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ORIGINAL ARTICLE

Inhibition of cytochrome P450 2B6 by Astragalus extract mixture HT042

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Abstract

Astragalus extract mixture (AEM) HT042 is a functional food approved by the MFDS (Korean FDA) for increasing height. It comprises a mixture of three standardized extracts from *Astragalus membranaceus* root, *Eleutherococcus senticosus* stem, and *Phlomis umbrosa* root. In this study, drug–functional food interaction was analyzed using six major human cytochrome P450 enzymes. The inhibitory effect of AEM HT042 on P450 activities was studied using a P450–NADPH P450 reductase reconstitution system. Among the six P450 enzymes (1A2, 2A6, 2B6, 2D6, 2C9, and 3A4), only P450 2B6 activity was markedly decreased by AEM HT042 addition. The bupropion hydroxylation activity of P450 2B6 was analyzed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS). A calculated IC_{50} value of 10.62 μg/ml was obtained. To identify the inhibitory compounds in the mixture, four active compounds in AEM HT042 were analyzed. Shanzhiside methylester exhibited inhibitory effects on P450 2B6, whereas formononetin, eleutheroside E, and sesamoside did not affect P450 2B6 activity at all. Our results suggest that shanzhiside methylester in AEM HT042 is responsible for the inhibitory effect on P450 2B6 metabolism. Characterization of the inhibitory effect on P450 can help determine the safe administration of functional foods along with many clinical drugs that are metabolized by P450.

Keywords Cytochrome P450 · P450 2B6 · Astragalus · Shanzhiside methylester · UPLC–MS/MS

Introduction

Human cytochrome P450 enzymes are involved in major drug metabolism processes and are involved in the metabolism of three-fourths of the marketed drugs [1]. There are fifty-seven genes encoding individual human P450 enzymes, and sixteen among them are classified as P450 enzymes for xenobiotic substrates [1]. Because P450 2B6 constitutes 2–10% of the entire P450 amount in the human liver, it was not a focus of attention before the recent introduction of several new clinical drugs that are P450 2B6 substrates [2–7].

Many drugs display significant alternations in their metabolism with co-administration of other drugs, and these effects are mainly because of the altered metabolism of P450 enzymes [8, 9]. Inhibition of P450 enzymes is a central mechanism for metabolism-based drug–drug interactions [10]. Interestingly, many herbal medicines and functional foods as well as drugs can change the catalytic activity of human drug-metabolizing enzymes or their expression levels; in particular, this has emerged as an important issue in medicines metabolized by P450 [11–13].

Astragalus extract mixture (AEM) HT042 has been approved by the Ministry of Food and Drug Safety, Korea (MFDS) as a functional food to improve the height growth of children [14, 15]. It comprises a mixture of three herbal components, *Astragalus membranaceus* root, *Eleutherococcus senticosus* stem, and *Phlomis umbrosa* root. AEM HT042 is known to improve longitudinal bone growth rates via chondrocyte proliferation and hypertrophy in growth plates [16, 17]. The mechanism of AEM HT042 includes stimulation of growth hormone secretion [18], which induces an increased in serum IGF-1 and IGFBP-3 levels [15].

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In this study, the inhibitory effect of human cytochrome P450 enzymes by AEM HT042 was evaluated. Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analysis was used to determine the inhibitory effect of HT042 on P450 2B6. Since AEM HT042 has recently been a widely consumed herb, our results are important, which suggest that metabolic variations of medicines metabolized by P450 owing to coadministration with AEM HT042 need to be addressed carefully.

Materials and methods

Chemicals

Bupropion, hydroxybupropion, formononetin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Shanzhiside methylester was purchased from Target Molecule Corp (Wellesley Hills, MA, USA). Sesamoside was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, Sichuan, China). All chemicals were of the highest grade commercially available. Ni^{2+}-nitrilotriacetate (NTA) agarose was purchased from Qiagen (Hilden, Germany). Astragalus extract mixture HT042 was kindly provided by NeuMed Inc. (Seoul, Korea) in proportion to the production procedure registered by the Korean Ministry of Food and Drug Safety (MFDS). Manufacturing process and quality management of HT042 registered in MFDS indicated that standardized HT042 contains shanzhiside methyl ester 0.15%, eleutheroside E 0.36%, and formononetin 0.008% [19]. *Escherichia coli* DH5α cells were purchased from Invitrogen (Carlsbad, CA, USA). Rat NADPH-P450 reductase was heterologously expressed in *E. coli* (HMS173) and purified as described previously [20, 21].

Expression and purification of P450 enzymes

The pCW vector containing P450 enzyme (1A2, 2A6, 2B6, 2C9, 2D6, and 3A4) genes was used to express the P450 holoenzyme [22, 23]. Expression of recombinant P450 enzymes was performed with some modifications as described previously [2]. The cloned plasmid was transformed into *E. coli* DH5α cells. One colony picked from an LB-agar plate was inoculated into 5 ml of LB medium containing 50 μg/ml ampicillin and was incubated in the shaking incubator at 250 rpm and 37 °C. After overnight incubation, the cultures were transferred into 500 ml of Terrific broth medium containing 50 μg/ml ampicillin. The expression cultures were incubated with shaking at 250 rpm and 37 °C. Recombinant protein expression of P450 enzymes was induced by 1.0 mM isopropyl β-D-thiogalactoside, 0.5 mM 5-aminolevulinic acid, 1.0 mM thiamine, trace elements and 1 μg/ml of chloramphenicol, and incubating at 30 °C with shaking at 200 rpm for 40 h, followed by harvesting using centrifugation.

The obtained bacterial pellets after harvesting were resuspended in TES buffer (pH 7.4) with lysozyme and incubated at 4 °C for 30 min. After the cells were centrifuged at 5000×*g* for 20 min, the pellets were sonicated in 180 ml of sonication buffer comprising 10 mM potassium phosphate (pH 7.4), 20% glycerol (v/v), 6.0 mM magnesium acetate, 100 μM dithiothreitol, 200 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor tablets (Roche, Branford, CT). The fractions were centrifuged at 5000×*g* for 20 min. The supernatant was ultracentrifuged at 10^5×*g* for 1 h, followed by solubilization in 180 ml of solubilization buffer containing 1% CHAPS (w/v), 100 mM potassium phosphate (pH 7.4), 20% glycerol (v/v), 1.0 mM EDTA, and 10 mM β-mercaptoethanol. After ultracentrifugation, solubilized proteins were loaded onto a Ni^{2+}-nitrilotriacetic acid (NTA) agarose column following a pre-equilibrium with 100 mM potassium phosphate buffer (pH 7.4) including 5 mM imidazole. Samples were washed once with 100 mM potassium phosphate buffer (pH 7.4) including 20 mM imidazole and elution was carried out with potassium phosphate buffer (pH 7.4) and 20% glycerol including 300 mM imidazole at 4 °C.

Inhibition assays of P450 enzymes

P450 inhibitory assays were performed using evaluation of the individual P450 biomarker enzyme reactions: ethoxyresorufin deethylation for P450 1A2, coumarin hydroxylation for P450 2A6, bupropion hydroxylation for P450 2B6, diclofenac hydroxylation for P450 2C9, dextromethorphan *O*-demethylation for P450 2D6, and midazolam hydroxylation for 3A4. The P450 reaction mixtures included 100 pmol P450 enzymes, 200 pmol purified rat NADPH-P450 reductase (NPR), 30 μg sonicated 1,2-dilauroyl-*sn*-glycero-3-phosphorylcholine (DLPC), 100 mM potassium phosphate buffer (pH 7.4), and varying concentrations of AEM HT042 (in 50% MeOH). Pre-incubations were performed for 3 min at 37 °C, and then 100 μM P450 enzyme substrates were added. After another pre-incubation for 3 min at 37 °C, adding the NADPH-generating system (0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1.0 IU glucose 6-phosphate dehydrogenase mL^{-1}) initiated the reaction. Incubations were performed for 20 min at 37 °C and reactions were stopped by addition of 1 ml CH_2Cl_2. After intense vortexing, the mixture was centrifuged at 3000 rpm for 15 min and transferred to a new test tube, followed by evaporation under a stream of nitrogen gas. The dried product was dissolved in 50 μl of CH_3CN and transferred to glass vials for analysis. The metabolic products in solutions were then determined by fluorescence or mass spectrometry analysis and IC_{50}

values were then estimated using Graph-Pad Prism software (GraphPad, Inc., La Jolla, CA, USA).

Analysis of P450 metabolic activities

P450 metabolic analysis for P450 1A2 and P450 2A6 was performed using fluorescence analysis [24, 25]. Excitation and emission wavelengths were used with 540 and 586 nm for P450 1A2 ethoxyresorufin deethylation and 350 and 453 nm for P450 2A6 coumarin hydroxylation, respectively [24, 25].

P450 metabolic analysis for P450 2B6, P450 2C9, P450 2D6, and P450 3A4 was performed using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). The samples of P450 metabolism were injected into an ACQUITY UPLC™ BEH C18 column (50 × 2.1 mm, 1.7 µM) equipped with Waters ACQUITY UPLC™ and Waters Quattro Premier™. The mobile phase consisted of 0.1% formic acid in water: acetonitrile (90:10 v/v) (A) and 0.1% formic acid in acetonitrile, with a flow rate of 0.3 ml/min. Mobile phase B was applied at 20% during the first 0.5 min and % B was increased from 20% to 90% for 2.5 min.% B was maintained at 90% for 1 min, followed by returning to 20% of mobile phase B and was kept constant for 2 min [3]. Analytes were observed using positive electrospray ionization (ESI^+) and the multiple reaction mode (MRM). The source temperature was 150 °C, the desolvation temperature was 350 °C, the desolvation gas flow rate was 700 L/h and the cone gas flow rate was 50 L/h. The column temperature was 40 °C. For analysis of P450 2B6 bupropion hydroxylation, the positive ionization transitions of bupropion (m/z 240 > 184) and hydroxybupropion (m/z 256.1 > 238.1) were monitored at collision energies of 20 and 14 eV. The peak areas were calculated using QuanLynx software (Waters).

Binding titrations of P450 2B6

Purified P450 2B6 enzymes were diluted to 1 µM with 100 mM potassium phosphate buffer (pH 7.4) and placed in two glass cuvettes with the same volume. Spectral changes (350–500 nm) were subsequently determined while adding various concentrations of shanzhiside methylester, using the CARY 100 Varian spectrophotometer. The concentration of shanzhiside methylester was plotted against the difference in absorbance between the maximum and minimum wavelength. Binding affinities to shanzhiside methylester were measured using GraphPad Prism software.

Results

Bupropion hydroxylation by P450 2B6

The catalytic activity of P450 2B6 was measured by hydroxylation of bupropion using UPLC–MS/MS analysis (Fig. 1). The retention times of bupropion and 6-hydroxybupropion were 1.73 and 1.24 min, respectively (Fig. 1). Bupropion substrate with an MRM of 240 > 184 and hydroxybupropion with an MRM of 256.1 > 238.1 were detected in the chromatogram (Fig. 1). Reconstitution of P450 2B6 successfully

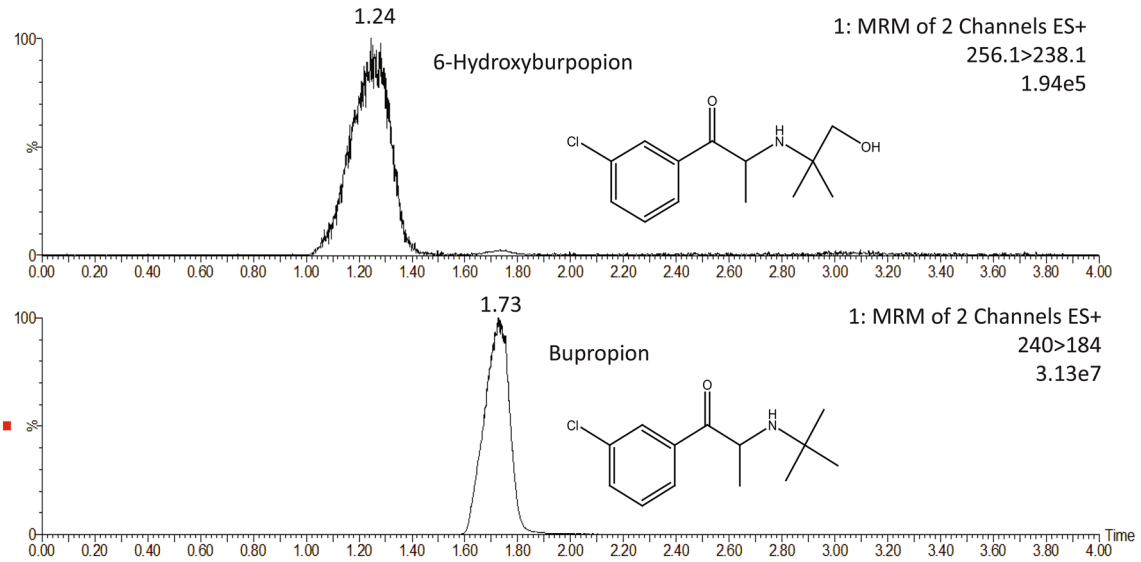

Fig. 1 UPLC–MS/MS analysis of bupropion hydroxylation by P450 2B6. Chromatograms of hydroxybupropion and bupropion in the multiple reaction mode (MRM). The positive ionization transitions of bupropion (m/z 240 > 184) and hydroxybupropion (m/z 256.1 > 238.1) were monitored

converted bupropion to hydroxybupropion as a major metabolite.

Inhibition of P450 enzymes by AEM HT042

The inhibitory effects of AEM HT042 on six P450 enzymes (P450 1A2, 2A6, 2B6, 2C9, 2D6, and 3A4) were examined using specific substrates for individual P450 enzymes. No significant inhibition was observed in P450 1A2, 2A6, 2C9, 2D6, and 3A4 (Fig. 2). However, AEM HT042 displayed significant inhibition in the metabolism of bupropion by P450 2B6 (Fig. 2). The calculated IC_{50} value of AEM HT042 on the bupropion hydroxylation of P450 2B6 was 10.6 ± 0.4 μg/ml (Fig. 2).

Inhibition of P450 2B6 by components of AEM HT042

To identify the chemical identity responsible for P450 2B6 inhibition by AEM HT042, the major chemicals [formononetin (in DMSO), shanzhiside methylester (in MeOH), eleutheroside E (in DMSO), and sesamoside (in H_2O)] of the individual herbal components of AEM were analyzed for P450 inhibition (Fig. 3). Only shanzhiside methylester caused highly decreased activity (27% of control at the highest concentration) of P450 2B6, whereas the other three chemicals did not induce any inhibition (Fig. 3). An IC_{50} value of 567 ± 11 μM was determined for shanzhiside methylester on P450 2B6 activity (Fig. 3).

Competitive inhibition by shanzhiside methylester

Spectral binding titration analysis of P450 2B6 was performed with shanzhiside methylester. Shanzhiside methylester induced a typical type I binding spectral change (Fig. 4). This suggested that shanzhiside methylester occupies the active site of P450 enzyme and can act as a competitive inhibitor of the P450 2B6 substrate. The binding affinity of shanzhiside methylester to P450 2B6 was calculated with a K_d value of 12 ± 4 μM.

Discussion

A recent increase in the number of clinical drugs metabolized by P450 2B6 has elevated the significance of P450–drug interactions. The current study established the metabolic analysis of recombinant P450 2B6 enzyme using UPLC/MS–MS in the MRM (Fig. 1). Evaluation of P450 inhibition was determined with established UPLC/MS–MS

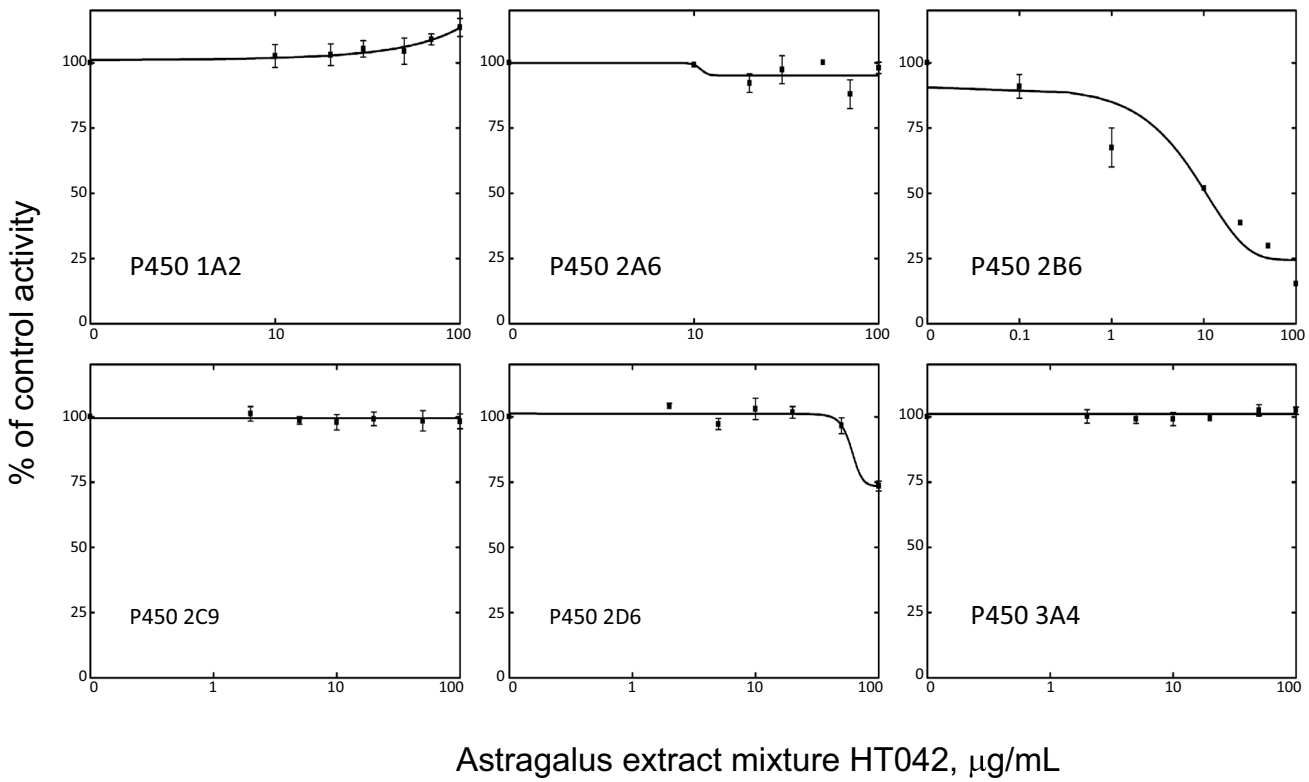

Fig. 2 Analysis of P450 1A2, 2A6, 2B6, 2C9, 2D6, and 3A4 inhibition by Astragalus extract mixture HT042. The calculated IC_{50} value of Astragalus extract mixture HT042 on P450 2B6 was 10.6 ± 0.4 μg/ml

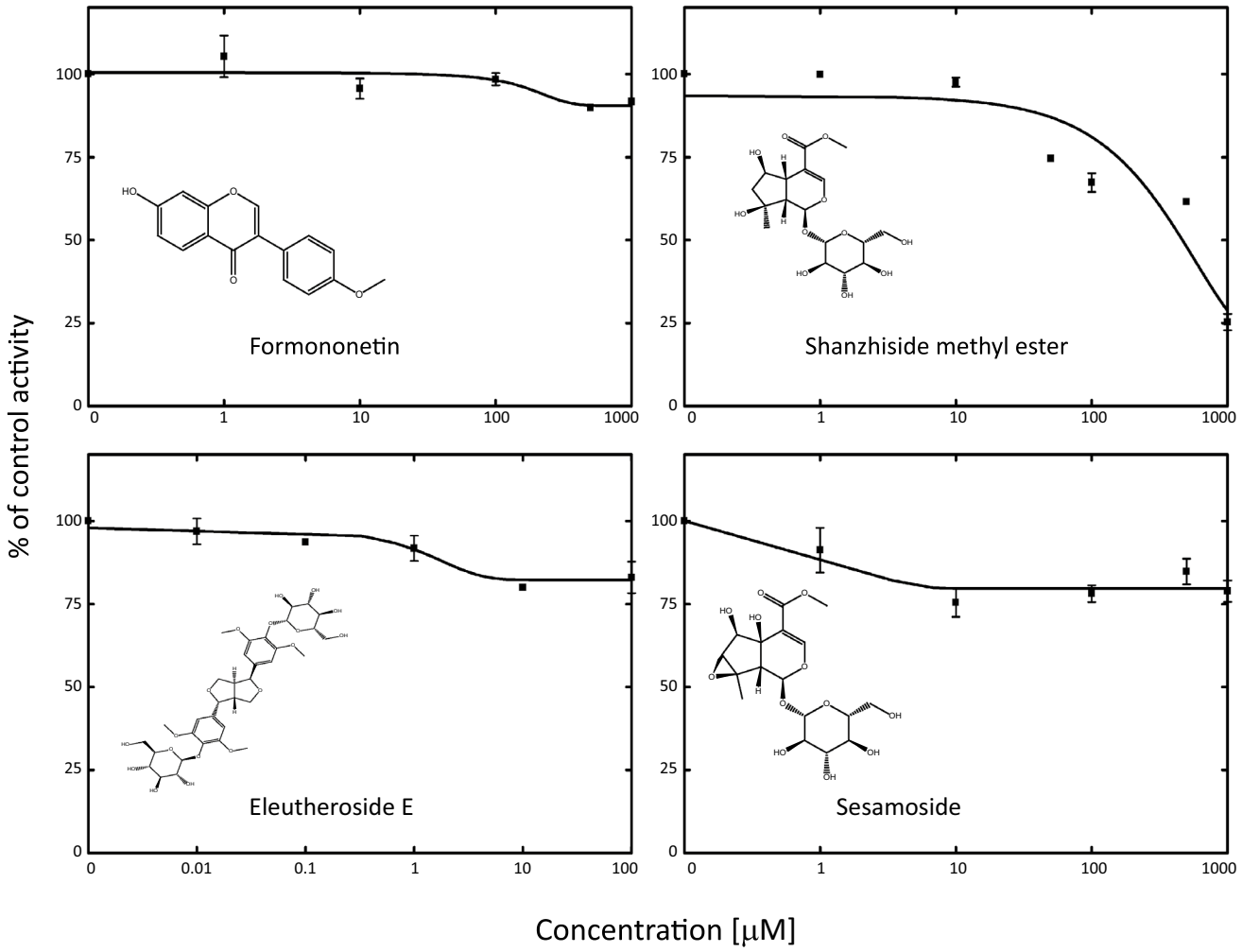

Fig. 3 Inhibition of P450 2B6 by formononetin, shanzhiside methylester, eleutheroside E, and sesamoside. Only shanzhiside methylester decreased the catalytic activity of P450 2B6 in a concentration-dependent manner. The calculated IC_{50} value of shanzhiside methylester was 567 ± 11 μM

conditions. Previously, we developed a cocktail approach for rapidly measuring the activities of seven cytochrome P450 enzymes simultaneously using GC–MS based analysis [26]. This optimized cocktail method successfully evaluated the effects of the decoction of a herbal medicine, Socheongryong-tangon cytochrome P450 enzymes [26].

AEM HT042 is a standardized mixture containing parts of the herbs *A. membranaceus*, *E. senticosus*, and *P. umbrosa*. AEM HT042 is consumed as a functional food to increase height growth in children [14]. Since many herbs and functional foods have potential capability to alter the catalytic activities of drug-metabolizing enzymes [11–13], we evaluated the inhibitory effect of AEM HT042 on six P450 enzymes, 1A2, 2A6, 2B6, 2C9, 2D6, and 3A4, in an in vitro reconstitution system (Fig. 2). The metabolic activities of P450 1A2, 2A6, 2C9, and 3A4 were not affected by AEM HT042. The catalytic activity of P450 2D6 decreased slightly at the highest concentration of AEM HT042. Only the catalytic activity of P450 2B6 showed a wide range of decrease in a concentration-dependent manner with an IC_{50} value of 10.6 ± 0.4 μg/ml. AEM HT042 contains four major chemical compounds, formononetin, shanzhiside methylester, eleutheroside E, and sesamoside. Among them, only shanzhiside methylester caused significant inhibition of P450 2B6 with an IC_{50} value of 567 ± 11 μM (Fig. 3). Considering the relatively high inhibitory concentration, we speculate that the P450 2B6 inhibitory effect of AEM HT042 is due to shanzhiside methylester. However, the clinical relevance of the AEM HT042 inhibitory effect may not be significant due to large human blood volume, and low gastro-intestinal absorption of functional foods. A previous study showed that shanzhiside methylester displayed biological activities such as inhibition of nuclear factor-κB to modulate ischemia and reperfusion, as well as an anti-fibrinolytic hemostatic

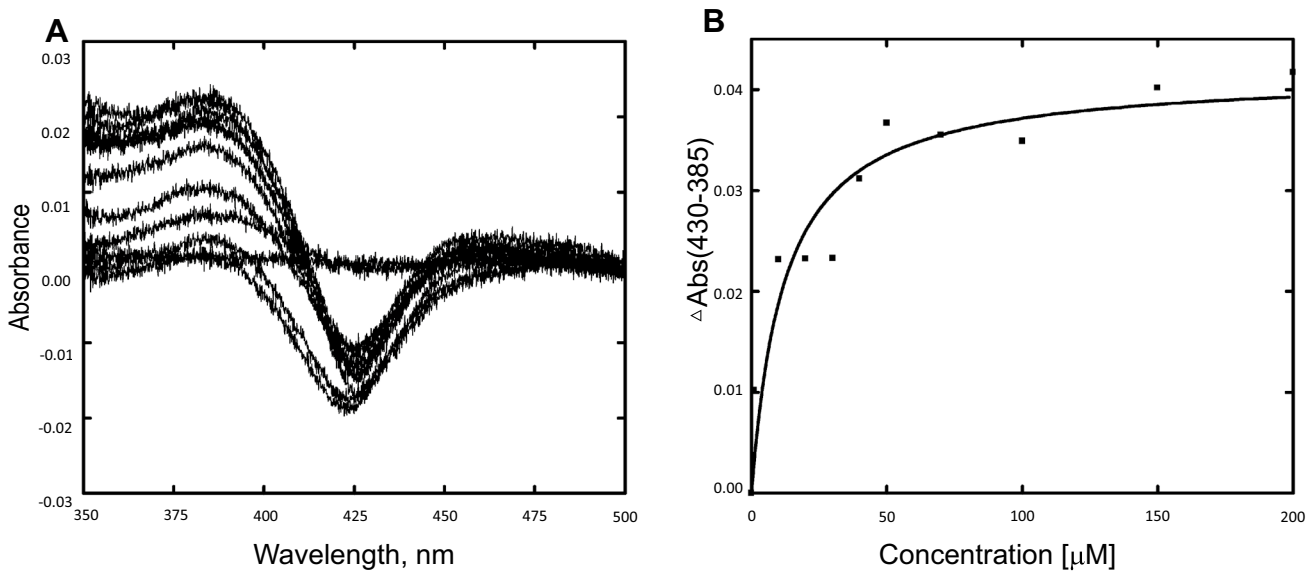

Fig. 4 P450 2B6 binding titration with shanzhiside methylester. The calculated K_d value of shanzhiside methylester, was determined as 12 ± 4 μM

effect [27, 28]. We examined the interaction of shanzhiside methylester with P450 2B6 by P450 binding spectral analysis. Shanzhiside methylester showed a good binding spectral change, indicating that this compound is located on the heme of P450 enzyme (Fig. 4). Tight binding affinity to P450 2B6 suggested that this chemical can compete with the substrate at the active site to decrease the metabolic rate of bupropion. In a previous study, we discovered the effect of monoterpenes on P450 2B6 catalytic activity using GC–MS analysis. The competitive inhibitory effect of α-terpinyl acetate on P450 2B6 suggests terpene–drug interactions [2].

Several studies have shown that the mechanism-based inactivation (MBI) of the human cytochrome P450 2B6 triggered adverse drug–drug interactions [29]. Clopidogrel, methadone, chlorpyrifos, imperatorin, and isoimperatorin have been identified as mechanism-based inactivators of P450 2B6 [30–33]. These experiments indicated that loss of heme is the key mechanism of P450 inactivation. Therefore, destruction of the prosthetic heme of P450 2B6 by inhibitory chemicals can be an important mechanism to inactivate the catalytic activity of P450 2B6 [29–32].

In conclusion, we observed the inhibitory effects of AEM HT042 on P450 2B6, and the results show that shanzhiside methylester binds to the active site of P450 2B6, thereby competing with other P450 2B6 substrates, implying that shanzhiside methylester is a competitive inhibitor. Thus, we demonstrate the presumed clinical effects of drug metabolism induced by P450 2B6. Our results also indicate a caution in drug therapy when drug–drug interactions or drug–herbal interactions are predicted in clinical practice. Future studies need to focus on obtaining toxicity information from drug and herbal treatments that affect P450 enzyme metabolism.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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