Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Pueraria candollei var. *mirifica*-Induced CYP1A1 and CYP1A2 Expression in Human Choriocarcinoma BeWo Cells

Waranya Chatuphonprasert, Tharita Kitisripanya[1], Waraporn Putalun[2], Isabella Ellinger[3], Kanokwan Jarukamjorn[2]

Division of Pre-clinic, Faculty of Medicine, Mahasarakham University, Maha Sarakham 44000, [1]Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, [2]Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand, [3]Center for Pathophysiology, Infectiology and Immunology, Institute for Pathophysiology and Allergy Research, Medical University of Vienna, 1090 Vienna, Austria

Submitted: 27-Apr-2020 Revised: 20-May-2020 Accepted: 14-Sep-2020 Published: 30-Nov-2020

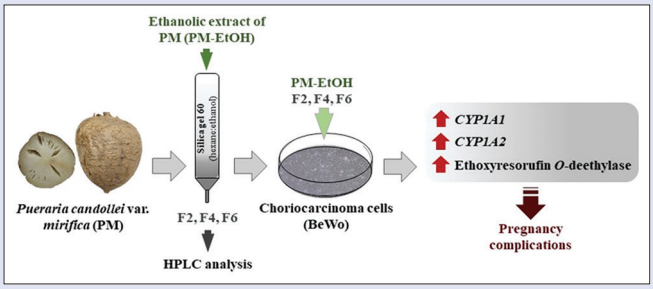
ABSTRACT

Background: The human placenta metabolizes many endogenous substances, drugs, and xenobiotics. Cytochrome P450 family 1 (CYP1) is expressed in both early- and full-term placenta. The Thai medicinal plant *Pueraria candollei* var. *mirifica* (PM) is traditionally consumed for rejuvenation and has neuroprotective, anti-osteoporotic, and antioxidant activities. **Objectives:** The objective of this study was to compare the effects of PM and the CYP1A inducer β-naphthoflavone (BNF) on the expression of *CYP1*, aryl hydrocarbon receptor (*AHR*), AHR nuclear translocator (*ARNT*), and the transporter *ABCG2*. **Materials and Methods:** Human choriocarcinoma BeWo cells were treated with BNF (10 μM), ethanolic extract of PM (PM-EtOH), or column chromatographic fractions of PM-EtOH (F2, F4, and F6) at 1, 10, and 100 μg/mL for 24 h. The mRNA expression of target genes was determined using real-time quantitative polymerase chain reaction. The activity of ethoxyresorufin-*O*-deethylase (EROD), a marker for *CYP1*, was measured at the RNA harvesting time point. **Results and Discussion:** PM-EtOH, F2, and F4 significantly induced EROD activity and expression of *CYP1A1* and *CYP1A2* while *CYP1B1* and *AHR* were slightly suppressed and *ARNT* was unchanged. *ABCG2* was slightly induced by F2. Therefore, the expression of CYP1 in BeWo cells appears to be independent of the *AHR*/*ARNT* regulatory pathway. **Conclusion:** The use of PM-containing products at high quantities or for long periods during pregnancy is of concern due to likely herb–drug interactions and toxicological risks through activation of *CYP1A1* and *CYP1A2* transcription.

Key words: Cytochrome P450, ethoxyresorufin-O-deethylase, nuclear receptor, placenta, transporter

SUMMARY

- The induction of *CYP1A1* and *CYP1A2* expression by the ethanolic extract of *Pueraria candollei* var. *mirifica* and its chromatographic fractions in BeWo cells are of concerns for risks of carcinogenesis and drug interactions, especially a use at high amount or in pregnancy.



Abbreviations Used: *AHR*: aryl hydrocarbon receptor; *ARNT*: AHR nuclear translocator; BNF: β-naphthoflavone; CYP: Cytochrome P450; EROD: ethoxyresorufin-*O*-deethylase; PM: *Pueraria candollei* var. *mirifica*; PM-EtOH: ethanolic extract of *Pueraria candollei* var. *mirifica*.

Correspondence:

Dr. Isabella Ellinger,
Center for Pathophysiology,
Infectiology and Immunology,
Institute for Pathophysiology and
Allergy Research, Medical University of
Vienna, Währinger Gürtel 18-20, 1090 Vienna,
Austria.
E-mail: isabella.ellinger@meduniwien.ac.at
Dr. Kanokwan Jarukamjorn
Faculty of Pharmaceutical Sciences,
Khon Kaen University, 123 Mitraparb Road,
Khon Kaen 40002, Thailand.
E-mail: kanok_ja@kku.ac.th
DOI: 10.4103/pm.pm_164_20

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Pueraria candollei var. *mirifica* (PM) is a Thai medicinal herb with several pharmacological activities, for example, antioxidation,[1,2] osteoporosis prevention,[3,4] and neuroprotection,[5] as well as anti-elastase and anti-collagenase activities useful for skincare products.[6] The human placenta produces the hormones and proteins necessary to maintain pregnancy and to support normal fetal development. In addition, the placenta metabolizes many exogenous substances, including drugs, environmental pollutants, and xenobiotics.[7] Cytochrome P450 family 1 (CYP1) comprises the most common metabolizing enzymes for xenobiotics and toxicants, and CYP1 enzymes are expressed in the placenta. The CYP1 family consists of three isoforms CYP1A1, CYP1A2, and CYP1B1.[8] In the human choriocarcinoma BeWo cell line, CYP1A1 and CYP1A2 are expressed and inducible.[9] CYP1B1 is commonly found in extrahepatic tissues such as lungs, lymphocytes, mammary glands, and placenta.[10,11] Polycyclic aromatic hydrocarbons (PAHs), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene (3MC), benzo[*a*]pyrene (B[*a*]P), or β-naphthoflavone (BNF), are broadly distributed environmental contaminants that cause several toxicities in mammals, including carcinogenesis, teratogenesis, and immune dysfunction.[12] PAHs induce the expression of various metabolizing enzymes through activation of the aryl hydrocarbon receptor (*AHR*),

Cite this article as: Chatuphonprasert W, Kitisripanya T, Putalun W, Ellinger I, Jarukamjorn K. *Pueraria candollei* var. *mirifica*-Induced CYP1A1 and CYP1A2 expression in human choriocarcinoma bewo cells. Phcog Mag 2020;16:S506-12.

which forms a dimer with the aryl hydrocarbon receptor nuclear translocator (*ARNT*) that binds to the aryl hydrocarbon response element upstream of the *CYP1* genes.[13] Breast cancer resistance protein (BCRP/MXR/ABCG2) is a member of the ATP-binding cassette (ABC) transporter superfamily that has been associated with multidrug resistance in many diseases.[14] ABCG2 is highly expressed in several human tissues, for example, colon, liver, small intestine, and placenta, and contributes to the protective function of those tissues.[14] In human placenta, ABCG2 is most abundantly expressed in the apical membrane of the placental syncytiotrophoblast[15] where it mediates the unidirectional transport of substrates, including PAHs, as an efflux pump.[16]

In experiments conducted *in silico* and in ICR mouse microsomes, five PM phytoestrogens (miroestrol, kwakhurin, deoxymiroestrol, methoxyisomiroestrol, and isomiroestrol) were shown to inhibit the activities of ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) enzymes, which act as markers for CYP1 activities.[17] Similarly, PM decreased the activities of benzyloxyresorufin *O*-dealkylase and pentoxyresorufin *O*-dealkylase, which represent CYP2B activity, in both regular diet- and high-cholesterol diet-fed male Wistar rats.[18] Miroestrol and deoxymiroestrol increased uterus weight and volume in female C57BL/6 mice[19] accompanied by induction of *Cyp2b9* expression and suppression of *Cyp1a2* expression in mouse hepatocytes.[20] To date, information about the effect of PM on the expression of *CYP1* and *ABCG2* in placenta models is limited. Therefore, the present study aimed to examine the effects of an ethanolic extract of PM on the expression of *CYP1* (*CYP1A1, CYP1A2,* and *CYP1B1*), the CYP upstream regulators, *AHR* and *ARNT*, and the transporter *ABCG2* in human choriocarcinoma BeWo cells.

MATERIALS AND METHODS

Chemicals and reagents

Authentic standards of isoflavonoids and chromenes were kindly provided by Prof. Waraporn Putalun (Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand). Dulbecco's modified Eagle's medium (DMEM) and cell culture supplements were supplied by Gibco® (Thermo Fisher Scientific, MD, USA). Ethoxyresorufin, resorufin standard, and resazurin were supplied by Sigma-Aldrich Chemical (St. Louis, MO, USA). Reverse transcription reagents, TaqMan™ probe and primers, and TaqMan™ gene expression assays were products of Applied Biosystems (California, USA). All other laboratory reagents and equipment were of the highest purity and standard quality from commercial suppliers.

The *Pueraria candollei* var. *mirifica* crude extract and the column chromatographic fraction preparation

PM tuberous roots (Reference specimen NI-PSKKU 007-010) were previously collected in Ubon Ratchathani, Thailand.[21] The dried powder of PM was extracted in absolute ethanol using a Soxhlet apparatus at 60°C for 3 h. The PM ethanolic extract (PM-EtOH) was filtered and evaporated at 60°C (2.26% yield). Then, the PM-EtOH extract was fractionated on a Silica Gel 60 column with hexane/ethanol mixtures of increasing polarity (from 0% to 100% of ethanol; 50 mL/h). A 5 mL aliquot of each fraction was analyzed by thin-layer chromatography (TLC), and the fractions with identical TLC patterns were pooled to obtain the F2, F4, and F6 fractions, which were subsequently evaporated. A 1 mg aliquot of PM-EtOH, F2, F4, and F6 was dissolved in 1 mL ethanol and filtered through a 0.2 μm-membrane for high-performance liquid chromatographic (HPLC) analysis.

High-performance liquid chromatographic condition

The HPLC was performed using a PerkinElmer Series 200 LC pump coupled with a reversed-phase C_{18} column (LiChroCART®, 125 mm × 4 mm, 5 μm particle size) and a PerkinElmer 785A UV/VIS detector. The detection was set at a wavelength of 280 nm. The flow rate was set at l mL/min. A 20 μL aliquot of each sample was subjected to HPLC. The gradient elution was programmed using a dual pumping system by varying the proportion of solvent A (1% acetic acid in water) and solvent B (100% acetonitrile) with a linear gradient program of 10%–15% B over 0–15 min, 15%–20% B over 15–35 min, 20%–35% B over 35–40 min, 35%–45% B over 40–50 min, 45%–80% B over 50–55 min, and finally maintained at 80% B for 5 min to elute any unwanted matrix. Then, the gradient elution program was set to 80%–10% B over 60–70 min and returned to the initial conditions.[22] A series of standard solutions was prepared by mixing the standards of isoflavonoids and chromenes to obtain the solutions with final concentrations of each isoflavonoid (puerarin, daidzin, genistin, daidzein, genistein, and kwakhurin) at 6.25 μg/mL and each chromene (isomiroestrol, miroestrol, and deoxymiroestrol) at 12.5 μg/mL.

Cell culture

BeWo cell clone b24 was cultured in high-glucose DMEM with 10% fetal calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.[23] BeWo cells (5×10^5 cells/well) were seeded in a 6-well plate for 24 h before treatment. The cells (n = 4–5 wells) were incubated with 0.2% dimethyl sulfoxide (DMSO, control), 10 μM BNF, or 1, 10, and 100 μg/mL of PM-EtOH, F2, F4, or F6 in phenol red-free medium for further 24 h.

Cell viability assessment

After treatment for 24 h, resazurin was added to the medium at a final concentration of 1 μM and incubated for 1 h at 37°C. The fluorescence intensity was measured at 530/590 nm excitation/emission. The percentage of cell viability was calculated compared to the control (0.2% DMSO).[24]

Ethoxyresorufin-*O*-deethylase activity assay

The EROD activity assay was modified from a previous report.[25] Ethoxyresorufin at the final concentration of 5 μM was incubated with the cells at 37°C for 3 h. Formation of resorufin in the medium was analyzed using spectrofluorometry at 530/590 nm excitation/emission and compared with a resorufin standard (10–200 nM). EROD activity was calculated as formation of resorufin (fmole) per min.

Total RNA isolation, cDNA synthesis, and real-time polymerase chain reaction determination

Total RNA was isolated using the peqGOLD TriFast™ Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). A 1 μg aliquot of total RNA was used to examine RNA integrity by 1.2% agarose gel electrophoresis in 1× Tris-borate-EDTA (TBE) buffer containing GelRed™ (Biotium, Koln, Germany) at 120 V for 20 min. Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, USA). Nontemplate control was a negative control using RNAse/DNAse-free water instead of any RNA sample. Thermal cycler was set to generate cDNA at a multiphase condition: 25°C (10 min), 37°C (120 min), 85°C (5 min), and 4°C (∞). For quantitative polymerase chain reaction (qPCR), the 7900HT Fast Real-Time PCR System and TaqMan™ Gene Expression Master Mix (Applied Biosystems, California, USA) in combination with the following TaqMan™ probes and primers of the

following genes: *CYP1A1* (Hs01054797_g1), *CYP1A2* (Hs00167927_m1), *CYP1B1* (Hs02382916_s1), *AHR* (Hs00907314_m1), *ARNT* (Hs01121918_m1), *ABCG2* (Hs01053790_m1), and two reference genes, ubiquitin (*UBC*; Hs00824723_m1) and TATA box-binding protein (*TBP*; Hs99999910_m1), were used. The changes in Ct (ΔCt) between the genes of interest and the geometric mean of the housekeeping genes and the changes in ΔCt between the treatments and the controls (ΔΔCt) were calculated. The fold change in expression between samples was calculated from $2^{-(\Delta\Delta Ct)}$.[26]

Statistical analysis

From the qPCR experiment, the average fold differences from each group (n = 4–5 per group) were plotted and analyzed using one-way analysis of variance followed by Tukey's *post hoc* test (SPSS version 23, Chicago, IL, USA). $P \leq 0.05$ was considered as statistically significant.

RESULTS

Quantitative analysis of isoflavonoids and chromenes in *Pueraria candollei* var. *mirifica* ethanolic extract and its column chromatographic fractions, F2, F4, and F6

An HPLC profile of pure isoflavonoids and chromenes is depicted in Figure 1. PM-EtOH (1 mg/mL) contained puerarin (71.53 ± 0.81 μg/mL), daidzin (3.01 ± 0.05 μg/mL), isomiroestrol (12.54 ± 0.52 μg/mL), daidzein (0.98 ± 0.02 μg/mL), and kwakhurin (4.82 ± 0.04 μg/mL). F2 contained daidzein (4.37 ± 0.01 μg/mL) and kwakhurin (25.94 ± 0.03 μg/mL). F4 had an HPLC profile similar to PM-EtOH and contained puerarin (116.98 ± 5.67 μg/mL), daidzin (5.43 ± 0.20 μg/mL), isomiroestrol (10.94 ± 0.24 μg/mL), daidzein (2.16 ± 0.02 μg/mL), and kwakhurin (5.70 ± 0.13 μg/m). F6 contained puerarin (86.47 ± 3.63 μg/mL) and daidzin (2.77 ± 0.05 μg/mL).

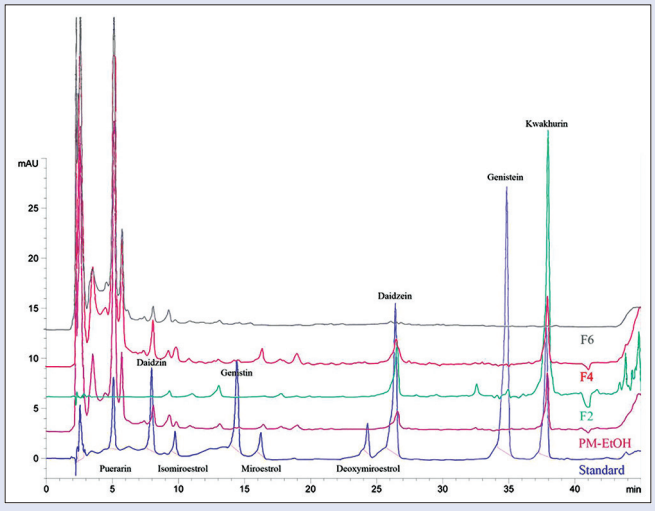


Figure 1: High-performance liquid chromatographic chromatogram of authentic standard isoflavonoids and chromenes, *Pueraria candollei* var. *mirifica* ethanolic extract, and its column chromatographic fractions F2, F4, and F6. Authentic standard isoflavonoids (puerarin, daidzin, genistin, daidzein, genistein, and kwakhurin) at 6.25 μg/mL and authentic standard chromenes (isomiroestrol, miroestrol, and deoxymiroestrol) at 12.5 μg/mL (blue line); *Pueraria candollei* var. *mirifica* ethanolic extract at 1 mg/mL (magenta line); the column chromatographic fraction number 2 of the *Pueraria candollei* var. *mirifica* ethanolic extract (F2) at 1 mg/mL (green line); the column chromatographic fraction number 4 of the *Pueraria candollei* var. *mirifica* ethanolic extract (F4) at 1 mg/mL (red line); (e) the column chromatographic fraction number 6 of the *Pueraria candollei* var. *mirifica* ethanolic extract (F6) at 1 mg/mL (gray line)

Effects of *Pueraria candollei* var. *mirifica* ethanolic extract and its column chromatographic fractions on ethoxyresorufin-*O*-deethylase activity

The CYP1A inducer BNF (10 μM) significantly induced EROD activity in BeWo cells [Figure 2]. PM-EtOH (at 10 and 100 μg/mL), F2 (at 1 and 10 μg/mL), and F4 (at 1, 10, and 100 μg/mL) extensively increased EROD activity. Likewise, F6 at the highest concentration of 100 μg/mL significantly elevated EROD activity. F2 was cytotoxic to BeWo cells at concentrations higher than 50 μg/mL, with <80% cell viability after treatment (data not shown), while other groups had >80% cell viability. Hence, only 1 and 10 μg/mL of F2 were further examined.

Effects of *Pueraria candollei* var. *mirifica* ethanolic extract and its column chromatographic fractions on the expression of *CYP1A1, CYP1A2, CYP1B1,* and *ABCG2* mRNAs

BNF (10 μM) significantly induced the expression of *CYP1A1* [143-fold, Figure 3a] and *CYP1A2* [10.7-fold, Figure 3b] mRNAs in BeWo cells. PM-EtOH, F2, and F4 extensively induced the expression of *CYP1A1* mRNA (47–215-fold) in a concentration-dependent manner, while F6 did not alter *CYP1A1* expression [Figure 3a]. Similarly, PM-EtOH, F2, and F4 significantly induced the expression of *CYP1A2* mRNA (3–7-fold), while F6 did not [Figure 3b]. In contrast, the expression of *CYP1B1* mRNA was suppressed by BNF, PM-EtOH, F2, and the lowest concentration of F6 (1 μg/mL) [Figure 3c]. The expression of *ABCG2* mRNA was significantly increased by F2 at a concentration of 10 μg/mL [Figure 3d], while all other treatments did not modify *ABCG2* expression.

Effects of *Pueraria candollei* var. *mirifica* ethanolic extract and its column chromatographic fractions on the expression of *AHR* and *ARNT*

The expression of *AHR* mRNA was significantly suppressed by PM-EtOH (10 and 100 μg/mL) and F6 (all concentrations) [Figure 4a].

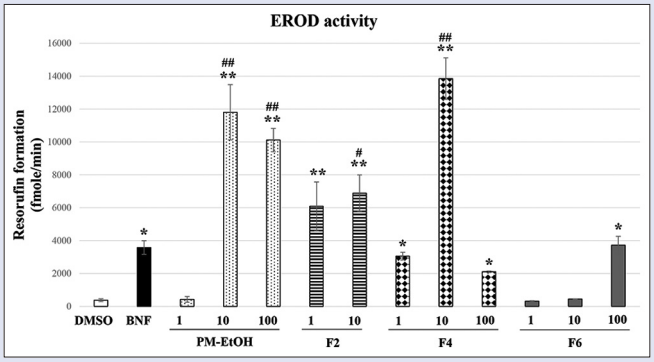


Figure 2: Effects of *Pueraria candollei* var. *mirifica* ethanolic extract and its column chromatographic fractions F2, F4, and F6 on ethoxyresorufin-*O*-deethylase activity. DMSO, 0.2% dimethyl sulfoxide; BNF, 10 μM β-naphthoflavone; PM-EtOH, *Pueraria candollei* var. *mirifica* ethanolic extract at 1, 10, and 100 μg/mL; F2, F4, F6, the column chromatographic fraction number 2, 4, and 6 of the *Pueraria candollei* var. *mirifica* ethanolic extract at 1, 10, and/or 100 μg/mL. $^{*}P < 0.05$, $^{**}P < 0.001$ versus dimethyl sulfoxide; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ versus BNF

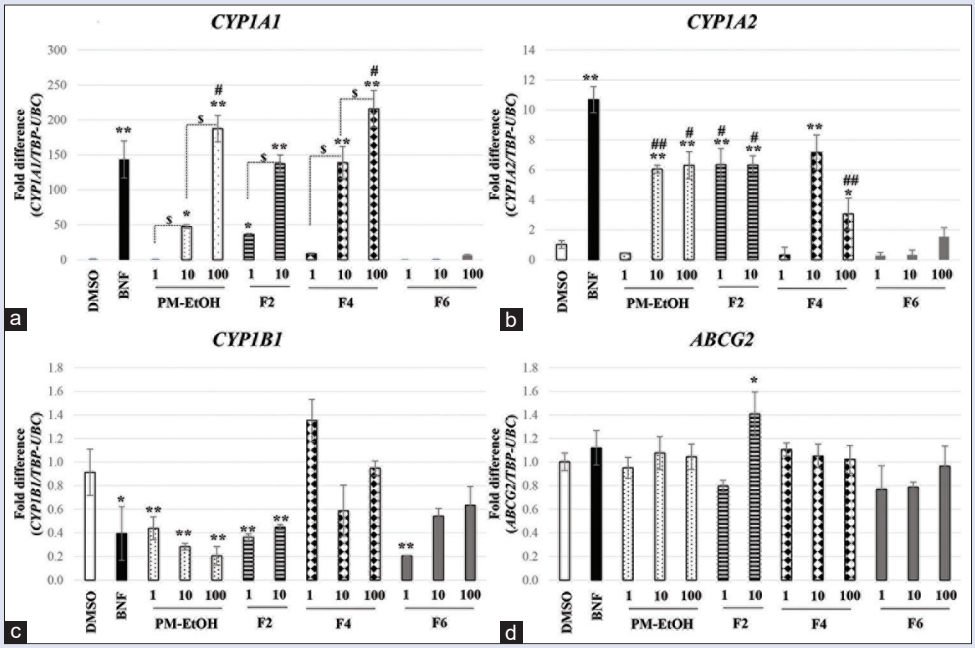


Figure 3: Effects of PM-EtOH and its column chromatographic fractions F2, F4, and F6 on the gene expression of *CYP1A1, CYP1A2, CYP1B1,* and *ABCG2*. Fold change in mRNA expression of the target genes, i.e., *CYP1A1* (a), *CYP1A2* (b), *CYP1B1* (c), and *ABCG2* (d), normalized to the reference genes TATA box-binding protein (TBP) and ubiquitin C (UBC). DMSO, 0.2% dimethyl sulfoxide; BNF, 10 μM β-naphthoflavone; PM-EtOH, *Pueraria candollei* var. *mirifica* ethanolic extract at 1, 10, and 100 μg/mL; F2, F4, F6, the column chromatographic fraction number 2, 4, and 6 of the PM-EtOH at 1, 10, and/or 100 μg/mL. $^{*}P < 0.05$, $^{**}P < 0.001$ versus dimethyl sulfoxide; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ versus BNF; $^{\$}P < 0.05$

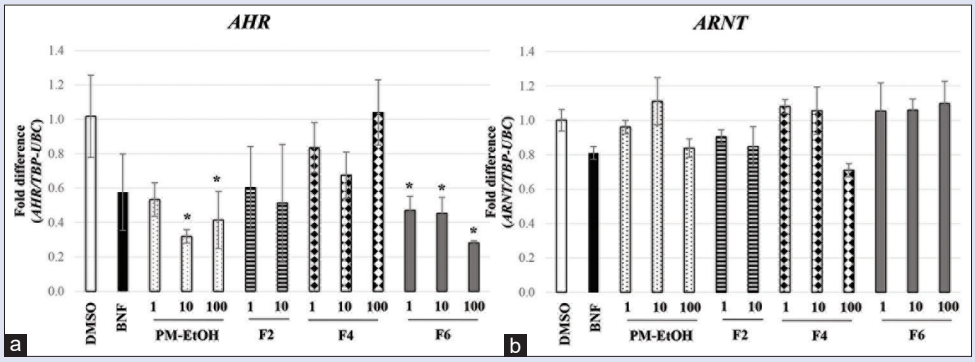


Figure 4: Effects of PM-EtOH and its column chromatographic fractions F2, F4, and F6 on the gene expression of aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT). Fold change in mRNA expression of the target genes, i.e., AHR (a) and ARNT (b), normalized to the reference genes TATA box-binding protein (TBP) and ubiquitin C (UBC). DMSO, 0.2% dimethyl sulfoxide; BNF, 10 μM β-naphthoflavone; PM-EtOH, *Pueraria candollei* var. *mirifica* ethanolic extract at 1, 10, and 100 μg/mL; F2, F4, F6, the column chromatographic fraction number 2, 4, and 6 of the PM-EtOH at 1, 10, and/or 100 μg/mL. $^{*}P < 0.05$ versus dimethyl sulfoxide

No significant changes were observed for the expression of *ARNT* mRNA following any treatment [Figure 4b].

DISCUSSION

Two chromenes, miroestrol and deoxymiroestrol, were not found in the PM-EtOH extract, presumably because they were lost during Soxhlet extraction, which was employed instead of maceration.[22,27] Miroestrol and deoxymiroestrol possess potent estrogenic effects,[28] and these two compounds should be avoided in pregnancy. In this study, the Soxhlet extraction yielded the isoflavonoid, puerarin, which has shown nephro- and retinoprotective activities in diabetic models.[29,30] In MCF-7 cells, an ethanolic extract of PM caused proliferation at 1 μg/mL and was antiproliferative at 100 μg/mL.[31] In this study, we chose the concentrations of the PM extracts (1, 10, and 100 μg/mL) to ensure >80% cell viability and prevent the inhibition of normal processes in cells. We determined *CYP1A1* mRNA expression at 24 h as a previous study that B[a]P induced *CYP1A1* in BeWo cells continuously from 8 to 96 h, reaching a maximum level of induction at 24 h.[32]

PAHs are ubiquitous environmental pollutants present wherever organic material is burned, for example, cigarette smoke, automobile exhaust, cooked foods, wood smoke, and a number of environmental products, such as coal, tar, petroleum, and cutting fluid.[33] Most PAHs are inducers of CYP1 (i.e., CYP1A1, CYP1A2, and CYP1B1), which is mainly responsible for formation of their toxic metabolites.[34,35] Such activation

may also occur at the placental level, and PAHs can induce placental enzymes including CYP1.[8] Here, we chose BNF, a PAH that has been used as a CYP1 inducer in several models.[36-38]

Increased CYP1A1 activity in placenta of female smokers has been associated with pregnancy complications, such as premature birth, intrauterine growth retardation, structural abnormalities, fetal death or placental abruption, and a risk of low birth weight, low birth length, and low head circumference.[39,40] In smokers, a significant increase in the levels of *CYP1A1* and *CYP1B1* transcripts has been observed.[41] EROD activity has been widely used as a marker for CYP1 activity.[42,43] EROD activity in BeWo cells has been shown to be sensitive to inducers, such as 3MC, 1,2-benzanthracene, and BNF (10–50 μM).[9] However, in this study, EROD activity [Figure 2] was not relative to the expression of *CYP1B1* mRNA [Figure 3c]. There was a report on different regulations of *CYP1A1* and *CYP1B1* expression in human medulloblastoma cells (UW228-3),[44] and in another choriocarcinoma cell line JEG3, TCDD induced the expression of *CYP1A1* and *AHR* mRNAs but not those of *CYP1B1* and *ARNT*.[8,45] Therefore, regulatory pathways of *CYP1A1* and *CYP1B1* in BeWo cells might be differently affected by xenobiotics. Moreover, EROD activity did not appear to be an appropriate enzymatic marker for CYP1B1 activity. The combination of dexamethasone and BNF additionally increased EROD activity,[9] and the transcriptional regulation of *AHR*-mediated pathways through dexamethasone induction has been reported.[46,47] However, another report demonstrated that cell-specific induction of *CYP1* was not related to the expression of *AHR*, *ARNT*, or estrogen receptors (ERs) in multiple human cell lines, namely HepG2 (hepatocellular carcinoma), ACHN (renal carcinoma), A549 (lung carcinoma), MCF-7 (breast carcinoma), LS-180 (colon carcinoma), HT-1197 (bladder carcinoma), HeLa (cervix of uterus adenocarcinoma), OMC-3 (ovarian carcinoma), and NEC14 (testis embryonal carcinoma).[34] In BeWo cells, the expression of *AHR* and *ARNT* mRNAs did not correlate with either *CYP1* expression or EROD activity. This is in contrast to placenta, where *CYP1A1* is transcriptionally upregulated through *AHR*.[40] Several studies have reported reactive oxygen species (ROS) production by PAH activating *Ahr* and inducing *Cyp1* expression, especially in mouse livers.[48-50] However, the expression of *AHR* and *ARNT* mRNAs did not correlate with *CYP1* expression in this study. Whether AhR-independent CYP1A activation is linked to ROS activation in BeWo cells remains to be demonstrated. Additional mechanisms involving transcription factors such as ER, androgen receptor, or nuclear factor-κB (*NF-κB*) could mediate the biological effects of *AHR* on *CYP1*.[51] Differential effects of PAHs have also been observed in JEG-3 and BeWo placental cells.[52] AHR-NF-κB interaction and AHR-ER cross-talk caused the inhibition of CYP1A1 and CYP1B1 proteins in BeWo cells, while CYP1A1 induction was not associated with NF-κB in JEG-3 cells.[52] Therefore, further examination of the other nuclear receptor-mediated pathways that are involved in the regulation of *CYP1* transcription in BeWo cells is required.

PAHs exposure has also been associated with epigenetic alterations, including cytosine methylation, genome instability, and subsequent cancer risk.[53] Hence, the induction of *CYP1A1* and *CYP1A2* and increased EROD activity caused by PM-EtOH and its fractions should be a concern to pregnant women exposed to this plant due to the increased risk to the fetus. Regarding the HPLC results [Figure 1], F6 (the fraction containing puerarin and daidzin) showed less potential to induce the expression of *CYP1A1* and *CYP1A2* mRNAs compared to F2 and F4. However, PM-EtOH and F6 both suppressed the expression of *AHR* mRNA, which is involved in many critical processes during pregnancy, including fetal development.[54]

We previously reported the inhibitory effects of PM on EROD and MROD activities in mouse liver microsomes,[17] an *in vitro* study that has a limitation because it can only reveal inhibitory effects. In rat livers, PM did not affect CYP1A1 and CYP3A but did suppress CYP1A2, CYP2B1/2B2, and CYP2E1.[18] *ABCG2*, also known as the *BCRP*, is highly expressed in placental syncytiotrophoblasts at the apical membrane.[15] To date, the physiological role of *ABCG2* in the placenta is not clear. Recent data suggest that *ABCG2* plays a key role in protecting the fetus against toxic xenobiotics, endogenous substances, drugs, and metabolites by efflux pumping them across the placental barrier.[15] Some flavonoids (e.g., silymarin, hesperetin, quercetin, and daidzein) increased the activity of *BCRP*/*ABCG2* in BCRP-overexpressing cell lines.[55] Miroestrol and deoxymiroestrol, the two potent chromene phytoestrogens in PM, suppressed the expression of *BSEP* (bile salt export pump, *ABCB11*) and *MRP2* (multidrug resistance protein, *ABCC2*) mRNAs in mouse livers.[4] Information about the effect of PM and its constituents on other transporters is still limited. In this study, although miroestrol and deoxymiroestrol were not found in PM-EtOH and its fractions, F2 did induce *ABCG2* expression, which might increase the efflux of xenobiotic(s) from fetus to mother.

CONCLUSION

PM-EtOH, F2, and F4, which contained puerarin, daidzin, isomiroestrol, daidzein, and kwakhurin, significantly induced EROD activity, corresponding to an increase in the expression of *CYP1A1* and *CYP1A2* mRNAs in BeWo cells. However, the induction of *CYP1A* was not dependent on *AHR*/*ARNT*. These findings suggest that the use of PM during pregnancy carries a risk of modified placental *CYP1A* activity.

Acknowledgements

WC sincerely thanks the Faculty of Pharmaceutical Sciences, Khon Kaen University, and Faculty of Medicine, Mahasarakham University, Thailand. The authors thank Dr. Glenn Borlace, Khon Kaen University, for English language assistance.

Financial support and sponsorship

Austrian South-East Asian University Partnership Network for Ernst Mach Grant to work at the Medical University of Vienna, Austria, and the Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology (Grant No. PANPB2561),

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Chatuphonprasert W, Udomsuk L, Monthakantirat O, Churikhit Y, Putalun W, Jarukamjorn K. Effects of *Pueraria mirifica* and miroestrol on the antioxidation-related enzymes in ovariectomized mice. J Pharm Pharmacol 2013;65:447-56.
2. Jearapong N, Chatuphonprasert W, Jarukamjorn K. Miroestrol, a phytoestrogen from *Pueraria mirifica*, improves the antioxidation state in the livers and uteri of β-naphthoflavone-treated mice. J Nat Med 2014;68:173-80.
3. Suthon S, Jaroenporn S, Charoenphandhu N, Suntornsaratoon P, Malaivijitnond S. Anti-osteoporotic effects of *Pueraria candollei* var. mirifica on bone mineral density and histomorphometry in estrogen-deficient rats. J Nat Med 2016;70:225-33.
4. Udomsuk L, Juengwatanatrakul T, Putalun W, Jarukamjorn K. Suppression of BSEP and MRP2 in mouse liver by miroestrol and deoxymiroestrol isolated from *Pueraria candollei*. Phytomedicine 2012;19:1332-5.
5. Sucontphunt A, De-Eknamkul W, Nimmannit U, Dan Dimitrijevich S, Gracy RW. Protection of HT22 neuronal cells against glutamate toxicity mediated by the antioxidant activity of *Pueraria candollei* var. mirifica extracts. J Nat Med 2011;65:1-8.
6. Chattuwatthana T, Okello E. Anti-collagenase, anti-elastase and antioxidant activities of *Pueraria candollei* var. mirifica root extract and *Coccinia grandis* fruit juice extract: An *in vitro* study. Europ J Med Plants 2014;5:318-27.

7. Isoherranen N, Thummel KE. Drug metabolism and transport during pregnancy: How does drug disposition change during pregnancy and what are the mechanisms that cause such changes? Drug Metab Dispos 2013;41:256-62.
8. Hakkola J, Pasanen M, Pelkonen O, Hukkanen J, Evisalmi S, Anttila S, *et al.* Expression of CYP1B1 in human adult and fetal tissues and differential inducibility of CYP1B1 and CYP1A1 by Ah receptor ligands in human placenta and cultured cells. Carcinogenesis 1997;18:391-7.
9. Avery ML, Meek CE, Audus KL. The presence of inducible cytochrome P450 types 1A1 and 1A2 in the BeWo cell line. Placenta 2003;24:45-52.
10. Zanger UM, Schwab M. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 2013;138:103-41.
11. Zhou SF, Wang B, Yang LP, Liu JP. Structure, function, regulation and polymorphism and the clinical significance of human cytochrome P450 1A2. Drug Metab Rev 2010;42:268-354.
12. Kasai A, Hiramatsu N, Hayakawa K, Yao J, Kitamura M. Direct, continuous monitoring of air pollution by transgenic sensor mice responsive to halogenated and polycyclic aromatic hydrocarbons. Environ Health Perspect 2008;116:349-54.
13. Shiizaki K, Kawanishi M, Yagi T. Modulation of benzo[a] pyrene-DNA adduct formation by CYP1 inducer and inhibitor. Genes Environ 2017;39:14.
14. Maliepaard M, Scheffer GL, Faneyte IF, van Gastelen MA, Pijnenborg AC, Schinkel AH, *et al.* Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res 2001;61:3458-64.
15. Mao Q. BCRP/ABCG2 in the placenta: Expression, function and regulation. Pharm Res 2008;25:1244-55.
16. Ebert B, Seidel A, Lampen A. Identification of BCRP as transporter of benzo[a]pyrene conjugates metabolically formed in Caco-2 cells and its induction by Ah-receptor agonists. Carcinogenesis 2005;26:1754-63.
17. Chatuphonprasert W, Jarukamjorn K, Putalun W, Juengwattanatrakul T. Inhibitory potentials of five phytoestrogens from *Pueraria candollei* var. *mirifica* on CYP1A1 and CYP1A2 proteins in mouse liver microsomes and *in silico*. J Appl Biopharm Pharmacokinet 2013;1:5-11.
18. Lawanprasert S, Phivthong-ngam L, Srichairat S, Niwattisaiwong N, Charoenkul K, Chaichantipyut C. Effects of *Pueraria mirifica* subchronic exposure on hepatic cytochrome P450 in rats fed with normal and high cholesterol diets. Thai J Pharmacol 2006;28:21-32.
19. Udomsuk L, Chatuphonprasert W, Monthakantirat O, Churikhit Y, Jarukamjorn K. Impact of *Pueraria candollei* var. *mirifica* and its potent phytoestrogen miroestrol on expression of bone-specific genes in ovariectomized mice. Fitoterapia 2012;83:1687-92.
20. Udomsuk L, Juengwatanatrakul T, Putalun W, Jarukamjorn K. Bimodal action of miroestrol and deoxymiroestrol, phytoestrogens from *Pueraria candollei* var. *mirifica*, on hepatic CYP2B9 and CYP1A2 expressions and antilipid peroxidation in mice. Nutr Res 2012;32:45-51.
21. Chatuphonprasert W, Jarukamjorn K, Putalun W. Regulation of cancer-related genes *Cyp1a1, Cyp1b1, Cyp19, Nqo1* and Comt-Expression in β-naphthoflavone-treated mice by miroestrol. J Pharm Pharmacol 2016;68:475-84.
22. 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.
23. Ellinger I, Schwab M, Stefanescu A, Hunziker W, Fuchs R. IgG transport across trophoblast-derived BeWo cells: A model system to study IgG transport in the placenta. Eur J Immunol 1999;29:733-44.
24. Chatuphonprasert W, Nawaratt N, Jarukamjorn K. Reused palm oil from frying pork or potato induced expression of cytochrome P450s and the SLCO1B1 transporter in HepG2 cells. J Food Biochem 2020;44:e13178.
25. Chatuphonprasert W, Rermraksakul P, Udomsuk L, Lao-ong T, Jarukamjorn K. Different profiles of hepatic alkoxyresorufin O-dealkylase activities in small rodents. J Appl Toxicol 2012;32:1002-7.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2-ΔΔCT method. Methods 2001;25:402-8.
27. Yusakul G, Udomsin O, Tanaka H, Morimoto S, Juengwatanatrakul T, Putalun W. Enzyme-linked immunosorbent assay by enhanced chemiluminescence detection for the standardization of estrogenic miroestrol in *Pueraria candollei* Graham ex Benth. Luminescence 2015;30:568-75.
28. Shimokawa S, Kumamoto T, Ishikawa T, Takashi M, Higuchi Y, Chaichantipyuth C, *et al.* Quantitative analysis of miroestrol and kwakhurin for standardisation of Thai miracle herb 'Kwao Keur' (*Pueraria mirifica*) and establishment of simple isolation procedure for highly estrogenic miroestrol and deoxymiroestrol. Nat Prod Res 2013;27:371-8.
29. Shen JG, Yao MF, Chen XC, Feng YF, Ye YH, Tong ZH. Effects of puerarin on receptor for advanced glycation end products in nephridial tissue of streptozotocin-induced diabetic rats. Mol Biol Rep 2009;36:2229.
30. Teng Y, Cui H, Yang M, Song H, Zhang Q, Su Y, *et al.* Protective effect of puerarin on diabetic retinopathy in rats. Mol Biol Rep 2009;36:1129-33.
31. 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.
32. Le Vee M, Kolasa E, Jouan E, Collet N, Fardel O. Differentiation of human placental BeWo cells by the environmental contaminant benzo(a)pyrene. Chem Biol Interact 2014;210:1-1.
33. Santodonato J, Howard P, Basu D. Health and ecological assessment of polynuclear aromatic hydrocarbons. J Environ Pathol Toxicol 1981;5:1-364.
34. Iwanari M, Nakajima M, Kizu R, Hayakawa K, Yokoi T. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: Chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch Toxicol 2002;76:287-98.
35. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J Biol Chem 2004;279:23847-50.
36. Sinha A, Muthiah K, Jiang W, Couroucli X, Barrios R, Moorthy B. Attenuation of hyperoxic lung injury by the CYP1A inducer β–naphthoflavone. Toxicol Sci 2005;87:204-12.
37. Chatuphonprasert W, Jarukamjorn K, Kondo S, Nemoto N. Synergistic increases of metabolism and oxidation-reduction genes on their expression after combined treatment with a CYP1A inducer and andrographolide. Chem Biol Interact 2009;182:233-8.
38. Lemaire P, Förlin L, Livingstone DR. Responses of hepatic biotransformation and antioxidant enzymes to CYP1A-inducers (3-methylcholanthrene, β-naphthoflavone) in sea bass (*Dicentrarchus labrax*), dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 1996;36:141-60.
39. Detmar J, Rennie MY, Whiteley KJ, Qu D, Taniuchi Y, Shang X, *et al.* Fetal growth restriction triggered by polycyclic aromatic hydrocarbons is associated with altered placental vasculature and AhR-dependent changes in cell death. Am J Physiol Endocrinol Metab 2008;295:E519-30.
40. Stejskalova L, Pavek P. The function of cytochrome P450 1A1 enzyme (CYP1A1) and aryl hydrocarbon receptor (AhR) in the placenta. Curr Pharm Biotechnol 2011;12:715-30.
41. Bruchova H, Vasikova A, Merkerova M, Milcova A, Topinka J, Balascak I, *et al.* Effect of maternal tobacco smoke exposure on the placental transcriptome. Placenta 2010;31:186-91.
42. Shimada T, Yamazaki H, Foroozesh M, Hopkins NE, Alworth WL, Guengerich FP. Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1. Chem Res Toxicol 1998;11:1048-56.
43. Ono S, Hatanaka T, Hotta H, Satoh T, Gonzalez FJ, Tsutsui M. Specificity of substrate and inhibitor probes for cytochrome P450s: Evaluation of *in vitro* metabolism using cDNA-expressed human P450s and human liver microsomes. Xenobiotica 1996;26:681-93.
44. Liu J, Wang Q, Wu DC, Wang XW, Sun Y, Chen XY, *et al.* Differential regulation of CYP1A1 and CYP1B1 expression in resveratrol-treated human medulloblastoma cells. Neurosci Lett 2004;363:257-61.
45. Koskela S, Hakkola J, Hukkanen J, Pelkonen O, Sorri M, Saranen A, *et al.* Expression of CYP2A genes in human liver and extrahepatic tissues. Biochem Pharmacol 1999;57:1407-13.
46. Mathis JM, Prough RA, Hines RN, Bresnick E, Simpson ER. Regulation of cytochrome P-450c by glucocorticoids and polycyclic aromatic hydrocarbons in cultured fetal rat hepatocytes. Arch Biochem Biophys 1986;246:439-48.
47. Prough RA, Linder MW, Pinaire JA, Xiao GH, Falkner KC. Hormonal regulation of hepatic enzymes involved in foreign compound metabolism. FASEB J 1996;10:1369-77.
48. Anandasadagopan SK, Singh NM, Raza H, Bansal S, Selvaraj V, Singh S, *et al.* β-Naphthoflavone-induced mitochondrial respiratory damage in Cyp1 knockout mouse and in cell culture systems: Attenuation by resveratrol treatment. Oxid Med Cell Longev 2017;2017. Available from: https://doi.org/10.1155/2017/5213186
49. Senft AP, Dalton TP, Nebert DW, Genter MB, Puga A, Hutchinson RJ, *et al.* Mitochondrial reactive oxygen production is dependent on the aromatic hydrocarbon receptor. Free Radic Biol Med 2002;33:1268-78.
50. Senft AP, Dalton TP, Nebert DW, Genter MB, Hutchinson RJ, Shertzer HG. Dioxin increases reactive oxygen production in mouse liver mitochondria. Toxicol Appl Pharmacol 2002;178:15-21.
51. Ohtake F, Baba A, Fujii-Kuriyama Y, Kato S. Intrinsic AhR function underlies cross-talk of

dioxins with sex hormone signalings. Biochem Biophys Res Commun 2008;370:541-6.

52. Drwal E, Rak A, Gregoraszczuk EL. Differential effects of ambient PAH mixtures on cellular and steroidogenic properties of placental JEG-3 and BeWo cells. Reprod Toxicol 2019;86:14-22.
53. Herbstman JB, Tang D, Zhu D, Qu L, Sjödin A, Li Z, *et al*. Prenatal exposure to polycyclic aromatic hydrocarbons, benzo[a]pyrene-DNA adducts, and genomic DNA methylation in cord blood. Environ Health Perspect 2012;120:733-8.
54. Jiang YZ, Wang K, Fang R, Zheng J. Expression of aryl hydrocarbon receptor in human placentas and fetal tissues. J Histochem Cytochem 2010;58:679-85.
55. Cooray HC, Janvilisri T, van Veen HW, Hladky SB, Barrand MA. Interaction of the breast cancer resistance protein with plant polyphenols. Biochem Biophys Res Commun 2004;317:269-75.