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ORIGINAL ARTICLE

Comparative Analysis of the Chemical Constituents from the Tuberous Root and Stem of *Pueraria candollei* var. *mirifica* and Evaluation of their Estrogenic Activity

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ABSTRACT

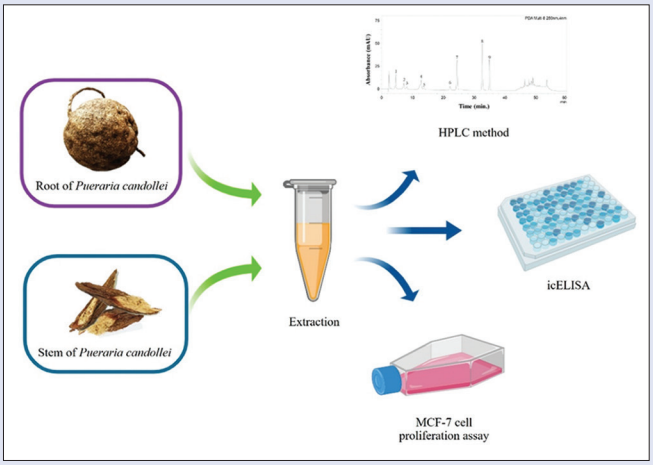
Background: *Pueraria candollei* var. *mirifica* (PC) is a tuberous plant enriched with bioactive phytoestrogens. PC is currently high demanded in the global markets as dietary supplement products, which resulted in a shortage of tuberous root from the natural resource and the field cultivation. **Materials and Methods:** We compare phytoestrogens contents from the tuberous root and stem of PC and their estrogenic activities on MCF-7 cells proliferation. **Results:** The root and stem of PC accumulated different contents of phytoestrogens. The highest amount of total isoflavonoids and total chromenes was found in the root bark of PC. However, the stem bark (10 μg/mL crude extract) which contains a high level of chromenes and low amount of isoflavonoids exhibited higher growth stimulation of MCF-7 cells (127.25% ± 4.43% relative proliferative effect [RPE] as compared to estradiol [10^{-10} M]) than root bark at the same concentration with 116.32% ± 3.59% RPE. After the solubilization of the ethanol extract with 10% (v/v) aqueous ethanol, the soluble fraction of crude extract of stem stimulated proliferation of MCF-7 cells without cell growth suppression effect at high concentration (100 μg/mL) as observed in the insoluble fraction of the extract. **Conclusion:** We suggested that the soluble part of crude extract might be less toxic than the ethanolic crude extract and its non-polar components. Our study opens the possibility of using the stem of PC as a new alternative source of bioactive phytoestrogens for dietary supplement products.

Key words: Chromenes, estrogenic activity, isoflavonoids, phytoestrogens, *Pueraria candollei* var. *mirifica*

SUMMARY

- Stem of *Pueraria candollei* var. *mirifica* could be used as a new alternative source of bioactive phytoestrogens.
- Partition extraction of the crude ethanolic extract with 10% ethanol had more safety to use than the ethanolic crude extract.

Abbreviations used: ANOVA: Analysis of variance; DMI: Deoxymiroestrol; E_2: Estradiol; icELISA: Indirect competitive enzyme-linked immunosorbent assay; HPLC: High-performance liquid chromatography; ISO: Isomiroestrol; MI: Miroestrol; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC: *Pueraria candollei*; RPE: Relative proliferative effect; SD: Standard deviation; YES: Yeast estrogen screen.



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INTRODUCTION

Pueraria candollei var. *mirifica* (PC) (known as *Pueraria mirifica*) is a famous plant in Thai traditional medicine. The tuberous root of this plant has been used for over 100 years regarding its estrogenic efficacy.[1] The phytochemical constituents of this plant including chromenes such as miroestrol (MI), deoxymiroestrol (DMI), and isomiroestrol (ISO) and isoflavonoid class such as genistein, daidzein (and their glycosides form, puerarin, genistin, and daidzin), and kwakhurin, have estrogenic activity.[2,3] Nowadays, the PC roots have been reported as strong estrogenic properties in the MCF-7 cells proliferation assays,[2,4,5] yeast estrogen screen test assays,[6] *in vivo* studies,[7-13] and clinical studies.[14-17] PC is presently used as the main ingredient in the health and cosmetic products that widely distributed in the global markets.

Although the tuberous roots of PC are rich in MI, DMI, ISO, daidzein, genistein, daidzin, genistin, kwakhurin, and puerarin, the agricultural processes take 5 years to grow until the decent size of PC root was

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achieved and then the root part was collected for herbal medicine manufacturing in industrial scale. The process will damage the wild ecosystems if the PC roots are collected from the natural resource. Moreover, the whole plant also was destroyed after root collecting process. Therefore, the new alternative sources containing phytoestrogens, specifically chromenes and isoflavonoids, are important at present. The other parts of the PC such as stems and leaves, which is leftover from harvesting the underground tubers, are the interesting parts for study on phytoestrogen accumulation and its estrogenic property. The leaves of PC have been reported accumulation of isoflavonoids; however, the yield of phytoestrogens in the leaf part was still lower than in tuberous root about two times.[18] For the stem part of the PC, there is no study reporting about their phytoestrogens and estrogenic activity. On the other hand, the stems of kudzu (*Pueraria lobata*), the plant whose root used widely in traditional Chinese, Japanese, and Korean medicines, were also composed of daidzein, genistin, daidzin, and puerarin as same as the root of *P. lobata*.[19] Thus, the stem of PC would be a promising alternative source of phytoestrogen.

In this study, we investigated the accumulation of bioactive compounds include chromenes and isoflavonoids of PC together with their estrogenic activities on MCF-7 cells proliferation. The results were compared between tuberous root and stem of PC. Thus, this research opens the possibility of the new, more economically alternative source of PC materials for natural phytoestrogen supply.

MATERIALS AND METHODS

Chemicals and reagents

Puerarin was obtained from Sigma-Aldrich (Missouri, USA). Genistin, genistein, daidzin, and daidzein were obtained from Fujicco Co., Ltd. (Kobe, Japan). MI, DMI, ISO, and kwakhurin were isolated from the tuberous root of PC and identified by previously described.[2,20] All chemicals reagents in our experiment were analytical grade products.

Plant materials

The tuberous root of PC was obtained from Khon Kaen Province, Thailand. The stems of PC were obtained from Kanchanaburi province, Thailand. Voucher specimens (NI-PSKKU 109-110) were deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand.

Sample preparation

The plant materials from the field cultivation were washed, sliced, and divided into root bark, root without bark, stem bark, a stem without bark and whole stem. All samples were completely dried at 50°C and ground into a fine powder. Ethanol (1 mL) was added into 200 mg of samples powders. Then, the sample was extracted with sonication for 20 min. After centrifugation, the extract was collected. The extraction process was repeated for 4 times. Finally, the extract was combined and evaporated at room temperature to give the crude ethanolic extract.

The crude ethanolic extract of the whole stem was collected and dried under vacuum in a rotary evaporator. For partition extraction, our preliminary studies show that 10% ethanol exhibited as an optimum condition for the extraction of phytoestrogens in PC (data not shown). Therefore, this concentration was chosen in this study. The crude ethanolic extract of 2 g amount was dissolved in 400 mL of 10% (v/v) ethanol in distilled water. This ratio of water and ethanol was used for separating the polar compounds from the crude extract. The sonicated for 60 min was applied to accelerate solubilization and then the mixture was transferred to 50 mL tube and centrifugation for 5 min at 4000 rpm (25°C). All soluble fraction of the extract was collected and concentrated under vacuum in a rotary evaporator to give the soluble crude extract. All precipitate remaining in the tube was collected, combined and dried to give the insoluble fraction of crude extract.

The determination of isoflavonoids by gradient high-performance liquid chromatography

Puerarin, daidzein, daidzin, genistein, genistin, and kwakhurin were analyzed by high-performance liquid chromatography (HPLC) method equipped with a gradient mobile phase system. This method was modified from the study previously reported.[21] The column oven was set at 30°C and the detection wavelength was set at 280 nm. All crude extracts were re-dissolved with 1 mL ethanol before each sample solution was injected (20 µL) by the autosampler. The gradient elution program was created by varying the proportion of solvent A (1.5% (v/v) acetic acid in water) and solvent B (100% acetonitrile). The flow rate was set at l mL/min and the column was eluted with a linear gradient program of 15%–20% B over 0–15 min, 20%–40% B over 15–40 min, 40%–100% B over 40–45 min, and then maintained at 100% B for 5 min to elute the unwanted matrix. Then, the gradient elution program was changed from 100% to 15% B over 5 min and maintained at 15% B for 5 min. Thus, the mobile phase system was returned to the initial conditions.

Determination of chromenes by indirect competitive ELISA

All crude extract solutions were investigated using an indirect competitive ELISA (icELISA) method to determine chromenes (MI, DMI, and ISO). The methods were modified from Yusakul *et al.*[22,23] and Kitisripanya *et al.*[24]

MCF-7 cells culture

Alpha-positive estrogen receptor human breast adenocarcinoma MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin) and cultured at 37°C in a humidified atmosphere of 5% CO_2.

MCF-7 cells proliferation assay

The MCF-7 cells were suspended in the estrogen-free medium (phenol red-free DMEM/F12 with 10% (v/v) charcoal-treated fetal bovine serum and antibiotics) and seeded into 96-well plates at a density of 7×10^3 cell/well. After 48 h of culture, the cells were exposed to 1×10^{-10} M estradiol (E_2) or different concentrations of test samples in 0.1% (v/v) ethanol in the estrogen-free medium. The medium was changed every 3 days. After 6 days of exposure, the cell proliferation was assessed. The cells were incubated with 10 µL/well of 5 mg/mL tetrazolium 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution and 100 µL/well of estrogen-free medium for 2 hr. Then, the solution (100 µL) of solubilizing agent (2% (v/v) glacial acetic acid, 40% (v/v) dimethylformamide, and 16% (w/v) sodium dodecyl sulfate in distilled water) was added and shook for 20 min and the absorbance was read at 595 nm. The cell response was defined as an increase in the frequency of proliferating cells (RPE%, relative proliferation effect):

$$RPE = (S/E) \times 100$$

Where S and E are the cell proliferation by the samples and 10^{-10} M estradiol.

Statistical analysis

The determination of phytoestrogen contents was performed in triplicate, and the assays of MCF-7 cell proliferation were performed in six replicates. The results are expressed as the mean ± SD. The difference

of value was investigated using one-way analysis of variance followed by Duncan's test ($P < 0.05$).

RESULTS AND DISCUSSION

Comparative analysis of bioactive compounds and estrogenic activities of PC tuberous root and stem

After extraction and re-dissolved with 1 mL ethanol, the crude ethanolic extracts of the root bark, the root without bark, the stem bark, the stem without bark and the whole stem of PC were analyzed for puerarin, daidzein, daidzin, genistein, genistin, and kwakhurin through the HPLC method equipped with gradient mobile phase system. The six isoflavonoids standard were well separated when they were monitored with ultraviolet of 280 nm [Supplementary Figure 1]. The HPLC fingerprints of these five PC crude extracts were complicated, but all peak of the isoflavonoids were still clearly separated from other substances. However, some PC samples showed an absence of some isoflavonoid(s). The amount of each isoflavonoid and total isoflavonoids that were found in each PC sample were calculated and shown in Figure 1A. In general, the highest mean of total isoflavonoids content was found in root bark of PC, following by root without bark, stem without bark, whole stem and the last one was stem bark. Daidzein in the root bark of PC was the major isoflavonoid that was much higher than that found in the root without bark and stems. Daidzin, the glycoside form of daidzein[25] was mostly found in root without bark, but it could not detect in stem bark. However, stem bark accumulated of daidzein, genistein and kwakhurin. Besides, it seems that kwakhurin was highly specific accumulated in the bark of this plant, such as root bark, stem bark and whole stem that included bark and wood. Stem without bark or wood of PC composed of puerarin as the major compound but it significantly lower concentration than root without bark. However, genistin was only found in root without bark. In the previous study[26] reported that root bark and root without bark of PC were determined of total isoflavonoids 3.45 ± 0.21–5.45 ± 0.17 mg/g dry wt. and 0.01 ± 0.00–1.36 ± 0.15 mg/g dry wt., respectively. Cherdshewasart and Sriwatcharakul[7] found that the tuberous root of *P. mirifica* accumulated of puerarin (0.14 ± 0.06–2.28 ± 0.88 mg/g dry wt.), daidzin (0.13 ± 0.03–1.78 ± 0.44 mg/g dry wt.), daidzein (0.20 ± 0.08 – 0.47 ± 0.33 mg/g dry wt.), genistin (0.11 ± 0.03–0.66 ± 0.11 mg/g dry wt.), and genistein (0.04 ± 0.02–0.25 ± 0.04 mg/g dry wt.). In addition, Yusakul *et al.*[27] reported that kwakhurin was accumulated only in root bark (0.05 – 0.21 mg/g dry wt.), but it could not be detected in root without bark of this plant.

In part of chromenes that were detected by an icELISA method. The results [Figure 2A] found that root bark showed the highest amount of MI, DMI, and ISO following by stem bark, whole stem, root without bark and lastly, stem without bark. Highly accumulation of MI, DMI, and ISO was obtained in root bark and stem bark of PC. The whole stem of PC was also found MI, DMI, and ISO, but its contents were significantly lower than root bark and stem bark. Previous studies that used icELISA method to determine chromenes, Yusakul *et al.*[22,23] reported accumulation of MI and DMI in root bark (25.23 ± 1.06–188.95 ± 9.25 μg/g dry wt. and 16.36 ± 1.00–195.04 ± 16.68 μg/g dry wt., respectively) and root without bark (0.09 ± 0.01 – 26.83 ± 2.03 μg/g dry wt. and 0.21 ± 0.01–32.28 ± 1.64 μg/g dry wt., respectively). Kitisripanya *et al.*[24] also found the accumulation of ISO in root bark (41.57 ± 7.06–404.96 ± 26.24 μg/g dry wt.).

The estrogenic effects of all-natural PC extract at concentrations of 0.1, 1, and 10 μg/mL crude extract were examined in MCF-7 cells. The proliferative effects were expressed as RPE% compared with estradiol (10^{-10} M, 100% RPE) [Figure 3A]. All extracts showed estrogenic activity with the dose-response relationship. Stem bark at

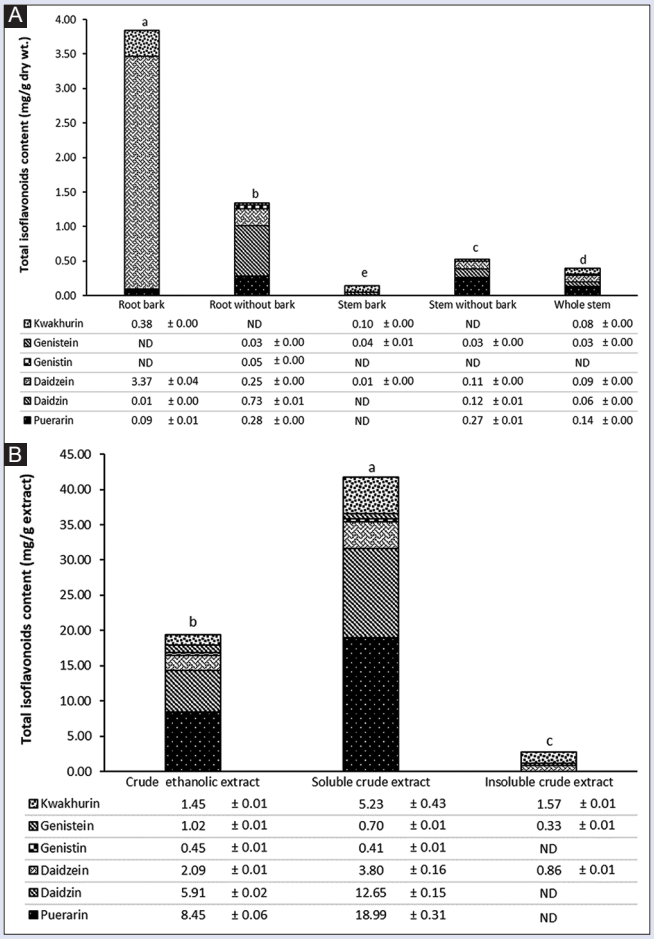

Figure 1: Isoflavonoids contents of (A) the root bark, the root without bark, the stem bark, the stem without bark and the whole stem of *Pueraria candollei* var. *mirifica*, (B) the crude ethanolic extract and its 10% ethanol-soluble and insoluble crude extract from *Pueraria candollei* var. *mirifica* whole stem. Data are the mean ± standard deviation from three replicates. Different letters indicated the statistically significant difference of total isoflavonoids content between sample at $P < 0.05$ by Duncan's multiple range test. ND: Not detected

the concentration of 10 μg/mL was the highest potent to stimulate the proliferation of MCF-7 cells. Stem bark at concentrations of 0.1 and 1 μg/mL and whole stem at a concentration of 0.1, 1, and 10 μg/mL could stimulate growth and proliferation of MCF-7 cells as similar as 10^{-10} M estradiol. In addition, root bark at concentration of 0.1, 1, and 10 μg/mL showed a higher level of proliferative effect than estradiol at 10^{-10} M. However, stem without bark at concentration of 0.1, 1, and 10 μg/mL exhibited lower estrogenic activities on MCF-7 cells than other samples at the same concentrations.

The proliferation effect on MCF-7 cells referred to the estrogenic activities of each sample. Stem bark contained the lowest total isoflavonoid contents, but it contained a high amount of three chromenes level in the second rank of all samples. The extract of stem bark at a concentration of 10 μg/mL showed higher stimulation of MCF-7 cells growth than root bark which accumulated the highest content of total isoflavonoids and chromenes. The root bark crude extract at concentration of 10 μg/mL might inhibit effect to MCF-7 cells proliferation. The high concentration of compounds might result in antiproliferative effect to cell. Similar to the previous study,[28] tuberous root extracts at concentrations of 10, 100, and 1,000 μg/mL

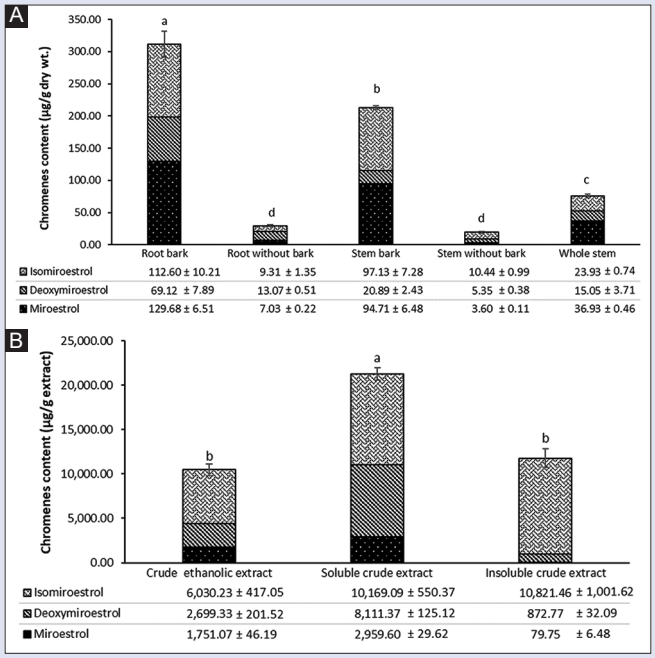

Figure 2: Chromenes contents of (A) the root bark, the root without bark, the stem bark, the stem without bark and the whole stem of *Pueraria candollei* var. *mirifica*, (B) the crude ethanolic extract and its 10% ethanol-soluble and insoluble crude extract from *Pueraria candollei* var. *mirifica* whole stem. Data are the mean ± standard deviation from three replicates. Different letters indicated the statistically significant difference of total chromenes content between sample at $P < 0.05$ by Duncan's multiple range test

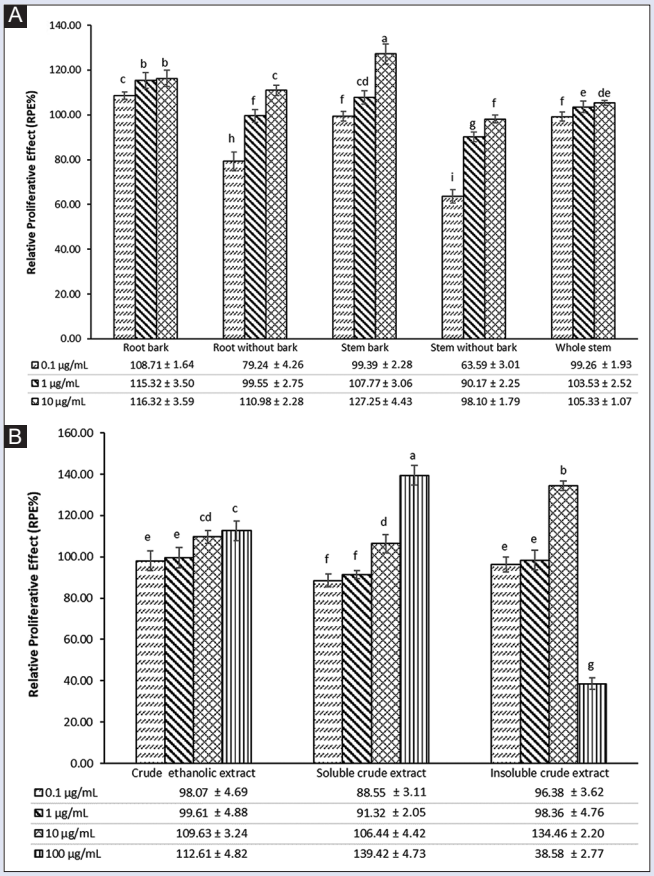

Figure 3: The MCF-7 cell proliferation effects of (A) *Pueraria candollei* var. *mirifica* extract from the root bark, the root without bark, the stem bark, the stem without bark, and the whole stem, (B) the crude ethanolic extract and its 10% ethanol-soluble and insoluble crude extract from *Pueraria candollei* var. *mirifica* whole stem. The proliferative effect is relative to E_2 (1×10^{-10} M estradiol, 100%), which was expressed as a relative proliferative effect. The results are expressed as mean ± standard deviation ($n = 6$). Different letters indicated the statistically significant difference at $P < 0.05$ by Duncan's multiple range test

exhibited cytotoxicity effect for MCF-7 cells. Between stem bark and root without bark of PC (the main materials used as phytoestrogen supplement products), stem bark had lower total isoflavonoids than root without bark about nine times. However, stem bark had higher total chromenes than the root without bark about 7 times; hence, stem bark extract exhibited higher RPE than the extract of root without bark at the same concentration. The comparative study of estrogenic activity of eight phytoestrogens isolated from PC[29] showed the rank order of potency using MCF-7 human breast cancer cells with the rage of DMI >MI>coumestrol>genistein>daidzein. Therefore, stem bark that had high DMI and MI levels could actively display estrogenic activity on MCF-7 cells proliferation. Consequently, stem bark could be used as an alternative source of phytoestrogens for health products.

Comparative analysis of bioactive compounds and estrogenic activities of stem extract after treatment

In previous results, stem bark showed a high amount of MI and DMI and exhibited strongly stimulate the proliferation of MCF-7 cells. In the correlation, the whole stem that included stem and wood of PC could stimulate growth and proliferation of MCF-7 cells, as well. Thus, the phytoestrogen contents and estrogenic activity of the whole stem were studied using extract treatment method. The ethanolic crude extract of PC whole stem (2.00 g, 100.00% w/w) was re-extraction in 10% (v/v) ethanol in distilled water and then, divided by centrifugation into soluble fraction (0.85 g, 42.50% w/w) and insoluble fraction of crude extract (0.75 g, 37.50% w/w). Each fraction was examined estrogenic property via MCF-7 human breast cancer cells and analyzed phytoestrogens content with the icELISA and HPLC methods. Six isoflavonoids of three samples were detected via the HPLC method [Supplementary Figure 2]. Crude ethanolic extract and 10% ethanol-soluble fraction composed of all isoflavonoids, but the insoluble fraction of the crude extract was found the only peak of daidzein, genistein, and kwakhurin. The major isoflavonoid of the crude ethanolic extract and the soluble fraction was puerarin [Figure 1B]. The amount of puerarin, daidzin and daidzein of the soluble fraction were higher than the crude ethanolic extract about 2 times. The most amount of genistin in the crude ethanolic extract was dissolved into the soluble fraction of crude extract part; therefore, it could not be detected in the insoluble fraction of the crude extract part. The major isoflavonoid of insoluble crude extract was kwakhurin. However, its content was lower than kwakhurin that found in the soluble fraction of crude extract.

Indirect competitive ELISA was used for quantitative analysis of MI, DMI, and ISO of the crude ethanolic extract and its soluble and insoluble fractions from the whole stem of PC [Figure 2B]. The most amount of MI and DMI from crude ethanolic extract were extracted into soluble fraction with 10% ethanol. Nevertheless, ISO contents that found in the fraction of soluble extract and insoluble were not a significant difference.

The proliferation and viability of MCF-7 cells were examined to determine estrogenic properties of crude ethanolic extract and its chemical components that were soluble and insoluble in 10% ethanol

at concentrations of 0.1, 1, 10, and 100 μg/mL [Figure 3B]. The soluble fraction of crude extract at a concentration of 100 μg/mL showed the highest stimulated of MCF-7 cells proliferation. The second rank order of estrogenic potency was 10 μg/mL of insoluble crude extract. Besides, it can be observed that insoluble crude extract at a concentration of 100 μg/mL presented a cytotoxic effect to MCF-7 cells. The crude ethanolic extract of the whole stem of PC at concentrations of 10 and 100 μg/mL exhibited cell growth similar to 10 μg/mL of the soluble fraction of extract. In addition, at the low concentrations (0.1 and 1 μg/mL), the RPEs of all extract were not significantly difference between both concentrations.

Our results displayed the yield of soluble crude extract and insoluble crude extract after solubilization from 2 g of crude ethanolic extract. The HPLC chromatograms showed that the most of non-polar substances at the last retention time (40–50 min) were removed from crude ethanolic extract into the insoluble part; however, there could be found a little amount in the soluble part. When considered on isoflavonoids and chromenes contents, most of the phytoestrogens were separated into the soluble part, except daidzein, genistein, kwakhurin, and ISO. These four chemicals were still found in the insoluble crude extract. The MCF-7 cells proliferation assays showed the highest estrogenic effect at 100 μg/mL of the soluble crude extract without cytotoxic effect on MCF-7 cells. In contrast, insoluble crude extract (100 μg/mL) of whole stem PC presented toxic property on this cell. From these consequences, it seems that the soluble fraction of crude extract was concentrated with highly effective phytoestrogens from crude ethanolic extract, while insoluble crude extract was accumulated toxic substances. Accordingly, we suggested that crude ethanolic extract and its portion of 10% ethanol-soluble fraction of PC whole stem could be alternative sources of rejuvenating products.

CONCLUSION

Root and stem of PC exhibited different accumulation of bioactive phytoestrogens. The estrogenic activities of root bark, stem bark, and whole stem were greater than or equal to 1×10^{-10} M estradiol; however, estrogenic activities of root without bark at low concentration (0.1 and 1 μg/mL) were lower than 1×10^{-10} M estradiol. After dissolving the crude ethanolic extract with 10% ethanol, the results suggest that cytotoxic chemicals in ethanolic crude extract from stem were removed to an insoluble fraction. Growth and proliferation of MCF-7 cells were still stimulated by ethanolic crude extract and soluble crude extract at high concentration (100 μg/mL), whereas the effects of growth suppression were observed in the insoluble part with the same concentration. It seems that the soluble crude extract had more safety to use than the ethanolic crude extract. Hence, we suggest that the stem of PC could be used as a new potential alternative source of phytoestrogens, which the source can be used instead of PC root for dietary supplement and cosmetic products. These sources will preserve this plant and reduce the problem of plant destroyed after root collecting process. Besides, the value of stem known as the waste product from harvesting is increased.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Kashemsanta MC, Lakshnakara SK. A new species of *Pueraria* (*Leguminosae*) from Thailand, yielding an estrogenic principle. Kew Bull 1952;7:549-51.
2. Chansakaow S, Ishikawa T, Sekine K, Okada M, Higuchi Y, Kudo M, *et al.* Isoflavonoids from *Pueraria mirifica* and their estrogenic activity. Planta Med 2000;66:572-5.
3. Ingham JL, Tahara S, Dziedzic SZ. A chemical investigation of *Pueraria mirifica* roots. Z Naturforsch C 1986;41:403-8.
4. 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;93:255-60.
5. Cherdshewasart W, Traisup V, Picha P. Determination of the estrogenic activity of wild phytoestrogen-rich *Pueraria mirifica* by MCF-7 proliferation assay. J Reprod Dev 2008;54:63-7.
6. Boonchird C, Mahapanichkul T, Cherdshewasart W. Differential binding with ERalpha and ERbeta of the phytoestrogen-rich plant *Pueraria mirifica*. Braz J Med Biol Res 2010;43:195-200.
7. Cherdshewasart W, Sriwatcharakul S. Major isoflavonoid contents of the 1-year-cultivated phytoestrogen-rich herb, *Pueraria mirifica*. Biosci Biotechnol Biochem 2007;71:2527-33.
8. Jaroenporn S, Urasopon N, Watanabe G, Malaivijitnond S. Improvements of vaginal atrophy without systemic side effects after topical application of *Pueraria mirifica*, a phytoestrogen-rich herb, in postmenopausal cynomolgus macaques. J Reprod Dev 2014;60:238-45.
9. 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;107:354-60.
10. 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;96:428-35.
11. 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;52:537-42.
12. Trisomboon H, Malaivijitnond S, Watanabe G, Cherdshewasart W, Taya K. The estrogenic effect of *Pueraria mirifica* on gonadotrophin levels in aged monkeys. Endocrine 2006;29:129-34.
13. Trisomboon H, Malaivijitnond S, Watanabe G, Taya K. Estrogenic effects of *Pueraria mirifica* on the menstrual cycle and hormone-related ovarian functions in cyclic female cynomolgus monkeys. J Pharmacol Sci 2004;94:51-9.
14. Chandeying V, Sangthawan M. Efficacy comparison of *Pueraria mirifica* (PM) against conjugated equine estrogen (CEE) with/without medroxyprogesterone acetate (MPA) in the treatment of climacteric symptoms in perimenopausal women: Phase III study. J Med Assoc Thai 2007;90:1720-6.
15. Masatomo N, Mitsuhiko M, Hiroyuki S. Efficacy on breast skin tension of the supplement containing *Pueraria mirifica* in healthy Japanese females: A randomized, double-blind, placebo-controlled study. Med Cons New-Remed 2017;54:29-36.
16. Masatomo N, Mitsuhiko M, Hiroyuki S. Efficacy on menopausal discomfort of the supplement containing *Pueraria mirifica* in healthy Japanese females: A randomized, double-blind, placebo-controlled study. Med Cons New-Remed 2017;54:37-45.
17. Suwanvesh N, Manonai J, Sophonsritsuk A, Cherdshewasart W. Comparison of *Pueraria mirifica* gel and conjugated equine estrogen cream effects on vaginal health in postmenopausal women. Menopause 2017;24:210-5.
18. Jungsukcharoen J, Dhiani BA, Cherdshewasart W, Vinayavekhin N, Sangvanich P, Boonchird C. *Pueraria mirifica* leaves, an alternative potential isoflavonoid source. Biosci Biotechnol Biochem 2014;78:917-26.
19. Mun SC, Mun GS. Dynamics of phytoestrogen, isoflavonoids, and its isolation from stems of *Pueraria lobata* (Willd.) Ohwi growing in democratic people's republic of Korea. J Food Drug Anal 2015;23:538-44.
20. Chansakaow S, Ishikawa T, Seki H, Sekine (née Yoshizawa) K, Okada M, Chaichantipyuth C. Identification of deoxymiroestrol as the actual rejuvenating principle of "Kwao Keur", *Pueraria mirifica*. The known miroestrol may be an artifact. J Nat Prod 2000;63:173-5.
21. Udomsin O, Juengwatanatrakul T, Yusakul G, Putalun W. Chromene stability: The most potent estrogenic compounds in White *Kwao Krua* (*Pueraria candollei var. mirifica*) crude extract. J Funct Foods 2015;19:269-77.
22. Yusakul G, Udomsin O, Juengwatanatrakul T, Tanaka H, Chaichantipyuth C, Putalun W. High performance enzyme-linked immunosorbent assay for determination of miroestrol, a potent phytoestrogen from *Pueraria candollei*. Anal Chim Acta 2013;785:104-10.
23. Yusakul G, Udomsin O, Juengwatanatrakul T, Tanaka H, Chaichantipyuth C, Putalun W. Highly

selective and sensitive determination of deoxymiroestrol using a polyclonal antibody-based enzyme-linked immunosorbent assay. Talanta 2013;114:73-8.

24. Kitisripanya T, Jutathis K, Inyai C, Komaikul J, Udomsin O, Tanaka H, *et al*. Development of an enzyme-linked immunosorbent assay for the detection of isomiroestrol, an identical marker, in White *Kwao Krua* using a monoclonal antibody. J Pharm Biomed Anal 2017;137:229-34.
25. Zubik L, Meydani M. Bioavailability of soybean isoflavones from aglycone and glucoside forms in American women. Am J Clin Nutr 2003;77:1459-65.
26. Yusakul G, Putalun W, Udomsin O, Juengwatanatrakul T, Chaichantipyuth C. Comparative analysis of the chemical constituents of two varieties of *Pueraria candollei*. Fitoterapia 2011;82:203-7.
27. Yusakul G, Togita R, Minami K, Chanpokapaiboon K, Juengwatanatrakul T, Putalun W, *et al*. An indirect competitive enzyme-linked immunosorbent assay toward the standardization of *Pueraria candollei* based on its unique isoflavonoid, kwakhurin. Fitoterapia 2019;133:23-8.
28. Cherdshewasart W, Kitsamai Y, Malaivijitnond S. Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. J Reprod Dev 2007;53:385-93.
29. 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;94:431-43.