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Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison with *Pueraria lobata*

Wichai Cherdshewasart [a,*], Subongkoj Subtang [b,c], Winai Dahlan [d]

[a] *Department of Biology, Faculty of Science, Chulalongkorn University, Phyathai Road, Patumwan, Bangkok 10330, Thailand*
[b] *Biotechnology Program, Faculty of Science, Chulalongkorn University, Phyathai Road, Patumwan, Bangkok 10330, Thailand*
[c] *Bureau of Community Technology, Department of Science Service, Ministry of Science and Technology, Rama-VI Road, Phyathai, Bangkok 10400, Thailand*
[d] *Faculty of Allied Health Science, Chulalongkorn University, Phyathai Road, Patumwan, Bangkok 10330, Thailand*

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Abstract

Pueraria mirifica tubers collected from 28 out of 76 provinces of Thailand and *Pueraria lobata* tubers collected from Guangzhou province, China were submitted to HPLC analysis with the established gradient system comprising 1.5% acetic acid and acetonitrile. Five major isoflavonoids, including puerarin, daidzin, genistin, daidzein and genistein, were adopted as authentic standards. *P. mirifica* tubers showed intra- as well as inter-provincial differences in isoflavonoid and total isoflavonoid contents. The difference in both cases should be mostly influenced by genetic and environmental factors. In comparison with *P. lobata*, *P. mirifica* population exhibited differences only with a lower amount of daidzein.

Keywords: *Pueraria mirifica*; *Pueraria lobata*; Isoflavonoid; HPLC analysis; Chemovariety

1. Introduction

Isoflavonoids, especially from soybeans, are attracting great interest in their anti-cancer properties as confirmed from *in vivo* [1–3] and cell culture studies [4–7] as well as other health benefits [8–10]. Attempts have also been made to evaluate isoflavonoid contents, especially daidzein and genistein, in other plants. Among the top ranked candidates, *Pueraria lobata* tuber was identified as a source of high levels of isoflavonoids, including puerarin, daidzin, genistin, daidzein and genistein [11,12] with therapeutic effects for the human body [13–15].

Pueraria mirifica Airy Shaw et. Suvatabhandu (Leguminosae), a Thai indigenous herb with the local name of White Kwao Krua, has long been consumed among Thai women for purposes similar to modern hormone replacement therapy. Puerarin, daidzin, genistin, daidzein and genistein were isolated from the plant tubers [16–20]. The estrogenic activity test *in vitro* of the tuberous extract required metabolic activation through specific cellular drug metabolizing enzymes [21]. *P. mirifica* cultivar Wichai-III tuberous extract, but not that from *P. lobata*, showed a biphasic response, with proliferation of MCF-7 at low concentration and antiproliferation at high concentration cells [22]. At high concentration, *P. mirifica* tuberous extract, and not that of *P. lobata*, also induced antiproliferation of HeLa cells [23]. Phytoestrogens from the same plant cultivar exhibited dose-dependent estrogenic effects on reproductive system, decreased FSH and LH serum levels, initiated vaginal cornification and increased uterine weight in ovariectomized rats [24], increased the length of the follicular phase and total menstruation cycle [25], and decreased FSH, LH, estradiol and progesterone and subsequently caused ovulation blockage in female monkeys [26]. The plant chemicals also decreased FSH and LH [27] and PTH [28] in aged menopausal monkeys. The findings confirmed that *P. mirifica* could be used as an alternative for estrogen. In a study with *Pueraria radix* (synonym: *P. lobata*) collected from many parts of Korea and submitted to estrogenic activity analysis in comparison with one sample of the Thai *P. mirifica* by means of uterotrophic assay, all collected tubers of *P. radix* expressed no uterotrophic effect but the Thai plant did. There was a conclusion that isoflavonoids in *P. radix* were less potent than the Thai *P. mirifica* [29]. In the other study, the crude extract and

* Corresponding author. Tel.: +66 2 2185033; fax: +66 2 2185034.
E-mail address: cwichai@sc.chula.ac.th (W. Cherdshewasart).

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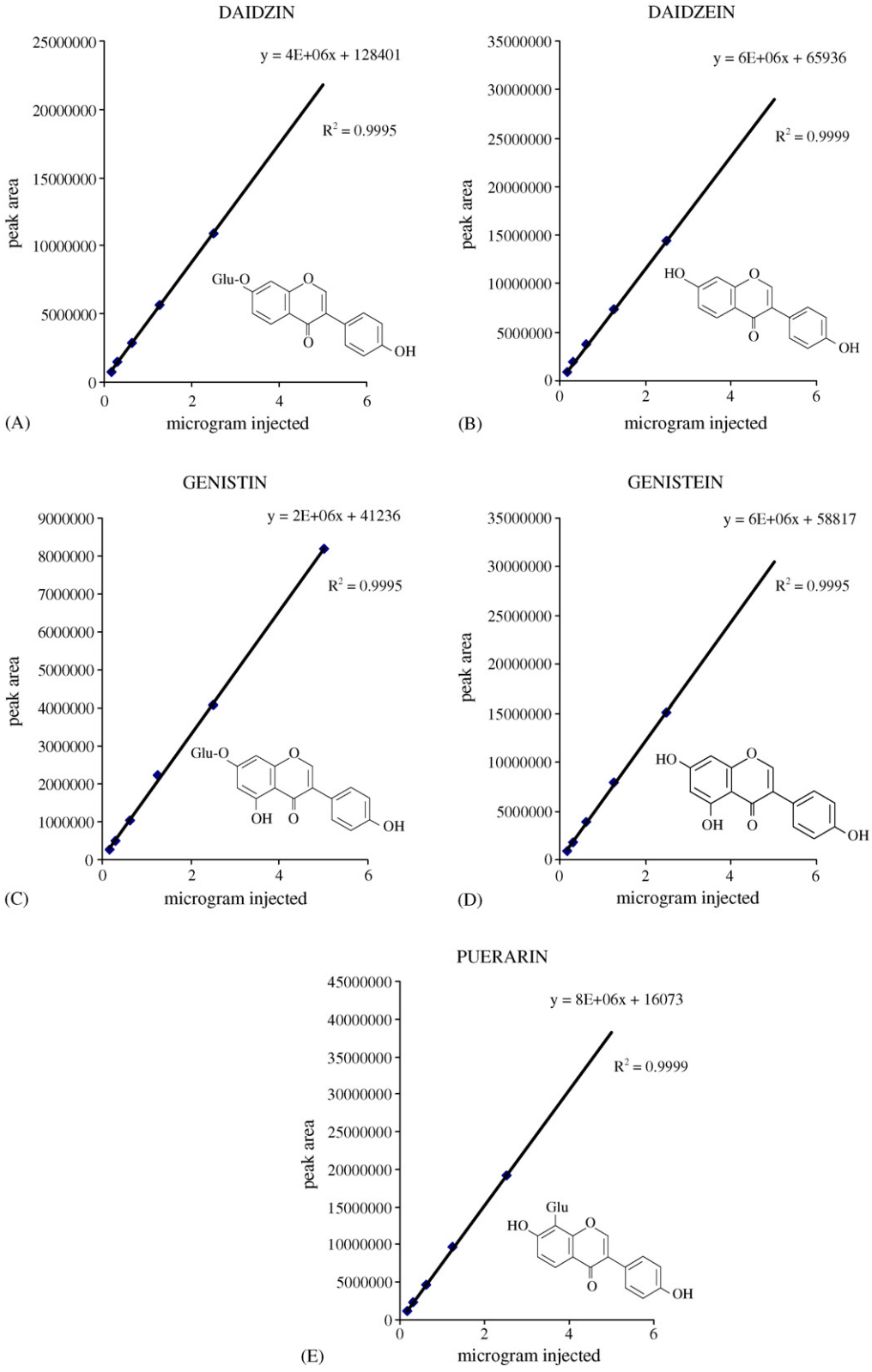

Fig. 1. Calibration curves and chemical structures of daidzin (A), daidzein (B), genistin (C), genistein (D) and puerarin (E).

sub-fraction of the Chinese *P. lobata* showed estrogenic activity in a recombinant yeast screening assay [30].

The domestic and global demand for raw materials derived from *P. mirifica* tubers has increased sharply since 1999 and resulted in heavy harvest of plant tubers from the forests in all studied provinces and districts of Thailand. We therefore initiated a study to evaluate the differences of isoflavonoid contents in mature tubers of wild collected *P. mirifica* from 28 out of 76

Table 1
Isoflavonid and total isoflavonoid contents in mg/100 g of *Pueraria mirifica* tuberous powder collected from 28 provinces of Thailand in comparison with *Pueraria lobata* from China (mean ± S.E.M.)

No.	Province	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
1	Kanchanaburi	45.25 ± 1.11*	50.24 ± 3.23*	85.69 ± 1.23*	13.92 ± 1.26*	3.19 ± 0.29*	198.29 ± 4.61*
2	Lamphun	33.18 ± 0.92	28.35 ± 0.68*	84.13 ± 0.54*	8.59 ± 0.09	0.76 ± 0.36	155.00 ± 1.42*
3	Chiang Mai	35.55 ± 3.57	27.39 ± 5.32*	58.00 ± 0.71*	8.38 ± 0.22	1.93 ± 0.54	131.25 ± 9.01*
4	Sakon Nakhon	87.05 ± 0.79*	11.48 ± 0.21	14.83 ± 0.22†	4.78 ± 0.37	1.42 ± 0.14	119.57 ± 1.39*
5	Mae Hong Son	36.99 ± 2.07	17.63 ± 1.74	55.44 ± 3.43*	7.52 ± 1.27	1.54 ± 0.08	119.12 ± 6.54*
6	Uthai Thani	10.85 ± 1.01	21.70 ± 0.84	50.17 ± 3.57	16.48 ± 1.35*	3.66 ± 0.16*	102.86 ± 6.53
7	Sukhothai	14.12 ± 0.94	25.09 ± 1.50*	51.43 ± 2.40	11.16 ± 0.85*	0.73 ± 0.23	102.52 ± 5.35
8	Lampang	34.65 ± 1.34	16.59 ± 0.08	33.30 ± 0.08	5.72 ± 0.09	1.54 ± 0.12	91.80 ± 1.72
9	Tak	29.06 ± 2.07	8.97 ± 0.99	43.86 ± 1.91	4.56 ± 0 .32	1.15 ± 0.19	87.60 ± 4.87
10	Saraburi	23.42 ± 1.21	17.92 ± 0.59	37.94 ± 3.42	4.86 ± 0.95	0.87 ± 0.46	85.01 ± 4.04
11	Ratchaburi	8.85 ± 0.36	15.39 ± 0.79	51.15 ± 1.75	6.84 ± 0.53	2.54 ± 0.15*	84.77 ± 2.67
12	Phitsanulok	35.24 ± 1.06	12.26 ± 0.13	26.53 ± 0.57	8.36 ± 0.23	1.63 ± 0.05	84.02 ± 1.91
13	Phetchaburi	13.19 ± 0.45	20.82 ± 1.78	37.56 ± 1.33	6.0 0 ± 0.24	1.13 ± 0.04	78.71 ± 3.15
14	Phrae	25.20 ± 1.54	10.55 ± 1.18	30.61 ± 0.81	5.45 ± 0.56	1.34 ± 0.14	73.16 ± 4.21
15	Lop Buri	19.50 ± 1.44	6.84 ± 0.09	39.47 ± 1.65	2.42 ± 0.79†	0.98 ± 0.09	69.21 ± 3.77
16	Chaiyaphum	15.83 ± 2.43	12.91 ± 1.44	29.48 ± 2.33	7.02 ± 0.89	1.89 ± 0.42	67.13 ± 5.47
17	Uttraradith	30.25 ± 0.44	13.69 ± 0.21	10.27 ± 0.19†	7.88 ± 0.18	0.00†	62.96 ± 1.03
18	Nakhon Sawan	13.34 ± 1.46	16.28 ± 1.64	27.71 ± 0.75	4.70 ± 0.37	0.72 ± 0.32	62.75 ± 3.24
19	Chiang Rai	20.02 ± 1.42	8.61 ± 1.12	29.58 ± 2.43	2.16 ± 0.08†	0.50 ± 0.28	60.87 ± 3.30
20	Nong Bua Lam Phu	12.65 ± 2.42	11.91 ± 3.02	23.65 ± 2.14	7.46 ± 0.96	1.91 ± 0.25	57.58 ± 3.61
21	Phayao	12.91 ± 0.99	8.46 ± 0.62	32.43 ± 1.35	3.03 ± 0.36	0.73 ± 0.30	57.56 ± 3.02
22	Prachuap Khiri Khan	10.42 ± 1.03	9.62 ± 0.44	30.31 ± 1.17	2.11 ± 0.36†	0.59 ± 0.07	53.05 ± 2.57
23	Chumphon	8.45 ± 0.22	7.38 ± 1.11	34.17 ± 4.81	2.64 ± 0.26	0.07 ± 0.06†	52.70 ± 5.46
24	Prachin Buri	12.42 ± 0.26	13.05 ± 0.65	16.69 ± 0.78†	4.28 ± 0.56	0.51 ± 0.09	46.94 ± 1.12
25	Phetchabun	9.40 ± 0.46	10.48 ± 0.67	15.54 ± 1.61†	8.11 ± 0.05	1.29 ± 0.02	44.83 ± 1.73†
26	Nakhon Ratchasima	13.09 ± 0.77	5.61 ± 0.07†	24.15 ± 1.42	1.20 ± 0.37†	0.21 ± 0.19†	44.27 ± 1.27†
27	Kamphaeng Phet	15.44 ± 1.14	7.01 ± 1.10	18.50 ± 4.45	2.31 ± 0.11†	0.46 ± 0.08	43.71 ± 4.02†
28	Nan	5.32 ± 0.44†	2.36 0 ± .22†	7.62 ± 1.36†	3.31 ± 0.31	0.00 ± 0.00†	18.61 ± 1.11†
Mean ± S.E.M.		23.01 ± 1.80	14.94 ± 1.07	35.39 ± 2.09	6.12 ± 0.40	1.19 ± 0.10	80.67 ± 4.11
Pueraria lobata		32.85 ± 0.72	21.9 ± 0.74	25.63 ± 0.86	10.34 ± 0.79*	0.81 ± 0.08	91.57 ± 3.18

* Greater than mean at $P < 0.05$.
† Less than mean at $P < 0.05$.

provinces and also 11 districts from 3 provinces of Thailand. The plant tuberous samples were collected during the summer of the same year to minimize the influence of climatic differences and were analyzed in comparison with *P. lobata* of Chinese origin. The results of the study would help evaluate the differences in isoflavonoid contents among plant tuberous samples collected from different sites in Thailand. The results may also indicate a possibility that the huge plant populations contain chemovariety.

2. Experimental

2.1. Plant materials

2.1.1. Inter-provincial study

P. mirifica tubers of at least 3 years old, counted from the tuberous annual rings, were randomly collected, taking 3 plants from each site, approximately in 1 km^2, in 28 from a total of 76 provinces of Thailand during March–April (local summer) in 2000 (Table 1). The collected sites were located in the latitude of 10.68° in the southern part to the latitude of 20.42° in the northern part of Thailand and in the longitude of 98.64° in the western part to the longitude of 103.66° in the north-eastern part of Thailand. Voucher specimens were identified by the author with the reference [31] in comparison with the voucher specimen no. BCU 11045 [22] and deposited at the Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The tuberous roots of *P. lobata* were collected from Guangzhou Province, China during April 2000. The voucher specimen was identified by Zhang Yam and deposited at the Agro-Biotechnical Research Institute, Academy of Agricultural Sciences, Guangdong, China. The tuberous powders were prepared as previously described [22].

2.1.2. Intra-provincial study

P. mirifica tubers were randomly collected as previously described in five districts in Chiang Mai province and three districts in Lampang province in the northern part of Thailand, and three districts in Kanchanaburi province in the western part of Thailand, during March–April of summer 2000. The three provinces were selected for this study because of the large forest area and abundant plant populations of *P. mirifica*.

2.2. Chemicals and equipment

Isoflavonoid standards, including puerarin, daidzin, genistin, daidzein and genistein (see Fig. 1), were purchased from Sigma,

USA. The organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. The water of over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA). HPLC system control and data processing were carried out by a Shimadzu instrument (Model CLASS-LC10 analytical workstation, Model SPD-10A ultraviolet–visible detector). The reversed phase C18 column (220 nm × 4.6 nm) was filled with 5 μm Spheri (Brownlee, USA). The filter set was Millipore membrane at 0.45 μm pore size with 13 mm diameter for the sample and 47 mm diameter for the mobile phase, of HA type for aqueous solution and HV type for organic solvent. The chromatography manager software was operated on a personal computer.

2.3. HPLC sample preparation

Five grams of tuberous powder was extracted twice at room temperature with 50 and 25 ml methanol (Merck), respectively, with the aid of sonication for 15 min. The supernatant was collected after filtration with Whatman #1 filter paper. The methanol extracts were evaporated to dryness *in vacuum*. The residue was dissolved in 10 ml methanol and the crude extract then filtered with a 0.45 μm pore size, 13 mm diameter membrane.

2.4. Quantitative HPLC

Methods for isoflavonoid analysis were modified from those previously described [32] by setting the linear gradient system for 45 min from 100:0 to 55:45 with 1.5% acetic acid:acetonitrile, with a flow rate of 1 ml/min for 45 min and analyzed at the wavelength of 254 nm. The standard isoflavonoids were serially diluted from 1:1 to 1:16 with methanol to establish the concentrations of 1/4, 1/8, 1/16, 1/32 and 1/64 mg/ml, to generate a five point calibration curve. Calibration curves were obtained for all isoflavonoids by plotting the standard concentration as a function of peak area from HPLC analysis of a 15 μl injection volume. The concentrations of standard were chosen to cover the range of isoflavonoid concentrations in the samples. The analyses of the samples were run in triplicate and identified by comparing the retention times and quantified for the amount using standard curves of peak area of the isoflavonoid standards.

2.5. Statistical analysis

The mean ± S.E.M. of isoflavonoid contents from the samples of *P. mirifica* and *P. lobata* were analyzed for statistical significance by the un-paired *t*-test at the significance level of $P < 0.05$.

3. Results and discussion

3.1. Calibration curves of standard isoflavonoids

Calibration curves of standard isoflavonoids were obtained for all standard isoflavonoids with high linearity, $R^2 > 0.995$ (Fig. 1). The established HPLC analysis for isoflavonoids in plant samples in this study, with a limit quantitation of

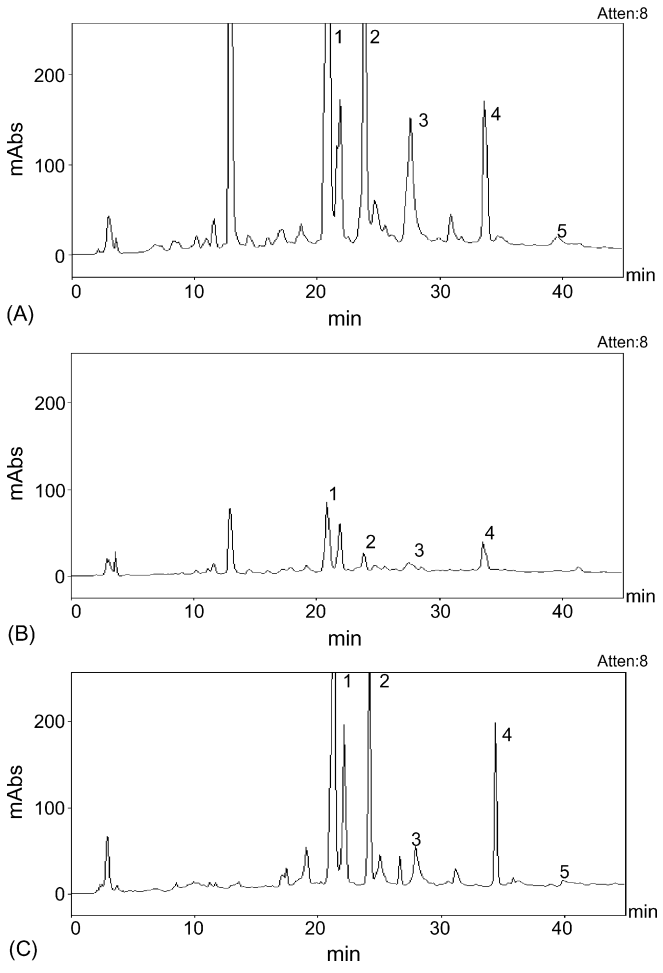

Fig. 2. HPLC isoflavonoid fingerprint of *Pueraria mirifica* tubers collected from Kanchanaburi province (A) showing maximum amount of total isoflavonoids, Nan province (B) showing minimum amount of total isoflavonoids and *Pueraria lobata* tubers collected from Ghanzhou province, China (C). (1) Puerarin, (2) daidzin, (3) genistin, (4) daidzein and (5) genistein.

0.5 mg/100 g, could demonstrate the difference of isoflavonoids among plant samples. It could be of practical use to screen for plants with high isoflavonoid content.

3.2. Inter-provincial study

P. mirifica tubers from 28 provinces showed inter-provincial diversity of isoflavonoids puerarin, daidzin, genistin, daidzein, genistein and total isoflavonoid as calculated from HPLC fingerprints (Table 1). HPLC fingerprints of *P. mirifica* tuber samples with the highest and lowest total isoflavonoid content were demonstrated (Fig. 2A and B) and compared with *P. lobata* analyzed under the same conditions (Fig. 2 C). There were common peaks including those representing puerarin, daidzin, genistin, daidzein and genistein in HPLC fingerprints of all analyzed samples. The results demonstrated that isoflavonoids were major components in the tubers and could represent differences among tubers collected from different provinces. The results (Table 1) showed a highly significant difference, with the maximum amount of 198.29 mg/100 g in tubers collected from Kanchanaburi province and the minimum amount of 18.61 mg/100 g

Table 2
Isoflavonoid and total isoflavonoid contents in mg/100 g of *Pueraria mirifica* tuberous powder collected from five districts of Chiang Mai province (mean ± S.E.M.)

District	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
Hod	35.55 ± 3.57*	27.39 ± 5.32*	58.00 ± 0.71	8.38 ± 0.22*	1.93 ± 0.54	131.25 ± 9.01*
Doi Tao	28.36 ± 3.33	28.70 ± 3.32	58.38 ± 7.96	4.05 ± 0.45	1.10 ± 0.41	120.60 ± 13.67
Doi Saket	17.17 ± 0.77	15.76 ± 0.71	44.12 ± 2.53	8.10 ± 0.53	1.66 ± 0.18	86.80 ± 1.84
Chiang Dao	10.96 ± 0.75†	12.37 ± 0.58	20.99 ± 3.12	4.33 ± 0.41	1.34 ± 0.11	49.98 ± 3.80
Chaiprakarn	16.13 ± 0.96	8.01 ± 0.36†	13.92 ± 2.16†	3.42 ± 0.49	0.79 ± 0.32	42.26 ± 2.94†
Mean ± S.E.M.	22.38 ± 2.82	19.30 ± 2.60	39.41 ± 5.29	5.73 ± 0.62	1.27 ± 0.16	88.11 ± 10.62

* Greater than mean at $P < 0.05$.
† Less than mean at $P < 0.05$.

from Nan province. The mean values of total isoflavonoid content of *P. mirifica* collected from each province were compared with the mean value of the *P. mirifica* population and resulted in finding of 5 and 4 samples exhibiting significantly higher and lower amounts, respectively, of total isoflavonoids as compared with the mean value of the *P. mirifica* population. Differences in isoflavonoid contents were observed within the plant population. There were 2, 4, 4, 3 and 3 samples that exhibited higher and 1, 2, 5, 5 and 4 samples exhibited lower levels of puerarin, daidzin, genistin, daidzein and genistein than the population mean, respectively. There was a great difference between the highest and lowest amount of isoflavonoids in the plant population as well. The maximum/minimum ratio of puerarin, daidzin, genistin, daidzein and genistein among the plant population were related to the total isoflavonoid contents. It was clear that not only individual isoflavonoids but also the total isoflavonoid contents of *P. mirifica* differed greatly among population.

The total isoflavonoid content of *P. lobata* was found to be 91.57 mg/100 g. There was no significant difference in average puerarin, daidzin, genistin, genistein and total isoflavonoid contents of the *P. mirifica* population compared with *P. lobata*. Only daidzein of *P. lobata* was found at higher concentration than the mean value of the *P. mirifica* population. It was also noted that the reported isoflavonoid contents in *P. lobata* also varied with the location of sample collection [29–30].

3.3. Intra-provincial study

There were differences in isoflavonoid and total isoflavonoid contents among collected samples from five districts of Chiang Mai province (Table 2), three districts of Lampang province (Table 3) and three districts of Kanchanaburi province (Table 4). It should be noted that isoflavonoid and total isoflavonoid contents of the tubers collected from Chiang Mai and its adjacent province, Lampang were not much different as compared with the same analyzed chemicals of the tubers collected from Kanchanaburi, which is located at a distance of approximately 700 km to the south of Chiang Mai province.

The results from plant samples collected for the intra-provincial study showed the same general trends as the inter-provincial study. They demonstrated clearly that there were great differences in isoflavonoid contents of the same plant species distributed across a vast area of Thailand. The collection season was limited to summer in this study when the soil was almost dried to minimize the influence of difference in the plant physiology. Most of the plants exhibited flowers and were defoliated. Since mature-sized tubers as regularly harvested by the villagers were collected from each area and a large-size plot of approximately 1 km^2 was applied for the collection of samples, they should be representative of the plant population in each collection site. It is likely that the significant difference in isoflavonoids found would be explained by differential expression of the genes in the isoflavonoid pathways and the influences of the ecological habitats of the plant samples *per se*. It is known that different climates and genotype can influence isoflavonoid contents in soybeans [33–35]. The influence of plant genes in the isoflavonoid pathways may outweigh environmental factors as it was noticed that in the intra-provincial study, many collecting sites were located at similar longitudes and latitudes, with the same type of habitat and soils, but the tubers nevertheless exhibited very different isoflavonoid contents.

Low values of S.E.M. were obtained in the chemical analysis data (Tables 1–4). This demonstrates clearly that the mature tubers of at least 3 years of age, collected from the same site, were almost homogeneous for isoflavonoid contents. This indicates that the mature plants within the same population were in the same physiological state with no influence from the age differences of the mature plants. Besides, the genes of the

Table 3
Isoflavonoid and total isoflavonoid contents in mg/100 g of *Pueraria mirifica* tuberous powder collected from three districts of Lampang province (mean ± S.E.M.)

District	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
Koh Ka	34.65 ± 1.34*	16.59 ± 0.07	33.30 ± 0.08	5.72 ± 0.10	1.54 ± 0.12	91.80 ± 1.72
Hangchat	11.77 ± 0.72†	14.66 ± 0.70	39.91 ± 2.04	19.32 ± 2.09*	2.22 ± 0.19	87.89 ± 4.36
Thern	20.85 ± 0.98	15.28 ± 1.14	29.03 ± 3.67	6.27 ± 0.68	2.54 ± 0.53	73.97 ± 5.01
Mean ± S.E.M.	22.42 ± 3.36	15.51 ± 0.48	34.08 ± 1.99	10.44 ± 2.31	2.10 ± 0.22	84.55 ± 3.35

* Greater than mean at $P < 0.05$.
† Less than mean at $P < 0.05$.

Table 4
Isoflavonoid and total isoflavonoid contents in mg/100 g of *Pueraria mirifica* tuberous powder collected from three districts of Kanchanaburi province (mean ± S.E.M.)

District	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
Srisawat	45.25 ± 1.11*	50.24 ± 3.23*	85.69 ± 1.23*	13.92 ± 1.25*	3.18 ± 0.29*	198.29 ± 4.61*
Sai Yoke	7.15 ± 0.51	16.61 ± 0.70	45.93 ± 0.76	6.73 ± 0.32	1.71 ± 0.10	78.14 ± 0.95
Thongpha-phum	8.33 ± 0.64	4.01 ± 0.50†	13.69 ± 2.85†	4.01 ± 0.23	0.62 ± 0.25	30.67 ± 4.27
Mean ± S.E.M.	20.25 ± 6.27	23.62 ± 6.97	48.44 ± 10.45	8.22 ± 1.53	1.84 ± 0.39	102.37 ± 25.00

* Greater than mean at $P < 0.05$.
† Less than mean at $P < 0.05$.

isoflavonoid pathways should not differ greatly in their expression. The results presented in this study demonstrate a great difference in isoflavonoid contents within the plant population collected from 28 provinces and should open the possibility of chemovariety within the plant population.

4. Conclusions

The difference in levels of individual and total isoflavonoid contents among tubers collected from different provinces in Thailand or within the same province but in different districts exhibited significant differences in the plants' active chemicals. Such differences may be influenced more by genotype than the plant age or environmental/habitat factors. The variability in isoflavonoid levels in mature tubers could seriously affect the quality of any tuber-derived materials or even research results derived from the plants. The established information of the isoflavonoid contents could therefore provide a helpful guideline for plant breeders acting as benchmark for selection for high isoflavonoid contents followed by clonal propagation for commercial plantations. It is possible to select for clones with higher isoflavonoid contents than *P. lobata*, resulting in development of commercial products with greater estrogenic activities. Furthermore, there is strong interest in setting up a comparative estrogenic activity study among the different plant samples to help select those with high estrogenic effects for the development of cosmetics, dietary supplements or pharmaceutical products. Furthermore, correlation analysis between isoflavonoid contents and bio-activity of the plant extracts could help characterize a chemical marker to identify plants with high estrogenic effects. The most interesting conclusion from this study is the genetic differences of the plants themselves. Chemovariety may also exist in the plant population. Molecular analysis at the level of RAPD could help finding the phylogenic relationship at the variety level. The analysis of some selected genes in the isoflavonoid pathways could enhance our understanding of the synthesis and storage of isoflavonoids in the plants and enable us to manipulate the whole plant or plant tissue to produce a higher amount of isoflavonoids with more estrogenic effects.

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