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Screening for inhibitory effect on nine CYP isoforms by 20 herbal medications

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We evaluated the potential of 20 herbal medications (HMs), commonly used in Korea, to inhibit the catalytic activities of several cytochrome P450 (CYP) isoforms. The abilities of 500 μg/ml of aqueous extracts of 20 HMs to inhibit phenacetin *O*-deethylation (CYP1A2), coumarin 6-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), rosiglitazone hydroxylation (CYP2C8), tolbutamide 4-methylhydroxylation (CYP2C9), *S*-mephenytoin 4'-hydroxylation (CYP2C19), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and midazolam 1'-hydroxylation (CYP3A) were tested using human liver microsomes. The HMs Woohwangcheongsimwon suspension and Hwanglyeonhaedok-Tang strongly inhibited CYP2B6 and CYP2D6 isoform activity, respectively. These results suggest that some of the HMs used in Korea have potential to inhibit CYP isoforms in vitro. Although the plasma concentrations of the active constituents of the HMs were not determined, some herbs could cause clinically significant interactions because the usual doses of those individual herbs are several grams of freeze-dried extracts.

Key words – Cytochrome P450, herbal medications, inhibition, drug interaction

Introduction

Herbal medicines have received great attention as an alternative to clinical therapy, and the consumption of herbal medicines in Asia, North America, and European countries has increased dramatically in recent years [2,5,16]. These herbs may be used either in their primary forms or combined into mixtures. Herbal medicines are largely unregulated, and many patients do not inform their physicians of the supplements they consume. Therefore, herb-drug interactions involving components of herbal medicines and clinically prescribed drugs present an increasing concern [2,4]. Herbal extracts usually contain a number of pharmacologically active constituents, including essential oils, tannins, coumarins, saponins, glycosides, flavonoids, terpenoids, anthraquinones, polyphenols, and alkaloids, all of which may potentially participate in herb-drug interactions [14,18]. Several medicinal herbs have been reported to cause herb-drug interactions, including St. John's wort (*Hypericum perforatum*), garlic (*Allium sativum*), licorice (*Glycyrrhiza glabra*), and ginseng (*Panax ginseng*) [9,18].

Cytochrome P450 (CYP) is a representative enzyme involved in hepatic drug metabolism, which is crucial for the elimination of many therapeutic drugs. Among the numerous CYP enzymes identified to date, nine human hepatic CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A) have been shown to a play dominant role in the metabolism of drugs and other xenobiotics [4,18]. Specially, CYP3A is the most important enzyme and is involved in the majority of the CYP-catalyzed metabolism [3]. Studies have shown that St. John's wort, an herbal medicine used to treat mild depression, is reported to decrease the blood concentrations of drugs by inducing hepatic CYP3A activity, thereby attenuating the efficacy of drugs such as cyclosporin, digoxin, indinavir, and simvastatin [6,10].

In Korea, patients frequently take both herbal medications (HMs) and conventional medicines without noticing any drug interactions. However, there are few reports that describe the possible interaction of therapeutic medicines and HMs prescribed commonly in Korea. Therefore, the effect of herbal medications on cytochrome P450 enzymes should be evaluated to avoid harmful drug interactions. In this study, we evaluated the potential of 20 HMs, frequently taken as over-the-counter drugs in Korea, to inhibit the catalytic activities of several CYP isoforms in order to assess the probability of herb-drug interactions.

Materials and Methods

Materials

Acetaminophen, bupropion, chlorpropamide, chlorzox-

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azone, coumarin, dextromethorphan, dextrorphan, 7-hydroxycoumarin, phenacetin, tolbutamide, β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (H161) were obtained from BD Gentest Co. (Woburn, MA). Rosiglitazone was kindly provided by GlaxoSmith Kline (Hertfordshire, UK). *S*-Mephenytoin, 4-hydroxymephenytoin, 6-hydroxychlorzoxazone, 1'-hydroxymidazolam, and midazolam were purchased from Ultrafine Chemical Co. (Manchester, UK). All other chemicals and solvents were of the highest grade available.

Preparations of herbal medications

Herbal medications were obtained from local pharmacy, and their available information is shown in Table 1. The herbal medications were extracted with distilled water. The concentration was calculated based on the extracts weight written on the each product sheets.

Enzyme assay

All incubations were performed in duplicate, and the mean values were used for analysis. The assays of phenacetin *O*-deethylase, coumarin 7-hydroxylase, bupropion 4-hydroxylase, rosiglitazone para-hydroxylase, tolbutamide 4-hydroxylase, *S*-mephenytoin 4-hydroxylase, dextromethorphan *O*-demethylase, chlorzoxazone 6-hydroxylase, and midazolam 1'-hydroxylase activities were determined as probe activities for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A, respectively, using cocktail incubation and tandem mass spectrometry, as described previously [11]. Briefly, incubation reaction was performed with 0.25 mg/ml human liver microsomes in a final incubation volume of 0.25 ml. The incubation medium contained 100 mM phosphate buffer (pH 7.4) with probe substrates. The incubation mixture containing various inhibitors (herbal extracts, 500 μg/ml) was pre-incubated for 10 min. After pre-incubation, an NADPH-generating system (including 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 1.0 unit/ml glucose-6-phosphate dehydrogenase) was added. After incubation at 37℃ for 15 min, the reaction was stopped by placing the incubation tubes on ice and adding 100 μl of ice-cold acetonitrile. The incubation mixtures were then centrifuged at 10,000×*g* for 5 min at 4℃. Aliquots of the supernatant were injected onto an LC/MS/MS system.

Table 1. The list of herbal medications used in this study

Number	Herbal medication /Product name	Pharmaceutical company
1	Galgeun-tang	Han-Zung
2	Bangpungtongseong-san	Han-Poong
3	Samso-eum	Han-Zung
4	Sosiho-tang	Han-Zung
5	Ssanghwa-tang	Han-Poong
6	Ojeok-san	Han-Poong
7	Yongdamsagan-tang	Han-Zung
8	Injinho-tang	Han-Zung
9	Palmi-won	Han-Zung
10	Pyeongwi-san	Han-Zung
11	Hwanglyeonhaedok-tang	Han-Zung
12	Socheongryong-tang	Ki-Wha
13	Banhasasim-tang	Ki-Wha
14	Baenongsan-geup-tang	Ki-Wha
15	Eun-gyo-san	Il-Sim
16	Woohwangcheongsim-won	Kwang-Dong
17	Galgeuntang-gacheongungsini	Ki-Wha
18	Geopungjibo-dan	Wales Korea
19	Cheonwangbosim-dan	Oriental
20	Yeolla-hwan	Il-Sim

LC/MS/MS analysis

The samples were analyzed using liquid chromatography-mass spectrometry with an QTrap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA) equipped with an electrospray ionization interface used to generate positive $[M+H]^+$ and negative ions $[M-H]^-$. Aliquots (5 μl) were injected onto a reverse-phase column (Luna C_{18}, 2.0 × 30 mm i.d., 5 μm particle size; Phenomenex, Torrance, CA) maintained at 40℃. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The mobile phase A was linearly increased from 10 to 80% over 0.1 min, held at 80% for an additional 2.5 min, and then immediately stepped back down to 10% for re-equilibration. The total run time was 6.5 min. After 0.6 min, the LC eluent was diverted from waste to the ion source of the mass spectrometer. The mobile phase was eluted using an HP 1100 series pump (Agilent, Wilmington, DE) at 0.2 ml/min.

The turboion-spray interface was operated in the positive ion mode at 5000 V and in the negative ion mode at -4000 V. The operating conditions were determined as follows: ion source temperature, 400℃; nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 80 V; ring voltage 400 V; colli-

sion gas (nitrogen) pressure, 3.58×10^{-5} Torr. Quantitation was performed by multiple reaction monitoring (MRM) of the $[M+H]^+$ ion and the related product ion for each metabolite, using an internal standard to establish peak area ratios. The MRM transitions and collision energies determined for each metabolite and internal standard are listed in Table 2. Quadrupoles Q1 and Q3 were set at unit resolution. The analytical data were processed by Analyst software (version 1.4, Applied Biosystems, Foster City, CA).

Data analysis

The CYP-mediated activities in the presence of inhibitors were expressed as percentages of the corresponding control values.

Results and Discussion

There have been some difficulties when patients are guided in taking medicines with herbal preparations concomitantly, since there have been rare information on herb-drug interactions arisen from drug metabolism or absorption. The herbal preparations are simply taken as over-the-counter in Asian countries such as China and Japan. In Korea, many patients also frequently take both HMs and conventional medicines without noticing any drug interactions. However, for the safe use of herbal medicines, it is important to clarify any CYP-mediated interactions between herbal medicines and drugs. To address this issue, the effects of aqueous extracts of 20 herbal medications, commonly used in Korea, on the catalytic activities of several CYP isoforms were examined. We didn't consider methanolic or ethanolic extracts of HMs because aqueous extracts are only commonly used herbal formulation for medication in Korea.

Focusing on drug metabolism-based drug interactions, in the present study, we screened an inhibitory effect of 20 kinds of herbal preparations (500 μg/ml) on nine CYP isoforms activities as a tool for assessing its clinical significances in herb-drug interaction. Most of herbal preparations didn't show significant inhibitory effect on CYP activities (Fig. 1). Two herbal preparations, Woohwangcheongsimwon suspension and Hwanglyeonhaedok-Tang, however showed potent inhibitory effect on CYP2B6 and CYP2D6 isoform activity, respectively.

Woohwangcheongsimwon suspension is one of the most widely used traditional Chinese prescription for the treatment of the emergency and acute treatment of stroke, numbness, hypertension, epilepsy and arteriosclerosis [12]. It strongly inhibited the metabolic activity of CYP2B6- catalyzed bupropion hydroxylase activity (Fig. 2). This in vitro data suggest that in vivo interaction studies of Woohwangcheongsimwon suspension remain to be further evaluated to rule out the possible inhibitory potential of Woohwangcheongsimwon suspension with CYP2B6 substrates such as bupropion [7] and efavirenz [17]. Woohwangcheongsimwon suspension had no effect on the activities of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A. These findings suggest that clinical interactions between Woohwangcheongsimwon suspension and these P450s would not be expected.

Hwanglyeonhaedok-tang is used as a therapy for various clinical symptoms associated with gastrointestinal disorders, inflammation, and cardiovascular diseases [8]. It potently inhibited the metabolic activity of CYP2D6-catalyzed dextromethorphan *O*-demethylation to 16% of control activity. This observation is supported by the in vitro drug interaction studies of berberine [1] which showed that berberine inhibits CYP2D6-catalyzed bufuralol 1'-hydroxylation. Berberine, a plant alkaloid, is purified chemical from Coptidis rhizoma and Phellodendri cortex, major con-

Table 2. MRM parameters for the major metabolites of the nine P450 probe substrates used in all the assays

P450 enzyme	Substrate	Concentration (μM)	Metabolite	Transition (*m/z*)	Polarity	Collision energy (*eV*)
1A2	Phenacetin	50	Acetaminophen	152 > 120	ES^+	25
2A6	Coumarin	5	7-Hydroxycoumarin	163 > 107	ES^+	35
2B6	Bupropion	50	Hydroxybupropion	256 > 238	ES^+	25
2C8	Rosiglitazone	10	p-Hydroxyrosiglitazone	374 > 151	ES^+	25
2C9	Tolbutamide	100	4-Hydroxytolbutamide	287 > 89	ES^+	60
2C19	*S*-Mephenytoin	100	4'-Hydroxymephenytoin	235 > 150	ES^+	25
2D6	Dextromethorphan	5	Dextrorphan	258 > 157	ES^+	60
2E1	Chlorzoxazone	50	6-Hydroxychlorzoxazone	184 > 120	ES^-	25
3A4	Midazolam	5	1-Hydroxymidazolm	342 > 203	ES^+	25
IS	Chlorpropamide			277 > 175	ES^+	25

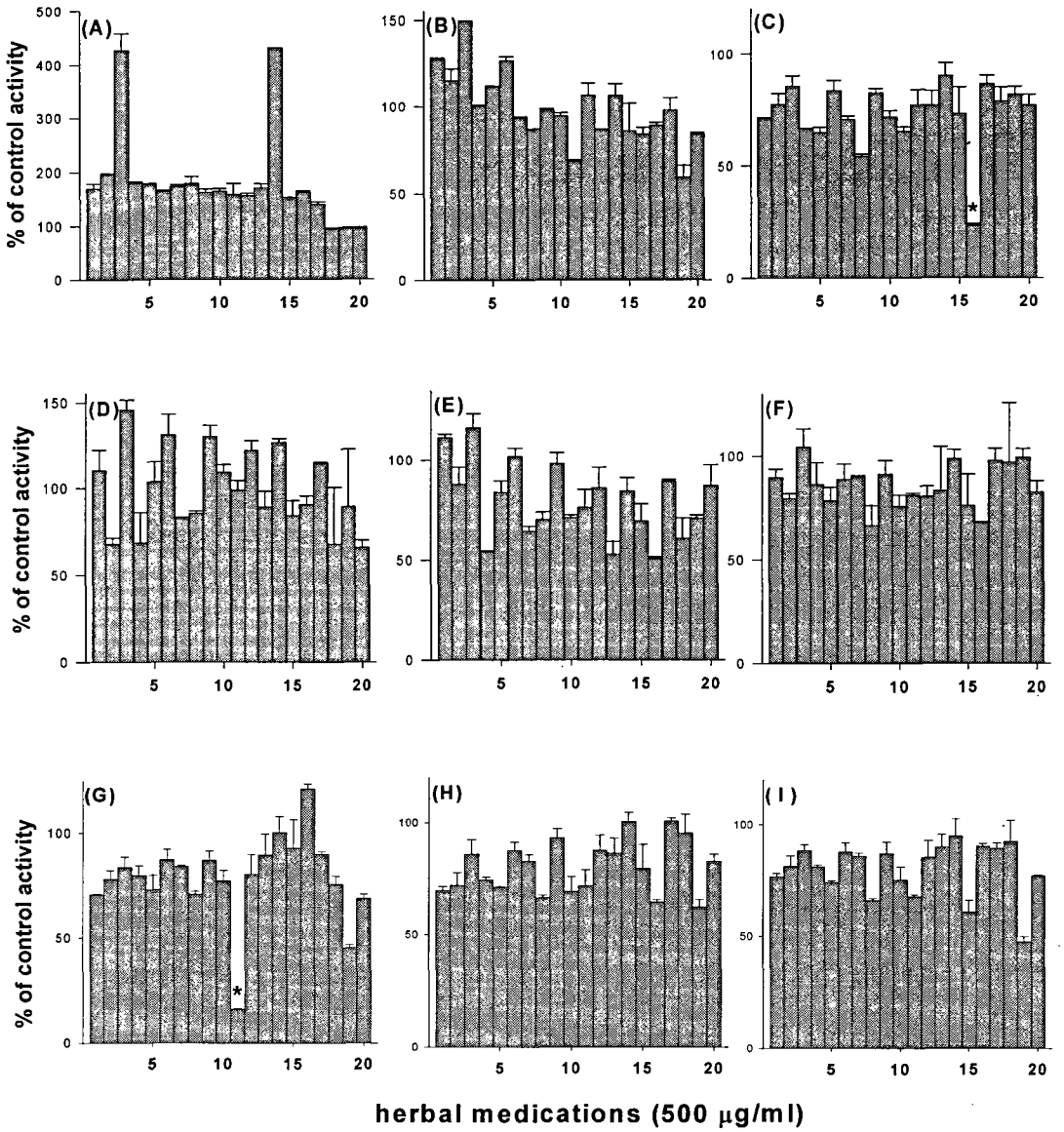

Fig 1. Inhibitory effects of 20 herbal medications (500 μg/ml) on nine CYP isoforms activities: (A) CYP1A2-catalyzed phenacetin *O*-deethylation, (B) CYP2A6-catalyzed coumarin 7-hydroxylation, (C) CYP2B6-catalyzed bupropion hydroxylation, (D) CYP2C8-catalyzed rosiglitazone *para*-hydroxylation, (E) CYP2C9-catalyzed tolbutamide 4-hydroxylation, (F) CYP2C19-catalyzed *S*-mephenytoin 4'-hydroxylation, (G) CYP2D6-catalyzed dextromethorphan *O*-demethylation, (H) CYP2E1-catalyzed chlorzoxazone 6-hydroxylation, (I) CYP3A-catalyzed midazolam 1'-hydroxylation. The asterisk in (C) and (G) denotes the inhibition by Woohwangcheongsimwon suspension and Hwanglyeondaedok-Tang on the activity of CYP2B6-catalyzed bupropion hydroxylation and CYP2D6-catalyzed dextromethorphan *O*-demethylation, respectively. (Table 1 shows the list of these herbal medications)

stituents of Hwanglyeonhaedok-Tang [8]. Although berberine has strong inhibitory effect on CYP2D6 activity, berberine is not the major constituent causing the inhibition of CYP2D6 isoform activity because its content in Hwanglyeonhaedok-Tang is very low (ca. 5.6% of the total amount) [13]. Therefore, other constituents may contribute to the inhibition of CYP2D6 activity by Hwanglyeondaedok-Tang. Further studies are needed to explore how the constituents of Hwanglyeondaedok-Tang contribute to the inhibition of human CYP2D6 isoforms. Hwanglyeondaedok-Tang did not show inhibitory effect on the activities of other P450 isoforms (Fig. 3).

Interestingly, Sosiho-Tang and Eungyosan resulted in activation of CYP1A2-mediated phenacetin *O*-deethylation.

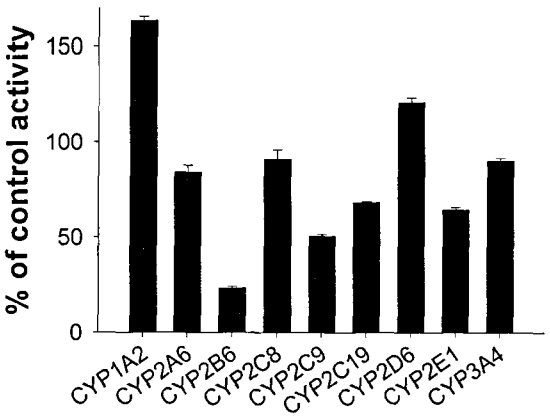

Fig 2. Inhibitory effect of 500 μg/ml Woohwangcheongsimwon on the activity of nine CYP isoforms.

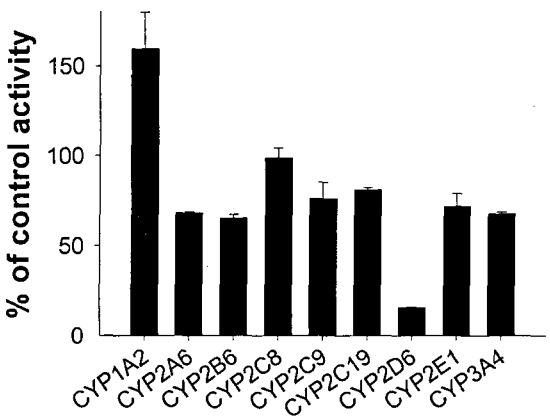

Fig 3. Inhibitory effect of 500 μg/ml Hwanglyeonhaedok-tang on the activity of nine CYP isoforms.

The activation of CYP1A2 by them is not an unusual finding, given that CYP1A2 is characteristically activated by certain compounds, such as α-naphthoflavone [15]. It is important to note, however, that activation of CYP1A2 activity in vitro does not necessarily translate into drug interactions in vivo. Therefore, the in vivo activation potential of them on phenacetin metabolism remains to be evaluated.

A comprehensive evaluation of the effects of herbal medications on nine CYP isoforms was conducted using in vitro human microsomal preparations. Our findings suggest that some herbal preparations, including Woohwangcheongsimwon suspension and Hwanglyeondaedok-Tang, could potentially inhibit the metabolism of co-administered drugs whose primary route of elimination is via CYP. Therefore, care must be exercised when co-administering such agents. Clinical trials to evaluate the effects of these herbal preparations on drug-metabolizing enzymes remain to be conducted.

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초록 : 고속 스크리닝 기법을 이용한 한약제제의 cytochrome P450 저해능 탐색

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본 연구는 우황청심원을 비롯한 상용되는 20종의 한약제제를 대상으로 9종의 시토크롬 동종효소에 대한 대사능의 저해정도를 고속 스크리닝 기법을 이용하여 탐색함으로써, 한약제제와 약물의 병용으로 인한 약물 상호작용 가능성을 평가하고자 하였다. 인체 간 마이크로좀 시료에 9종의 주요 시토크롬 약물대사효소의 지표약물과 NADPH-generating system 및 한약제제(500 μg/ml)를 첨가한 후 37℃에서 15분간 반응시켜 생성된 각각의 대사물을 LC/MS/MS를 이용하여 정량하여 시토크롬 동종효소 활성의 변화를 평가하였다. 그 결과 우황청심원 현탁액 및 황련해독탕 물 추출물이 각각 CYP2B6 및 CYP2D6 효소 활성을 선택적으로 강력하게 저해하였다. 이러한 결과는 약국에서 쉽게 구입할 수 있는 한약제제들 중 일부는 인체 간 시토크롬 활성 저해능을 가지고 있고, 이들 효소에 의해 대사되는 약물과의 병용 복용시 약물상호작용 발생 가능성이 있음을 의미한다. 향후 한약제제에서 저해능을 나타내는 주된 성분을 규명하여 이 성분의 저해능과 저해 기전을 살피는 노력이 필요할 것이다.