatives of icarin on human breast cancer MCF-7 cells. *Phytomedicine* 2005; 12: 735–741.
 31. **Chen W-F, Huang M-H, Tzang C-H, Yang M, Wong M-S.** Inhibitory actions of genistein in human breast cancer (MCF-7) cells. *Biochem Biophys Acta* 2003; 1638: 187–196.
 32. **Stroheker T, Picard K, Lhuguenot JC, Canivenc-Lavier MC, Chagnon MC.** Steroid activities comparison of natural and food wrap compounds in human breast cancer cell lines. *Food Chem Tox* 2004; 42: 887–897.
 33. **Chansakaow S, Ishikawa T, Seki H, Sekine K, Okada M, Chaichantipyuth C.** Identification of deoxymiroestrol as the actual rejuvenation principle of “Kwao Keur”, *Pueraria mirifica*, the known miroestrol may be an artifact. *J Nat Prod* 2000; 63: 173–175.
 34. **Matsumura A, Ghosh A, Pope GS, Darbre PD.** Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *J Steroid Biochem Mol Biol* 2005; 94: 431–443.