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Research Article
Plumbagin and *Plumbago indica* Differentially Modulated Cytochrome P450 and Transporter Profiles in BeWo and HepG2 Cells

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Abstract

Background and Objective: The medicinal herb *Plumbago indica* (PI) and its major constituent plumbagin have reported pharmacological properties but there is a lack of information about their herb-drug interactions. The effects of methanolic (PI-MeOH) and ethanolic (PI-EtOH) crude extracts of PI and plumbagin on the expression of cytochrome P450s (*CYP1A2*, *CYP2E1* and *CYP3A4*) and transporters (*ABCC1*, *ABCG2* and *SLC22A11*) were investigated in BeWo and HepG2 cells. **Materials and Methods:** BeWo or HepG2 cells were treated with 0.5-5 μM plumbagin or 25-500 μg mL^{-1} of PI-MeOH or PI-EtOH for 24 hrs. Total RNA was extracted and mRNA expression of CYPs and transporters were determined using RT-qPCR. **Results:** PI and plumbagin affected mRNA expression differently in the two tested cell types. In BeWo cells, all concentrations of PI-MeOH induced *CYP2E1*, 100 and 500 μg Ml^{-1} PI-MeOH and PI-EtOH up-regulated *CYP1A2*, *CYP3A4* and *ABCG2* and 500 μg mL^{-1} PI-EtOH induced *ABCG2* expression. Plumbagin suppressed *CYP1A2* and induced *SLC22A11* expression at the highest concentration, 5 μM. In HepG2 cells, 5 μM plumbagin and 500 μg Ml^{-1} PI-EtOH suppressed *CYP3A4* expression and 500 μg mL^{-1} PI-MeOH and PI-EtOH up-regulated *CYP1A2* and *CYP2E1* expression. *ABCC1* expression was induced by all treatments while *ABCG2* and *SLC22A11* were induced only by 500 μg mL^{-1} PI-MeOH and PI-EtOH. **Conclusion:** The use of PI or plumbagin supplements in large quantities or for long periods should be carefully considered due to the risk of herbal drug interactions via modulated expression of CYPs and transporters.

Key words: Herbal drug interaction, CYPs, metabolising enzymes, drug transporters, naphthoquinone, plumbagin, cytochrome

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plumbago indica Linn. (PI, family Plumbaginaceae) is a shrub widely distributed in Africa, Australia and Asia. The active component of PI is Plumbagin, a naphthoquinone isolated from the roots[1]. PI and plumbagin exhibit various pharmacological properties including activities against bacteria, influenza and cancer[1-3]. PI is an ingredient in Thai traditional and applied Thai traditional recipes for the treatment of dizziness, flatulence, colic, amenorrhea and help excretion of lochia or amniotic fluid after giving birth[4].

Cytochrome P450 (CYP) is a superfamily of metabolising enzymes abundantly expressed in the liver[5]. Human *CYP1A2*, *CYP2E1* and *CYP3A4* mediate the biotransformation of 42% of clinical drugs[6]. Various metabolising CYP enzymes are also present in the placenta. Not only CYPs but also transporters play an essential role in the disposition and effects of multiple drugs[7]. ATP Binding Cassette (ABC) transporters are crucial determinants of drug disposition in several organs which might be a major cause of drug resistance[8]. *ABCC1* encoded multidrug-resistant proteins transporters 1 (MRP1) and *ABCG2* encoded Breast Cancer Resistance Protein (BCRP) is constitutively expressed in the placenta and liver[9]. Solute carrier family 22 member 11 (*SLC22A11*), also known as an Organic Anion Transporter 4 (OAT4), mediates drug transport[10,11] and is expressed in kidneys and placenta.

Alteration of CYP expression by drug and/or supplement might consequently cause either more or less effectiveness to the co-administered drug(s). However, information regarding any impact of PI and plumbagin on the expression profile of CYPs and transporters is very limited. PI and plumbagin induced mRNA expression of hepatic *CYP1A2* and *CYP2E1* and lung *Cyp2f2* while suppressed hepatic *Cyp2c29* and *Cyp3a11/13* in mice[12,13]. HepG2 and BeWo are well-known *in vitro* models for human livers and placenta, respectively[14,15].

This study aims to investigate the effects of PI and plumbagin on the mRNA expression of selected CYPs and transporters in BeWo and HepG2 cells which are *in vitro* models of human placenta and liver.

MATERIALS AND METHODS

Study area: The study was carried out at the Research Group for Pharmaceutical Activities of Natural Products using Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand during July, 2019-March, 2020 and at the Pathophysiology of the Placenta Group, Institute for Pathophysiology and Allergy Research at the Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria during April-June, 2019.

Chemicals and reagents: Plumbagin was a product of LKT Laboratories (St. Paul, Minnesota, USA). A Lactate Dehydrogenase (LDH) detection kit was supplied by Sigma-Aldrich Chemical (St. Louis, Missouri, USA). ReverTraAce®, Thunderbird™ Probe qPCR Mix and other reagents for RT/qPCR were products of Toyobo Co., Ltd. (Osaka, Japan). TaqMan™ gene expression assays were products of Applied Biosystem™ (Waltham, Massachusetts, USA). Other laboratory chemicals and materials were provided by commercial suppliers with analytical or molecular grade.

***P. indica* extraction:** The root of PI was bought from Mor Tong-In Thai Traditional Medicine (Maha Sarakham, Thailand) in May, 2018. The plant materials were identified by Ms. Pornpimon Wongsuwan, a botanist, Faculty of Medicine, Mahasarakham University (the reference specimen no. PANPB-PI 2017-002). The PI root was dried at 50°C in an oven and then shredded and subjected to Soxhlet extraction with methanol (PI-MeOH) or ethanol (PI-EtOH) for 3 hrs, followed by evaporation of the solvent and freeze-drying into powder. Plumbagin content was determined using HPLC-UV[12]. Contents of chemical markers including total phenolic, total flavonoid, anthocyanin and tannin contributions were analysed according to the standard protocols[16].

Cell culture: BeWo and HepG2 cells were cultured in standard media and supplements[16,17] and seeded in 6 well plates (5×10^5 cells/well) for 24 hrs before treatment. The cells (every five replicates) were incubated with 0.2% dimethyl sulfoxide (DMSO, control), 5 µM ketoconazole (Keto) or rifampicin (Rifam), 0.5, 1 and 5 µM plumbagin, 25, 50, 100 and 500 µg mL^{-1} PI-MeOH or PI-EtOH in phenol red free medium for further 24 hrs. At 24 hrs after treatments, total RNA was isolated using the guanidinium thiocyanate-phenol-chloroform method[13]. Toxicity of treatment was assessed via LDH activity[16].

RT-qPCR: Total RNA was reverse transcribed using ReverTraAce® kit (Toyobo Co., Ltd., Osaka, Japan). Expression of *CYP1A2* (Hs00167927_m1), *CYP2E1* (Hs00559370_m1), *CYP3A4* (Hs00604506_m1), *ABCC1* (Hs02514106_s1),

ABCG2 (Hs01053790_m1) and *SLC22A11* (Hs00945829_m1) mRNAs were determined using RT-qPCR and normalized to reference genes, beta-actin (*ACTB*, Hs03023943_g1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02786624_g1), using the probe-primers of TaqMan™ gene expression assays (Applied Biosystem™, Waltham, Massachusetts, USA) with Thunderbird™ reagents (Toyobo Co., Ltd., Osaka, Japan). The relative fold expression was calculated using the delta-delta C_t method[17].

Statistical analysis: The results are expressed as mean±SD (n = five per group) and were analysed using one way analysis of variance (ANOVA) followed by Tukey's *post hoc* test (SPSS version 23, Chicago, IL, USA). $p \leq 0.05$ was considered as statistically significant.

RESULTS

Contents of plumbagin and chemical markers in the PI crude extracts and LDH toxicity: The data of Table 1 shows the contents of plumbagin and chemical markers in the PI crude extracts. Methanolic extract (PI-MeOH) revealed a higher yield (%) while ethanolic extract (PI-EtOH) contained slightly more plumbagin, phenolics, flavonoids and tannin. After 24 hrs of treatment, Keto and Rifam significantly increased LDH levels in BeWo and HepG2 cells (Fig. 1). Other treatments did not increase LDH level by more than 15 %, except plumbagin at 5 µM. Hence, the study was employed these concentration ranges.

Effects of plumbagin and PI extract on the mRNA expression of CYPs: Keto extensively induced expression of *CYP1A2* and *CYP3A4* but not *CYP2E1* in BeWo cells (Fig. 2a) and induced all tested CYPs (*CYP1A2, CYP2E1 and CYP3A4*) in HepG2 cells (Fig. 2b). Rifam induced only *CYP3A4* in both BeWo and HepG2 cells. The highest concentration of plumbagin (5 µM) suppressed the expression of *CYP1A2* in BeWo cells and *CYP3A4* in HepG2 cells. In BeWo cells, all tested concentrations of PI-MeOH significantly up-regulated *CYP2E1* expression while *CYP1A2* and *CYP3A4* expression was only induced by the higher tested concentrations of PI-MeOH. The highest concentration of PI-EtOH induced all tested CYPs (*CYP1A2, CYP2E1 and CYP3A4*) in BeWo cells but suppressed *CYP3A4* expression in HepG2 cells. The highest concentrations of both extracts induced *CYP1A2* and *CYP2E1* expression in HepG2 cells.

Effects of plumbagin and PI extract on the mRNA expression of drug transporters: Keto suppressed *SLC22A11* expression and Rifam suppressed *ABCC1*, *ABCG2* and *SLC22A11* expression in BeWo cells (Fig. 3a). The inverse effect was noted in HepG2 cells (Fig. 3b), Keto induced expression of all drug transporters and Rifam induced expression of *SLC22A11*. High concentrations of PI-MeOH and PI-EtOH up-regulated *ABCG2* expression in BeWo cells, whereas, in HepG2 cells, *ABCC1* expression was induced by all treatments including plumbagin and both extracts. The highest concentrations of PI-MeOH and PI-EtOH up-regulated expression of all tested drug transporters in HepG2 cells.

Table 1: Plumbagin and chemical marker contents in the *P. indica* extracts

Contents	PI-MeOH	PI-EtOH
Yield (%)	32.23±3.21	12.86±1.76
Plumbagin (% d. wt.)	0.15±0.03	0.21±0.14
Total phenolic (mg g^{-1} d. wt.)	0.97±0.06	1.62±0.30
Total flavonoids (mg g^{-1} d. wt.)	5.35±0.93	5.95±0.64
Anthocyanin (mg g^{-1} d. wt.)	0.45±14.75	0.39±0.00
Tannin contribution (%)	2.06±0.05	2.22±0.03

PI-MeOH: *P. indica* crude extract with methanol, PI-EtOH: *P. indica* crude extract with ethanol and d. wt.: Dry weight

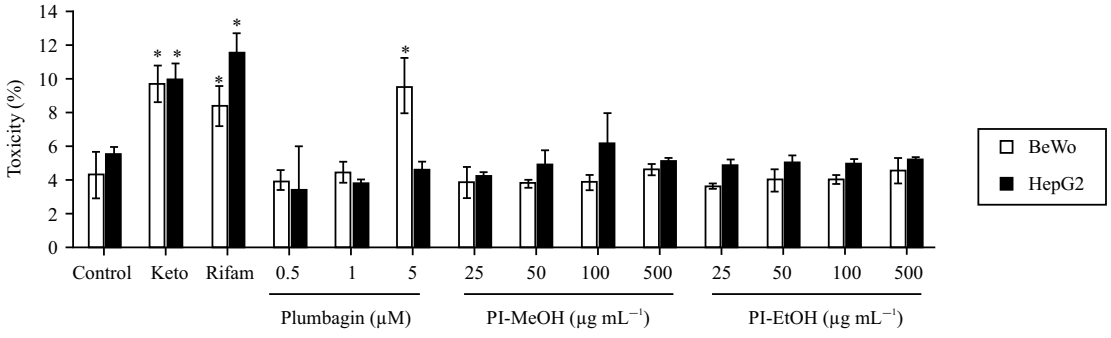

Fig. 1: Toxicity of treatments for 24 hrs using lactate dehydrogenase (LDH) assay

*p<0.05, vs. control

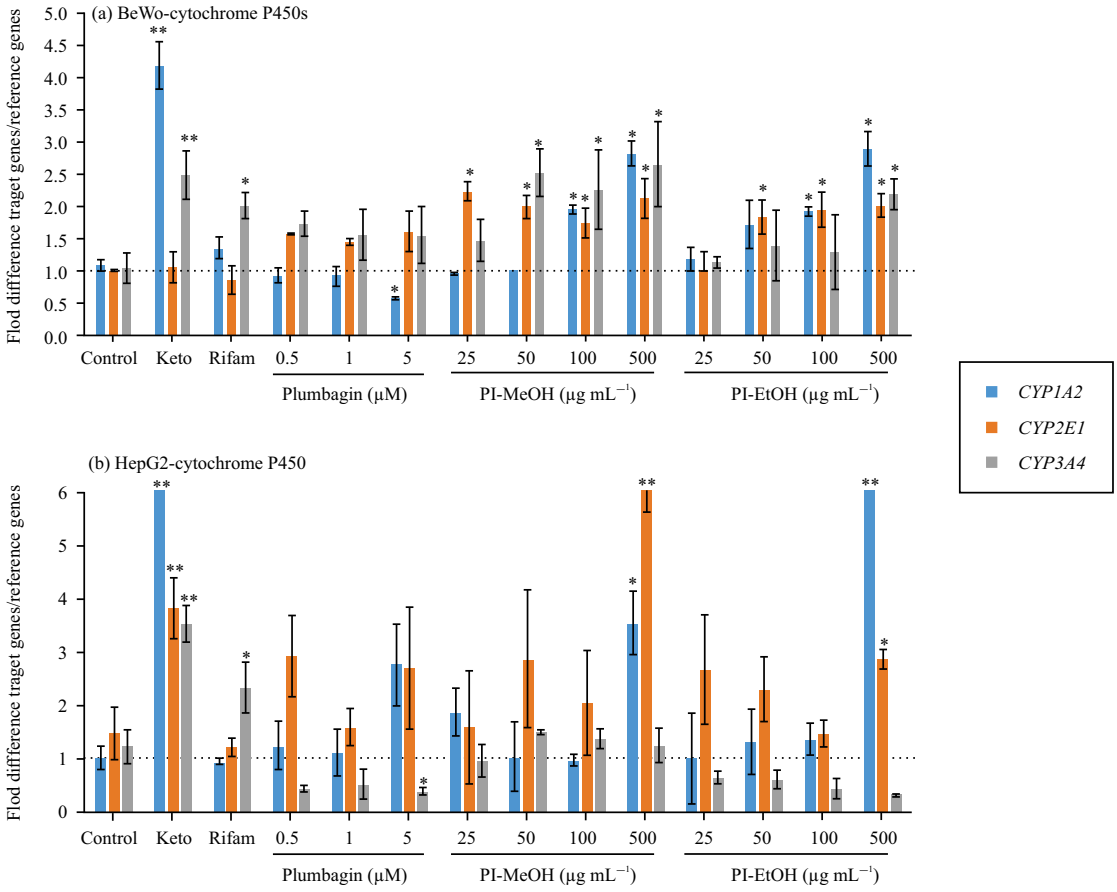

Fig. 2(a-b): Expressions of *CYP1A2*, *CYP2E1* and *CYP3A4*
(a) BeWo and (b) HepG2, *p<0.05, **p<0.001 vs. control

DISCUSSION

In this study, Keto and Rifam were employed as positive controls because they have been marked as a high alert for drug interaction[18]. Human *CYP1A2* is constitutively expressed in the liver[6] and is responsible for the metabolism of anti-depressants and anti-psychotics as well as anti-inflammatory, anaesthetic and analgesic drugs[19]. Induction of *CYP1A2* expression by high concentrations of both PI-MeOH and PI-EtOH in BeWo and HepG2 cells might influence the metabolism of those substrate drugs creating a risk of herb-drug interaction. Similarly, high concentration plumbagin suppressed *CYP1A2* expression in BeWo cells, which could reduce the metabolism of *CYP1A2* substrates leading to drug concentrations that are over the therapeutic range and result in more adverse effects and toxicity.

CYP2E1 is the main enzyme associated with oxidative stress, up-regulation of *CYP2E1* contributes to the generation of free radicals, leading to lipid peroxidation and mitochondrial damage[6]. Induction of *CYP2E1* expression in either BeWo or HepG2 cells by high concentrations of PI-MeOH and PI-EtOH might lead to negative effects and disadvantages such as an increased risk of paracetamol or ethanol toxicity[20]. Correspondingly, *CYP1A2* and *CYP2E1* expression were found to be induced by PI-MeOH and plumbagin in mouse livers[12].

CYP3A4 metabolises a large and diverse range of molecules that includes over 50% of clinical drugs including bronchodilators and antiviral, antibacterial, antifungal, lipid-lowering and anti-hypertensive drugs[6]. Expression of *CYP3A4* is normally at nominal levels in the placenta compared to the liver, which might support the different effects of PI and plumbagin in BeWo (placental) and HepG2 (hepatic) cells in the current study[21]. Induction of *CYP3A4* expression in BeWo cells by PI-MeOH and PI-EtOH might lessen the levels of co-administered drugs in the placenta. On the contrary, *CYP3A4* suppression by plumbagin and PI-EtOH in HepG2 cells could increase hepatic concentrations of co-administered drugs.

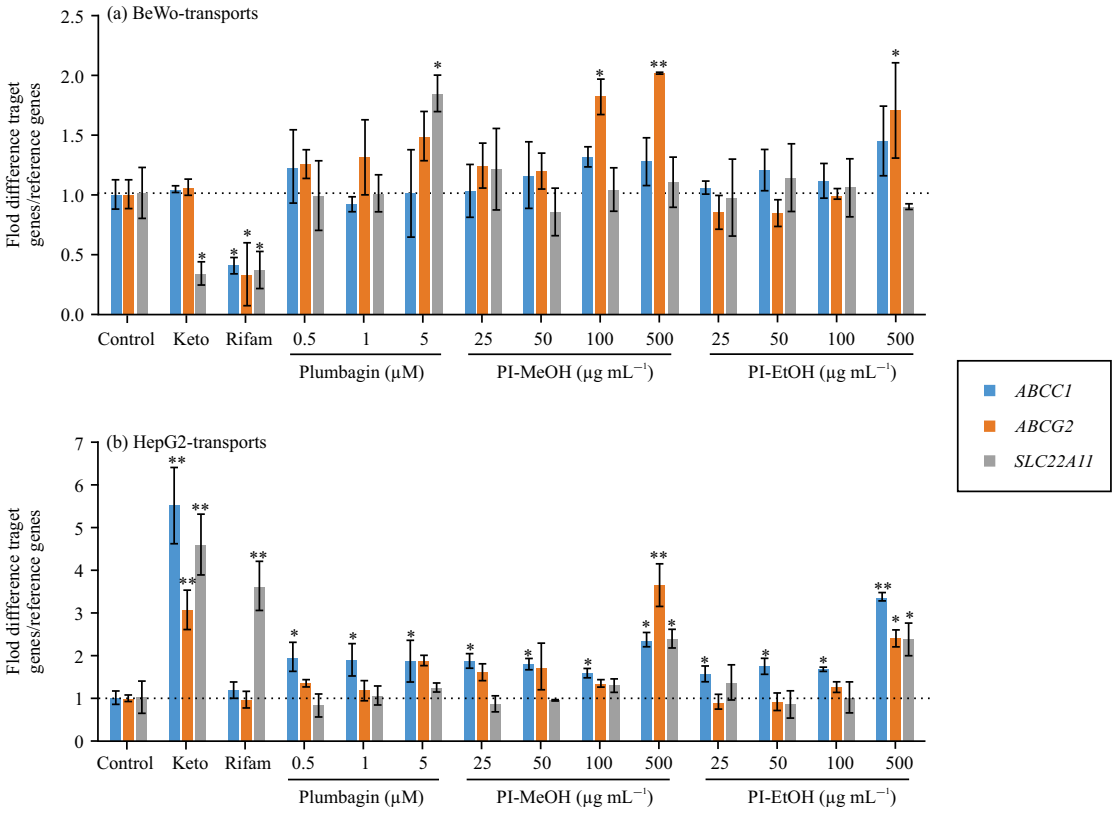

Fig. 3(a-b): Expressions of *ABCC1*, *ABCG2* and *SLC22A11*
(a) BeWo and (b) HepG2, *p<0.05, **p<0.001 vs. control

ABCC1 and *ABCG2* play a key role in protecting the foetus against endogenous substances, drugs, xenobiotics and metabolites by efflux pumping them across the placental barrier and expression of these transporters is a critical determinant for chemo-resistance in hepatocellular carcinoma[9,22]. Some polyphenols from herbal plants have been shown to inhibit the expression of *ABCC1* and *ABCG2*[23]. However, in the current study, plumbagin, PI-MeOH and PI-EtOH induced *ABCC1* expression in HepG2 cells and *ABCG2* expression in BeWo cells, particularly at high concentrations. This might cause herb-drug interactions by an increase in efflux pumping of co-administered drugs, leading to treatment failure. *SLC22A11* or OAT4, is involved in the uptake-transportation of drugs into placental syncytiotrophoblasts while its hepatic expression is nominal[11,24]. Plumbagin and PI did not change *SLC22A11* expression in BeWo cells but high concentrations of PI-MeOH and PI-EtOH up-regulated expression of *SLC22A11* in HepG2 cells. Currently, it is unclear how *SLC22A11* responds in either the liver or HepG2 cells[10].

In previous studies in mice, both PI and plumbagin induced mRNA expression of hepatic *CYP1A2* and *CYP2E1* and lung *Cyp2f2* while suppressing hepatic *Cyp2c29* and *Cyp3a11/13*[12,13]. The current findings demonstrate that the modulatory impact of crude extract of *P. indica* on CYPs and transporters was not dependent solely on plumbagin content. Other phenolic compounds, flavonoids and tannins present in PI might also cause herb-drug interaction[25]. The effects of plumbagin and *P. indica* on CYPs and transporters should be investigated in placental and hepatic cell models as prolonged consumption of either plumbagin or *P. indica* in large amounts runs the risk of previously unidentified herb-drug interactions.

CONCLUSION

PI induced *CYP1A2*, *CYP2E1* and *CYP3A4* expression in BeWo and HepG2 cells but suppressed *CYP3A4* expression in HepG2 cells. PI and plumbagin induced *ABCC1, ABCG2* and *SLC22A11* expression in both BeWo and HepG2 cells. Hence, the use of PI and/or plumbagin containing supplements in high quantities or for long periods is a risk for herb-drug interactions via altered regulatory expression of these important CYPs and drug transporters.

SIGNIFICANCE STATEMENT

This study revealed the potentials of plumbagin or *P. indica* to modify the expression profiles of CYPs and transporters in BeWo and HepG2 models. These might be a beneficial impact, in terms of precaution on herbal drug interaction, for further development of plumbagin or *P. indica* as herbal supplements.

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