RESEARCH ARTICLE

ScienceAsia 34 (2008): **371–376**

doi: 10.2306/scienceasia1513-1874.2008.34.371

Isoflavone content of rodent diets and its estrogenic effect on vaginal cornification in *Pueraria mirifica*-treated rats

Nontakorn Urasopon[a,b,c], **Yuzuru Hamada**[d], **Kazuo Asaoka**[e], **Ubon Poungmali**[c], **Suchinda Malaivijitnond**[c,*]

[a] Biological Science Ph.D. Program, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Animal Science, Faculty of Agriculture, Ubon Rajathanee University, Ubon Ratchathani 34190, Thailand
[c] Primate Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[d] Section of Morphology, Primate Research Institute, Kyoto University, Inuyama, Aichi, 484-8506, Japan
[e] Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi, 484-8506, Japan

[*] Corresponding author, e-mail: suchinda.m@chula.ac.th

Received 24 May 2008
Accepted 9 Oct 2008

ABSTRACT: *Pueraria mirifica* or White Kwao Krua has been extensively studied for its estrogenic effects on reproductive organs and bones using rodents as experimental animals. Commercial rodent diets are usually formulated with soybean products and therefore deliver a high dose of isoflavone phytoestrogens. Using high performance liquid chromatography, we determined the quantities of five major isoflavones (puerarin, daidzin, genistin, daidzein, and genistein) in five lots of standard rodent diets, a soybean-free diet, and two lots of *P. mirifica* 'Wichai-III'. The concentrations of total isoflavones were 38.6–72.4 mg/100 g in the standard rodent diets, 6.1 mg/100 g in the soybean-free diet, and 123.2–157.3 mg/100 g in the *P. mirifica*. While absent in the rodent diets, puerarin accounted for about half of the isoflavone content in *P. mirifica*. The levels of genistein and genistin in *P. mirifica* were very low compared to the level found in the standard rodent diets. Given the same dose of 50 mg/kg BW/day of *P. mirifica* for 14 days, rats fed with standard rodent diet showed a significantly higher percentage of cornified cells than those fed with soybean-free diet. These findings suggest the potential presence of phytoestrogens in standard rodent diets and its liability to be a confounding factor in estrogenic or phytoestrogenic research.

KEYWORDS: puerarin, genistein, phytoestrogens, white kwao krua, HPLC

INTRODUCTION

Phytoestrogens are produced by plants and function in a similar way to endogenous estrogen. The three main classes of phytoestrogens are isoflavones, coumestans, and lignans. Isoflavones are predominantly found in soybeans and other legumes. Coumestans are found at high concentrations in clover, alfalfa, and soy sprouts. Lignans occur in oil seed, whole cereals, legumes and fruits[1]. Recently, the phytoestrogen-rich plants, *Pueraria* spp. and related plants, have been the subject of interest for researchers, especially with respect to their estrogenic properties[2–11]. *Pueraria* spp. have been widely used in oriental herbal medicines in China, Korea, Japan, and Thailand. In particular, *Pueraria mirifica* Airy Shaw et Suvatabhandu (Leguminosae), which contains large amounts of isoflavones, has been extensively studied for its content[8,12] and estrogenic effects on reproductive organs[5–8] and bones[10,11,13].

Most in vivo studies evaluating the estrogenic effects of phytoestrogens in *P. mirifica* used rodents as experimental animals[5–11]. However, the diets fed to experimental rodents usually contain soybean products as a protein source. A number of laboratories in the US and Europe have reported that commercially available rodent dietary formulations contain variable but significant levels of phytoestrogens, especially daidzein and genistein[14–18]. They therefore deliver large daily doses of isoflavones to experimental rodents. This was shown by the isoflavone levels in the serum of adult rats (2613 ± 873 ng/ml) and mice (2338 ± 531 ng/ml) exceeding the animals' endogenous estrogen levels by 30 000–60 000 times[19].

Dagen et al[14] conducted an experiment to evaluate the estrogenic effect of genistein in ovariectomized (OVX) rats by determining the uterine weight. They

reported that when given the same dose of genistein treatment, rats fed with soybean diet exhibited a larger uterine weight than rats fed with a phytoestrogen-free diet. The phytoestrogen content in rodent diets is dependent upon the supplier[20]. It was therefore suspected that the presence of endogenous phytoestrogens may disturb the response of animals to exogenously administered estrogenic substances[16,21]. Hence, the testing of estrogenic activity of estrogenic or phytoestrogenic substances in experimental rodents should be designed so that the dietary-derived phytoestrogens in the experiment are controlled. This is also true for testing the effect of *P. mirifica*.

There are no available data on the phytoestrogen content in rodent diets supplied by Thai companies. The purpose of the present study is to determine the concentrations of five major isoflavone phytoestrogens (puerarin, daidzin, daidzein, genistin, and genistein) in five lots of standard and one lot of soybean-free rodent diets available in Thailand, and also in *P. mirifica* 'Wichai-III.' The effect of the isoflavone content of rodent diet on vaginal proliferation was also assessed in rats treated with 50 mg/kg BW/day of *P. mirifica*.

MATERIALS AND METHODS

Chemicals, rodent diets, and *Pueraria mirifica*

Puerarin, daidzin, genistin, daidzein, and genistein standards with purity of 80, 95, 95, 98, and 98%, respectively, were purchased from Sigma and Fluka. Acetonitrile and acetic acid (HPLC grade) were purchased from Wako Pure Chemical Industries. Absolute ethanol was purchased from Katayama Chemicals.

The rodent diets were supplied by S.W.T. Co., Ltd., Samutprakarn, Thailand. Five lots of standard rodent diet (C.P. 082; Lot Nos. 2, 10, 18, 21, and 24) and one lot of soybean-free rodent diet (C.P. 082/SBF; Lot No. 050119) were analysed for their isoflavone contents. C.P. 082 contains 26% soybean meal and 8% full-fat soybean in terms of total raw weight. The other basic ingredients of C.P. 082 and C.P. 082/SBF diets were yellow corn, rice, rice by-products, fish meal, corn gluten meal, vegetable oil, vitamins, and minerals.

The tuberous roots of *P. mirifica* 'Wichai-III' were collected from Chiang Dao District, Chiang Mai Province. The voucher specimen of *P. mirifica* (No. BCU 11045) was deposited at the herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University. Two lots of *P. mirifica* (Lot Nos. 990609 and 990611 collected on 9 and 11 June 1999, respectively) were used to prepare 100-Mesh *P. mirifica* powder as described previously[3,9]. The powder was kept in a desiccator until analysis.

Isoflavone analysis

In the extraction step, 1 g of ground rodent diet or 100-Mesh *P. mirifica* powder was mixed with 4 ml of 70% ethanol (which has been shown to be the most suitable extraction solvent owing to its superior efficiency and low cost and toxicity[20]). The mixture was incubated in an incliner oven at 20 °C for 14 h and then centrifuged at 2800 rpm for 15 min. The supernatant was collected and stored at −20 °C until HPLC analysis. The precipitate was again extracted with 4 ml of 70% ethanol in the same way as mentioned above, two more times, decreasing the incubation time to 6 h. Supernatants collected from the three extractions (200 µl each) were pooled together and then dried using a centrifugal concentrator (Tomy) for 4 h at room temperature. The dry samples were re-dissolved with 200 µl of 0.4% acetic acid in ultrapure distilled water and analysed for isoflavone content. The amount of isoflavones analysed was adjusted to 100%. The remaining supernatant from each extraction was used to evaluate the extraction efficiency, calibrating to the three extractions pooled samples.

A 5 µl injection volume of extracted solution was analysed for five isoflavones using a high performance liquid chromatograph (HPLC, LC-9A, Shimadzu). Chromatography was performed in a 4.6 mm × 150 mm column (ODS-80 TM, TOSOH) at an ambient temperature of approximately 17 °C. The mobile phase consisted of solution A (100:0.4 v/v of ultrapure distilled water:acetic acid) and solution B (100:0.4 v/v of acetonitrile:acetic acid). A linear gradient was maintained for 60 minutes from 20% to 100% of solution B in solution A with a flow rate of 1 ml/min. The isoflavone content in the samples was analysed by comparing the retention times and quantifying the amounts using the peak area of the standard curves of the isoflavone standards. An elution of each isoflavone standard of puerarin, daidzin, genistin, daidzein, and genistein was monitored by a UV spectrophotometer (Shimadzu) at 260 nm. Calibration curves were obtained for all isoflavones by plotting the standard concentrations as a function of peak area from HPLC analysis of a 5-µl injection volume using a Chromatopac machine (Shimadzu). The serial concentrations of standards at 0, 0.05, 0.1, 0.2, and 0.4 µg were chosen to cover the range of isoflavone concentrations in the samples. The analyses of the samples were run in duplicate for both the extraction and HPLC analysis, and the data were averaged.

Investigation of vaginal cornification

Female 8-week-old Wistar rats were obtained from the National Laboratory Animal Centre, Mahidol University. They were housed 5 animals per cage in a room with controlled temperature (25 ± 1 °C) and lighting (lights on 0600–1800 h). The animals were fed with the rodent diets 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.

Rats were divided into 2 groups (10 rats/group), and fed with standard rodent diet (C.P. 082; Lot No. 18) or soybean-free rodent diet (C.P. 082/SBF; Lot No. 050119). They were ovariectomized under ether anaesthesia on the first day of the experiment. The experimental schedule was separated into three 14-day periods: pre-treatment, treatment, and post-treatment. Rats were gavaged with 50 mg/kg BW/day of *P. mirifica* 'Wichai-III' between 1000–1100 h during the treatment period. Vaginal smears were checked daily between 0900–1000 h in all rats. The vaginal epithelial cells observed under the microscope were classified into 3 types: leukocyte cells (L), nucleated cells (O), and cornified cells (Co). A total of 100 epithelial cells were randomly counted, and the percentage of cornified cells (%Co) was calculated[5,22]. Differences of %Co between groups were examined using an unpaired t-test (5% significance level). The Statistical Packages for Social Science (version SPSS/PC 11.0) was used.

RESULTS AND DISCUSSION

Isoflavone extraction

Five isoflavone standards, puerarin, daidzin, genistin, daidzein, and genistein, analysed by HPLC were eluted at 21.72, 24.77, 29.02, 34.58, and 39.06 min, respectively (Fig. 1D). Calibration curves of standard isoflavones were obtained with high linearity, $R^2 =$ 0.9881–0.9997. The sensitivity of the established HPLC analysis for isoflavones in rodent diets and *P. mirifica* samples is approximately 0.005 µg. Comparing the three extractions for the five isoflavones (Fig. 2), the efficiency was high for the first extraction (72.0–74.4%). Most of the remaining isoflavones could be recovered by a second extraction (19.6–22.0%), and only some of isoflavones remained for a third extraction (5.8–6.8%).

Isoflavone content of rodent diets

The total amount of the four isoflavones found in the Thai rodent diet (Fig. 1, Table 1), although highly

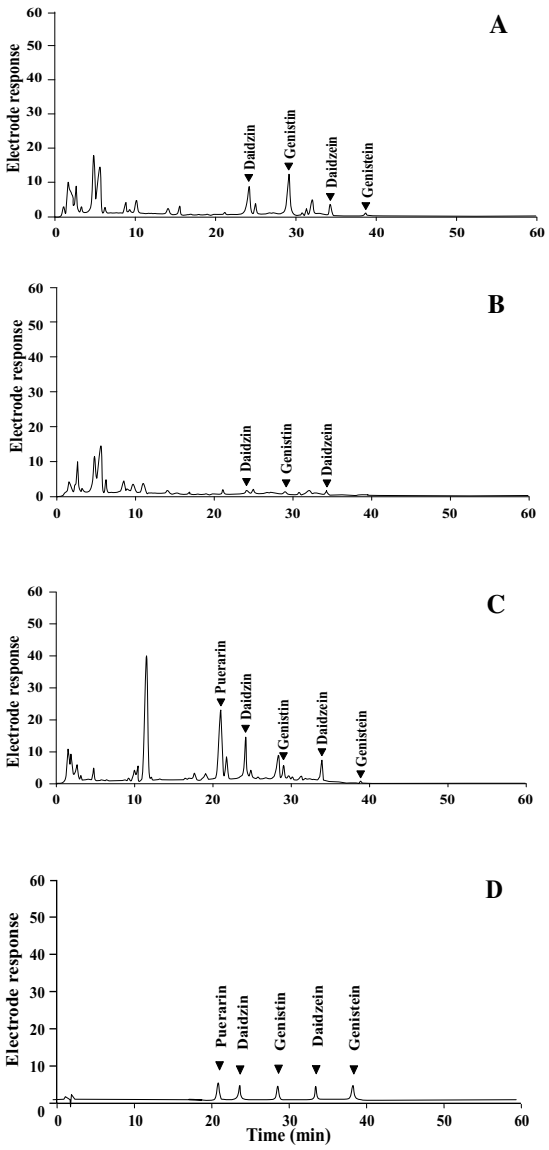

Fig. 1 HPLC fingerprints showing isoflavone content of (A) standard rodent diet (C.P. 082 lot no. 18), (B) soybean-free rodent diet (C.P. 082/SBF), (C) *Pueraria mirifica* (Lot No. 990611), (D) 0.1 µg of each isoflavone standard.

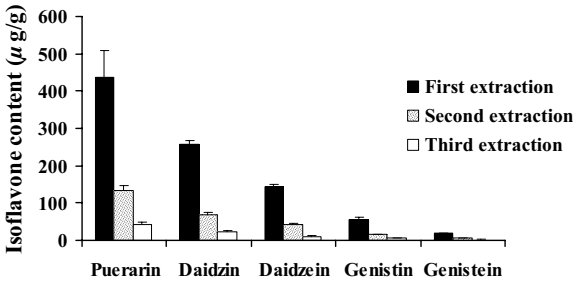

Fig. 2 Isoflavone content in *P. mirifica* Lot No. 990611.

variable, was comparable to those reported in the US and German rodent diets (10–54 mg/100 g of diet)[14]. The major isoflavones found in soybean-based rodent diets were the glycoside isoflavones (daidzin and genistin) which accounted for 82–86% of the total isoflavones. This was also reported for the US rodent diet in which 70–72% of the isoflavones were in the glycoside form[19].

Fully mature adult (100-day-old) female rats are 230–270 g in body weight[8], and are generally fed 15 g of rodent diet per day[23]. Thus they receive 21.5–40.2 mg/kg BW/day of isoflavones (6.8–14.6 and 11.5–21.4 mg/kg BW/day of daidzin and genistin, respectively). These amounts are comparable with the effective oral dose in preventing bone loss of 50 mg/kg BW/day of daidzin or genistin[19]. Likewise, the amounts of daidzein and daidzin (9.3–20.2 mg/kg BW/day), were typically higher than the effective oral dose of daidzein used for preventing bone loss, 10 mg/kg BW/day, in OVX rats[24]. In addition, the amount of genistein (0.2–0.8 mg/kg BW/day) from the standard rodent diets was considerably higher than the dose of genistein, 0.1 mg/kg BW/day, that causes a significant increase of the bone calcium content in elderly female rats[25]. Thus the standard rodent diet available in Thailand contains more than enough isoflavones to prevent the bone loss caused by endogenous estrogen deficiency. We should also emphasize the fact that OVX rats, the preferred animal in bone loss studies, are hyperphagic[26] and take in larger amounts of isoflavones by means of diet in general.

Isoflavone content of *P. mirifica*

Five isoflavones were found in large amounts in *P. mirifica*, with puerarin accounting for about half of the total (Table 1). The total isoflavone content found was comparable to that reported by a previous study (187.1 mg/100 g of *P. mirifica*)[8] and in the range of other cultivars of *P. mirifica* collected in Thailand (18.61–198.29 mg/100 g of *P. mirifica*)[12].

Cherdshewasart et al[12] reported that *P. mirifica* collected from different locations (and considered to be different cultivars) during March and April showed a high variation in the amounts of the five isoflavones, and this was thought to be due to differences in climate and genes. Our study showed that the two lots of *P. mirifica* 'Wichai-III', although of the same cultivar and grown in the same location, still showed a significant difference in the isoflavone content. However, this intra-cultivar variation is lower than the variation between different cultivars (or inter-cultivar variation). Nevertheless, it demonstrates that products made from the same cultivar of *P. mirifica* should still be calibrated for their isoflavone content.

Comparison of vaginal cornification

Most of the vaginal epithelial cells during the pre-treatment period after ovariectomy are leukocyte cells, and only 10–30% of the cells were cornified (Fig. 3). The percentage of cornified cells rose above 50% on day 7 in standard diet fed rats and on day 8 in soybean-free diet fed rats when they were treated with 50 mg/kg BW/day of *P. mirifica*. Although the patterns of %Co were the same between two groups of rats, the %Co in standard rodent diet fed rats was

Table 1 Isoflavone contents (mg/100 g sample) determined by HPLC.

Samples	Isoflavones (mg/100 g sample)					
	Puerarin	Daidzin	Daidzein	Genistin	Genistein	Total
Rodent diet (C.P. 082)						
Lot no. 2	nd	20.7 ± 0.6	10.2 ± 3.4	38.6 ± 2.6	1.4 ± 0.7	70.9 ± 3.0
Lot no. 10	nd	12.2 ± 0.2	4.5 ± 0.6	20.7 ± 0.9	1.2 ± 0.3	38.6 ± 2.9
Lot no. 18	nd	26.2 ± 0.9	9.1 ± 1.0	36.2 ± 1.1	0.9 ± 0.6	72.4 ± 3.7
Lot no. 21	nd	13.7 ± 0.8	5.4 ± 0.3	22.7 ± 0.8	0.8 ± 0.5	42.5 ± 0.8
Lot no. 24	nd	18.8 ± 1.8	10.0 ± 0.2	28.8 ± 3.5	0.4 ± 0.1	58.0 ± 5.6
Rodent diet (C.P. 082/SBF)						
Lot no. 050119	nd	0.9 ± 0.1	3.0 ± 0.1	2.2 ± 0.1	nd	6.1 ± 0.2
Pueraria mirifica						
Lot no. 990609	86.5 ± 5.4	39.9 ± 2.4	22.8 ± 0.5	7.9 ± 0.4	0.3 ± 0.1	157.3 ± 8.7
Lot no. 990611	61.0 ± 4.8	34.9 ± 0.6	19.4 ± 0.8	7.6 ± 0.3	0.3 ± 0.1	123.2 ± 6.6

nd = not detected

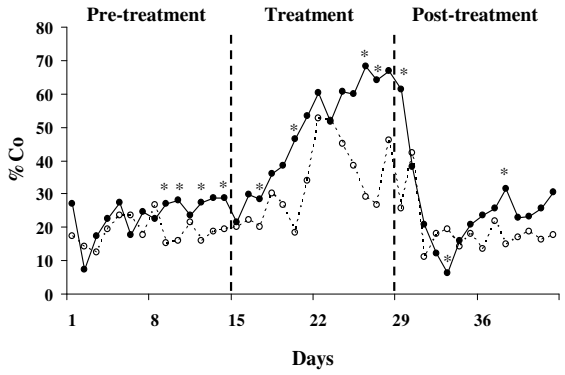

Fig. 3 Percentage vaginal cornification (%Co) in rats fed with a standard rodent diet (●) and rats fed with a soybean-free diet (○), when both groups were gavaged the same dose of 50 mg/kg BW of *Pueraria mirifica* for 14 days during treatment period. * = $p < 0.05$.

significantly higher than that in soybean-free diet fed rats, starting from day 9 of the pre-treatment period until day 10 of the post-treatment period.

P. mirifica at the doses of 100 and 1000 mg/kg BW/day were reported to have a significant estrogenic effect, inducing a cornification of the vaginal epithelium and an increase of uterine weight[5,8,9] in OVX rats fed with a soybean-based rodent diet, and the effects were not observed in rats fed with 10 mg/kg BW/day of *P. mirifica*. Our study is the first to report that *P. mirifica* at the dose lower than 100 mg/kg BW/day, (that is, 50 mg/kg BW/day), also induced vaginal cornification in OVX rats. The greater degree of vaginal cornification in soybean-based rodent diet fed rats compared to those of soybean-free diet fed rats concurs with the increase of uterine weight observed by Dagen et al[14]. Rats fed with the soybean diet exhibited a higher uterine weight than rats fed with a phytoestrogen-free diet when both groups also received the same dose of genistein treatment[14]. This implies that the estrogenic effect of phytoestrogens in *P. mirifica* on vaginal epithelial cells is enhanced by dietary phytoestrogens. The 50 mg/kg BW/day of *P. mirifica* contains 0.062–0.077 mg of the five isoflavones. The isoflavone intake (21.5–40.2 mg/kg BW/day) from the soybean-based rodent diet found in the present study is therefore much higher than that from *P. mirifica* treatment. However, the OVX rats fed only a soybean-based rodent diet during the pre-treatment period did not show the vaginal cornification. Thus, it is possible either that the estrogenic effects that occurred during treatment period are due to the isoflavones in *P. mirifica* combined with the isoflavones in the rat diet, or due to the puerarin isoflavone found in *P. mirifica* which is undetectable in the rat diets. It was reported that the *P. mirifica* tuberous root contains at least 13 known substances classified as phytoestrogens[12,27,28]. It is also probable that other phytoestrogens that we have not determined here such as miroestrol[27] could play a role.

CONCLUSIONS

In conclusion, the use of rats as experimental animals for evaluating the effects of estrogen or estrogen-like substances should specify whether the animals are fed with soybean-based diets, because the high isoflavone contents in those diets could influence the results. For example, if the effects of genistein were examined, the soybean-free diet in which no genistein could be detected in our study should be used. The findings from the present study give a caution to researchers to be aware of the confounding effects of phytoestrogen contents in soybean-based rodent diets.

Acknowledgements: The authors thank Dr. Wichai Cherdshewasart for providing the *P. mirifica*. This work was financially supported by grants from the Thailand Research Fund (PHD/0006/2547), the Primate Research Unit, Chulalongkorn University, and the Japanese Society for the Promotion of Science (Fund No. 14204083).

REFERENCES

1. Cornwell T, Cohick W, Raskin I (2004) Dietary phytoestrogens and health. *Phytochemistry* **65**, 995–1016.
2. Boue SM, Wiese TE, Nehls S, Burow ME, Elliott S, Carter-Woenties CH, Shih BY, McLachlan JA, Cleveland TE (2003) Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *J Agr Food Chem* **51**, 2193–9.
3. Cherdshewasart W, Cheewasopit W, Picha P (2004) 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* **93**, 255–60.
4. Cherdshewasart W, Cheewasopit W, Picha P (2004) Anti-proliferation effects of the white (*Pueraria mirifica*), red (*Butea superba*) and black (*Mucuna collettii*) Kwao Krua plant on the growth of HeLa cells. *J Sci Res Chula Univ* **29**, 27–31.
5. Cherdshewasart W, Kitsamai Y, Malaivijitnond S (2007) Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. *J Reprod Dev* **53**, 385–93.
6. Jaroenporn S, Malaivijitnond S, Wattanasirmkit K, Trisomboon H, Watanabe G, Taya K, Cherdshewasart W (2006) Effects of *Pueraria mirifica*, an herb containing

phytoestrogens, on reproductive organs and fertility of adult male mice. *Endocrine* **30**, 93–101.
7. Jaroenporn S, Malaivijitnond S, Wattanasirmkit K, Watanabe G, Taya K, Cherdshewasart W (2007) Assessment of fertility and reproductive toxicity in adult female mice after long-term exposure to *Pueraria mirifica* herb. *J Reprod Dev* **53**, 995–1005.
8. Malaivijitnond S, Kiatthaipipat P, Cherdshewasart W, Watanabe G, Taya K (2004) Different effects of *Pueraria mirifica*, a herb containing phytoestrogens, on LH and FSH secretion in gonadectomized female and male rats. *J Pharmacol Sci* **96**, 428–35.
9. Malaivijitnond S, Chansri K, Kijkuokool P, Urasopon N, Cherdshewasart W (2006) Using vaginal cytology to assess the estrogenic activity of phytoestrogen-rich herb. *J Ethnopharmacol* **107**, 354–60.
10. Urasopon N, Hamada Y, Asaoka K, Cherdshewasart W, Malaivijitnond S (2007) *Pueraria mirifica*, a phytoestrogen-rich herb, prevents bone loss in orchidectomized rats. *Maturitas* **56**, 322–31.
11. Urasopon N, Hamada Y, Cherdshewasart W, Malaivijitnond S (2008) Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats. *Maturitas* **59**, 137–48.
12. Cherdshewasart W, Subtang S, Dahlan W (2007) Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison with *Pueraria lobata*. *J Pharmaceut Biomed Anal* **43**, 428–34.
13. Trisomboon H, Malaivijitnond S, Suzuki J, Hamada Y, Watanabe G, Taya K (2004) Long-term treatment effects of *Pueraria mirifica* phytoestrogens on parathyroid hormone and calcium levels in aged menopausal cynomolgus monkeys. *J Reprod Dev* **50**, 639–45.
14. Degen GH, Janning P, Diel P, Bolt HM (2002) Estrogenic isoflavones in rodent diets. *Toxicol Lett* **128**, 145–57.
15. Murphy PA, Farmakalidis E, Johnson LD (1982) Isoflavone content of soya-based laboratory animal diets. *Food Chem Toxicol* **20**, 315–7.
16. Boettger-Tong H, Murthy L, Chiappetta C, Kirkland JL, Goodwin B, Adlercreutz H, Stancel GM, Makela S (1998) A case of a laboratory animal fed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. *Environ Health Perspect* **106**, 369–72.
17. Casanova M, You L, Gaido KW, Archibeque-Engle S, Janszen DB, Heck HA (1999) Developmental effects of dietary phytoestrogens in Sprague-Dawley rats and interactions of genistein and daidzein in rat estrogen receptors alpha and beta in vitro. *Toxicol Sci* **51**, 236–44.
18. Thigpen JE, Setchell KD, Ahlmark KB, Locklear J, Spahr T, Caviness GF, Goelz MF, Haseman JK, Newbold RR, Forsythe DB (1999) Phytoestrogen content of purified, open and closed formula laboratory animal diets. *Lab Anim Sci* **49**, 530–6.
19. Brown NM, Setchell KD (2001) Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. *Lab Investig* **81**, 735–47.
20. Rostagno MA, Palma M, Barroso CG (2004) Pressurized liquid extraction of isoflavones from soybeans. *Anal Chim Acta* **522**, 169–77.
21. Thigpen JE, Setchell KD, Goelz MF, Forsythe DB (1999) The phytoestrogen content of rodent diets. *Environ Health Perspect* **107**, A182–3.
22. Terenius L (1971) The Allen Doisy test for estrogens reinvestigated. *Steroids* **17**, 613–8.
23. Kijkuokool P, Parhar IS, Malaivijitnond S (2006) Genistein enhances N-nitrosomethylurea-induced rat mammary tumorigenesis. *Canc Lett* **242**, 53–9.
24. Picherit C, Coxam V, Bennetau-Pelissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, Barlet JP (2000) Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. *J Nutr* **130**, 1675–81.
25. Gao HY, Yamaguchi M (1998) Zinc enhancement of genistein's anabolic effect on bone components in elderly female rats. *Gen Pharmacol* **31**, 199–202.
26. Breitman PL, Fonseca D, Cheung AM, Ward WE (2003) Isoflavones with supplemental calcium provide greater protection against the loss of bone mass and strength after ovariectomy compared to isoflavones alone. *Bone* **33**, 597–605.
27. Pope GS, Grundy HM, Jone HEM, Tait SAS (1958) The estrogenic substance (miroestrol) from the tuberous roots of *P. mirifica*. *J Endocrinol* **17**, 15–7.
28. Chansakaow S, Ishikawa T, Sekine K, Okada M, Higuchi Y, Kudo M, Chaichantipyuth C (2000) Isoflavonoids from *Pueraria mirifica* and their estrogenic activity. *Planta Med* **66**, 572–5.