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Potential Inhibitory and Inducing Effects of Triphala Formulation on Cytochrome P450 Enzymes

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Abstract

Triphala formulation is one of the most common traditional medicines used for several health conditions. The study aimed to investigate the inhibitory and inducing potentials of Triphala formulation, including its active compounds gallic acid and ellagic acid, on cytochrome P450 (CYP450) enzymes. The inhibitory effects of Triphala formulation, gallic acid and ellagic acid on the 5 major human CYP450 enzymes were evaluated using a bioluminescent CYP450 inhibition assay. Evaluation of inducing effect of Triphala formulation on CYP1A2 and CYP3A1 mRNA expression following daily oral doses of 1,000 and 5,000 mg/kg body weight for 28 days was evaluated using RT-PCR. Gallic acid potently inhibited CYP1A2, CYP2C9 and CYP2C19 with a non-competitive nature, while the inhibitory potencies on CYP2D6 and CYP3A4 were weak. Chronic dosing of Triphala formulation at 1,000 and 3,000 mg/kg body weight significantly induced mRNA expression of CYP1A2 but not CYP3A1. Results suggest the propensity of metabolic drug interactions when Triphala formulation was concurrently administered with other conventional drugs or herbal medicines.

Keywords: CYP450 inhibition, CYP450 induction, Triphala formulation, Gallic acid, Ellagic acid

Introduction

The use of herbal products as complementary and alternative medicines for the prevention and treatment of various diseases has been increasing worldwide, including in Thailand [1,2]. This has been particularly prominent with the rise in the global burden of noncommunicable diseases and the ageing society [3,4]. Elderly patients are using herbal drugs along with conventional prescription drugs for their multiple illnesses. Herbal products have commonly been considered safe. Nevertheless, due to their diverse and complex chemical constituents, potential adverse interactions are a tremendous public health concern when they are concurrently administered with other herbal medicines or prescription drugs. These interactions, remarkedly cytochrome P450 (CYP450)-mediated interactions, may lead to an enhancement of toxicity and/or attenuation in the clinical efficacy of the co-administered drugs. Human CYP450 constitutes a superfamily of haemoproteins that catalyze a broad range of endogenous compounds, as well as xenobiotics, including drugs (70 - 80 % of all currently prescribed drugs), environmental pollutants and dietary products [5,6]. Although CYP450 is ubiquitously expressed in a number of organs, most are expressed at the highest levels in the liver [7]. The significance of individual CYP450 enzymes in human drug metabolism varies, with the CYP3A, CYP2D and CYP2C subfamilies being responsible for the metabolism of 50, 25 and 15 % of current clinical drugs, respectively [8].Inhibition of CYP450 enzymes could increase the risk of toxicity as a consequence of increased plasma concentrations of the co-administered drugs, particularly for drugs with a narrow therapeutic index [8,9]. The inducing effect of herbal medicines or drugs on these CYP450 enzymes usually decreases the plasma concentration of certain drugs, leading to reduced efficacy of the drug or treatment failure [10,11].Serious clinical interactions have been reported with *Allium sativum* (garlic), *Piper nigrum* (pepper), *Curcuma longa* (turmeric), *Panax ginseng* (ginseng) and *Hypericum perforatum* (St. John's wort) following co-administration with prescription medicines [12,13]. In a previous study, 14 plants

commonly used in Thai traditional medicine, *i.e.*, *Curcuma longa, Piper nigrum, Rhinacanthus nasutus, Piriformospora indica, Liometopum sinense, Zingiber zerumbet, Atractylodes lancea, Carthamus tinctorius, Hibiscus sabdariffa, Stevia rebaudiana, Saussurea lappa, Amanita sinensis, Helicteres isora* and *Rheum palmatum*) were found to potently inhibit CYP3A4-mediated testosterone 6-β hydroxylation activity *in vitro* at the concentration of 50 μg/mL of the crude extract [14].The *in vitro* CYP450 inducing potential of St. John's wort (CYP3A4), common valerian (CYP3A4 and CYP2D6) and Ginkgo biloba (CYP1A2 and CYP2D6) were found to be most potent among all herbs. Clinically, St. John's wort is a potential inducer of CYP3A4, CYP2E1 and CYP2C19 as well as P-glycoprotein (P-gp) transporter [15,16]. In addition, garlic supplements was shown to induce CYP3A4 and P-gp, resulting in a significant decrease of bioavailability of saquinavir may result in induce CYP3A4 and P-gp [17].

Triphala formulation is one of the most common Ayurvedic (King of Rasayana) and traditional Thai medicines used for several ailments and health conditions, such as chronic constipation, detoxification of the colon, food digestive problems, hypertension, hypercholesteremia, diabetes mellitus, upper respiratory diseases, peptic ulcers, mouth ulcers, anaemia, abdominal pain, skin wounds, obesity and cancer [18-22]. Experimental studies have confirmed several pharmacological activities of Triphala formulation, *i.e.*, appetite stimulation, antioxidant, anti-inflammatory, antibacterial, antimutagenic, antineoplastic, chemoprotective and lipid- and blood sugar-lowering activities [23-25]. No acute and subacute toxicity was observed in rats following 2,000 and 5,000 mg/kg body weight dose levels [26]. Triphala formulation consists of dried pericarps of the fruits from three plants: *Terminalia chebula* Retz., *Terminalia bellirica* (Gaertn.) Roxb. and *Emblica officinalis* Gaertn, mixed in equal proportions (1:1:1) [25]. The primary chemical constituents are the 2 polyhydroxy phenolic compounds gallic acid (7 - 15 %) and ellagic acid (2.4 %) [27,28] (**Figures 1(A)** and **1(B)**). Gallic acid is also found in other natural products, such as black and green tea, grapes, wines, strawberries, bananas and other fruits [29]. A wide range of health benefits of gallic acid has been reported, including anticancer, anti-inflammatory, cardioprotective and antidiabetic properties [30]. Ellagic acid has been reported to exert anti-inflammatory, antiproliferative, antiangiogenic, anticarcinogenic and antioxidative properties in a variety of diseases [31]. In previous studies, gallic acid and ellagic acid were shown to interfere with CYP3A4 and CYP2D6 enzymes in rat liver microsomes [32,33]. However, their inhibitory potential on human CYP450 enzymes remains unclear. Results of a clinical study in healthy Thai subjects suggested that Triphala formulation was safe and exhibited significant immunostimulatory effects on human cellular immune response, especially cytotoxic T cells and natural killer (NK) cells following a daily dose of 1,050 mg ethanol extract for 14 days [34]. The present study aimed to investigate the inhibitory propensity of gallic acid and ellagic acid on the 5 major human CYP450 isoforms, *i.e.,* CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 using an *in vitro* bioluminescent CYP450 assay. The inducing activity of Triphala formulation on the 2 inducible CYP450, *i.e.,* CYP1A2 and CYP3A1 were evaluated in rats**.**

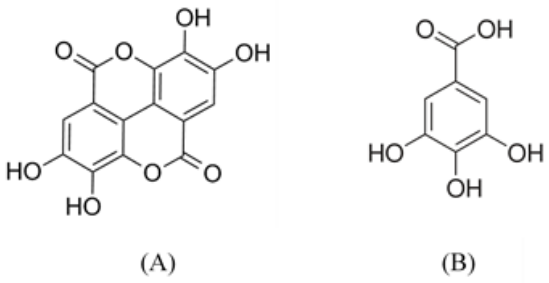

Figure 1 Chemical structures of the 2 major constituents of Triphala formulation: (A) Ellagic acid and (B) gallic acid.

Materials and methods

Chemicals

Commercial standards of gallic acid, ellagic acid, apigenin and the selective CYP450 inhibitors α-naphthoflavone and quinidine were purchased from Sigma Chemicals (St. Louis, MO, USA). Sulfaphenazole and troglitazone were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Ketoconazole was purchased from Tokyo Chemical Industries (Tokyo, Japan). Water, acetonitrile, methanol and formic acid were purchased from Fisher scientific (London, UK). TRIzol™ reagent, SuperScriptTM III Reverse Transcriptase kit and DEPC-treated water were purchased from Invitrogen

Life Technologies Inc. (Carlsbad, CA, USA). SYBR™ Green Real-time PCR Supermix was purchased from Bio-Rad Laboratories Inc. (CA, USA). Other reagents were purchased from conventional commercial sources.

Plant extract

The Triphala capsule formulation (water extract) was prepared by the Pharmaceutical Chemistry and Natural Products Research Unit, Faculty of Pharmacy, Mahasarakham University, Thailand. The plant materials were collected from Doi Saket district, Chiang Mai Province, Thailand. All materials were dried at 60 ℃ for 48 h in the hot-air oven after thoroughly washed with tap water and the seeds were removed from each fruit. All dried materials were powdered using the electric blender and passed through sieve No.14. The powdered plant material (15 g) in equal proportion (1:1:1) was extracted by boiling in 1,000 mL of distilled water for 1 h, then filtered through Whatman No.1 filter paper and freeze-dried. The Microbial contamination was controlled using gamma-irradiation. The gallic acid and ellagic acid content of Triphala formulation were 133.80 ± 2.94 and 24.59 ± 0.80 mg/g (mean + SD) of the extract, respectively.

Animals

Males and females of Wistar rats, aged between 5 - 7 weeks, weighing 150 to 180 g, were used in the study. All were obtained from the National Laboratory Animal Center, Thailand. The experiment was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals [35]. Animals were acclimatized for 1 week before the start of the study. All were housed in stainless-steel cages at the Laboratory Animal Center, Thammasat University under controlled light of 12 h dark/light cycle, at the temperature of 22 ± 2 ℃ and relative humidity of 30 - 70 %. Animals were fed with stock diets and water ad labitum. During drug administration, animals were observed daily for abnormal signs and symptoms. The study protocol was prepared and ethical approval of the study conduct was obtained from the Ethics Committee for Animal Research, Thammasat University, Thailand (No. 019/2559).

***In vitro* luminogenic CYP450 inhibition assay**

The inhibitory potentials of gallic acid and ellagic acid on CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 enzymes were investigated using P450-GloTM Screening Systems (Promega Corp, Madison, USA). The system consists of recombinant human CYP1A2 (rCYP1A2), CYP2C9 (rCYP2C9), CYP2C19 (rCYP2C19), CYP2D6 (rCYP2D6) and CYP3A4 (rCYP3A4) enzymes, which are produced by a baculovirus expression system, specific luminogenic CYP450 substrates (luciferin-ME, luciferin-H, luciferin-H EGE, luciferin-ME EGE and luciferin-IPA), negative control membranes without CYP450 activity, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) regeneration system, reaction buffer, luciferin detection reagent and luciferin-free water. The assay was performed according to the manufacturer's protocols (Technical Bulletin, P450-Glo Assays, Promega Corp., Madison, USA) [36,37] in a white opaque 96-well plate. Each test compound (12.5 µL of gallic acid or ellagic acid) was prepared using luciferin-free water to obtain final concentrations of 5, 50, 100, 250, 500, 1,000 and 2,000 µM. The concentration ranges of the selective CYP450 inhibitors (12.5 µL) were: 0 - 5 µM ∝-naphthoflavone (CYP1A2 inhibitor), 0 - 50 µM sulfaphenazole (CYP2C9 inhibitor), 0 - 50 µM troglitazone (CYP2C19 inhibitor), 0 - 25 µM quinidine (CYP2D6 inhibitor) and 0 - 12.5 µM ketoconazole (CYP3A4 inhibitor). Luciferin-free water (12.5 µL) was added to the untreated and minus-P450 control wells. A control reaction mixture containing the luminogenic CYP450-specific substrate, control membranes and potassium phosphate buffer (12.5 µL each) was added to the minus-P450 control wells. The reaction mixture (12.5 µL) containing human CYP450 membrane preparations, a luminogenic CYP450-specific substrate and potassium phosphate buffer was added to other wells. Each plate was pre-incubated at 37 ℃ for 10 min. Reactions were then initiated by adding the NADPH regeneration system (25 µL) and incubated for 10 (CYP1A2 and CYP3A4), 20 (CYP2C19 and CYP2C9) or 30 (CYP2D6) min. At the end of the incubation period, an equal volume of the luciferin detection reagent (50 µL) was added at room temperature (25 ℃). The luminescence of all of the samples was measured using a microplate reader after 20 min of incubation to stabilize the luminescent signal. Each experiment was performed as three independent assays. The total light produced is directly proportional to the CYP450 enzyme activity. The signals from untreated CYP450 reactions signify the total CYP450 activity (100 %). Modulation of the CYP450 activity by the test compounds was determined by comparing the alterations from the average net signal of untreated CYP450 reactions with the changes observed from the test compound. The IC50 (concentration that inhibits 50 % of the CYP450 activity) values were estimated from concentration-response curves using CalcuSyn software (BIOSOFT, Cambridge, UK).

Based on the IC50 values, the mechanisms of inhibition, including the enzyme kinetic parameters of gallic acid on CYP1A2, CYP2C9 and CYP2C19 were determined using least-squares linear regression of the inverse of the substrate concentration versus the inverse reaction velocity (Lineweaver - Burk plot) and of the substrate concentration versus the inverse reaction velocity (Dixon plot). An increasing concentration of gallic acid (0 - 200 µM) was incubated with various concentrations of specific luminogenic CYP450 substrates (Luciferin-ME for CYP1A2, Luciferin-H for CYP2C and Luciferin-H EGE for CYP2C19). The mean values were used to estimate the Vmax (maximum enzyme velocity) and Km (concentration of the substrate at 50 % maximum velocity) [38]. The Ki (inhibition constant) value for each CYP450 enzyme was determined using non-linear regression according to the equation:

$$v = (Vmax\ S) / (Km\ (1 + I/Ki) + S)$$

where, I is the concentration of gallic acid; Ki is the inhibition constant; S is the concentration of the substrate; and Km is the substrate concentration at half the maximum velocity (Vmax) of the reaction.

***Ex vivo* evaluation of inducing activity of Triphala formulation on CYP1A2 and CYP3A1 mRNA expression**

Animals (40 rats) were randomly divided into 4 groups (5 males and 5 females for each group). Rats in the treated groups were fed with Triphala formulation at a daily oral dose of 1,000, 3,000 and 5,000 mg/kg body weight for 28 days. The control group received a daily oral dose of distilled water (2 mL) for 28 days. At the end of the experiment, all animals were euthanized with isoflurane under euthanasia machine, and liver organs were removed for investigation of the inducing effect of Triphala formulation on CYP1A2 and CYP3A1 using RT-PCR.

Preparation of RNA from liver samples: Rat liver samples were washed with ice-cold saline (0.9 % NaCl), and total RNA was isolated using TRIZOLTM reagent according to the manufacturer's protocol (TRIzol™, Invitrogen Co. Ltd., Carlsbad, CA, USA). Briefly, 100 mg of liver sample was homogenized in 1 mL of TRIzol reagent and thoroughly mixed with 0.2 mL of chloroform and incubated at 25 ℃ for 3 min. The upper aqueous phase was separated through centrifugation (12,000 xg for 15 min, 4 ℃) and transferred to a new tube. RNA was extracted by mixing the suspension with 0.5 mL of isopropyl alcohol and incubated at room temperature (25 ℃) for 10 min. After centrifugation (12,000 xg for 10 min, 4 ℃), the RNA pellets were precipitated and washed with 1 mL of 75 % ethanol. The pellets were separated through centrifugation (7,500 xg, 5 min, 4 ℃), air-dried and dissolved in 20 µL of DEPC-treated water. The concentration of RNA was measured using NanoDrop Spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA).

Preparation of the first-strand cDNA: The cDNA was prepared using SuperScript^{TM} III First-Strand Synthesis System for RT-PCR according to Invitrogen's protocol. Briefly, total RNA (1,000 ng) from liver cells, 50 µM oligo(dT)20 (0.5 µL), 10 mM dNTP mix (0.5 µL) and DEPC-treated water (up to 5 µL) were incubated at 65 ℃ for 5 min and chilled on ice for 2 min. The cDNA was synthesized in a total volume of 5 µL reaction mixture containing 10x RT Buffer (200 mM Tris-HCL (pH 8.3), 500 mM KCL (1 µL), 25 mM $MgCl_2$ (2 µL), 0.1 M DTT (1 µL), 40 U/µL RNaseOutTM Recombinant RNase Inhibitor (0.5 µL) and 200 U/µL Superscript III Reverse (0.5 µL). The reaction was terminated by heating at 85 ℃ for 5 min, and the sample was placed on ice for 2 min.

Table 1 Primer sequences for determination of CYP3A1 and CYP1A2 mRNA expression.

Gene	Primer sequence
CYP3A1	Forward 5'-ACCTGGGTGCTCCTAGCAAT-3' Reverse 5'-GCACAGTGCCTAAAAATGGCA-3'
CYP1A2	Forward 5'-TGGTGGAATCGGTGGCTAAC-3' Reverse 5'-GACCGGGAAGAAGTCCACTG-3'
GAPDH	Forward 5'-GGAGAGTGTTTCCTCGTCCC-3' Reverse 5'-ATGAAGGGGTCGTTGATGGC-3'

Quantification of CYP1A2 and CYP3A1 mRNA expression: The forward and reverse primers for the selected genes used in the study are shown in **Table 1**. The platinum SYBRTN Green qPCR Supermix-UDG was used for RT-PCR analysis using iCycler IQ machine (BioRad Laboratories Inc., Hercules, CA, USA). The reaction mixture (20 µL) consisted of 50 ng/µL of cDNA, platinum SYBRTM

Green qPCR Supermix-UDG mixture, 10 µM forward primer, 10 µM reverse primer and sterile double distilled water. The PCR cycles for CYP1A2, CYP3A1 and GAPDH consisted of denaturation at 95 ℃ for 10 min, followed by 40 cycles of amplification at 95 ℃ for 15 s, and annealing at 60 ℃ for 1 min. Each RT-PCR was performed in duplicate. Ct value (threshold cycle), which is the intersection between an amplification and threshold line, was generated to reflect the relative measure of the concentration of target in the RT-PCR reaction. The delta-delta Ct method was used to calculate CYP1A2 and CYP3A1 mRNA expression level relative to control, and the housekeeping gene GAPDH was used for normalization of CYP1A2 and CYP3A1 mRNA expression. The delta-delta Ct calculation for the relative quantification of the target gene was as follows:

$\Delta Ct\ (1) = [Ct\ (CYP1A2 \text{ or } CYP3A1) - Ct\ (GAPDH)]$

$\Delta Ct\ (2) = [Ct\ (\text{control for, } CYP1A2 \text{ or } CYP3A1) - Ct\ (\text{control for } GAPDH)$

$\Delta\Delta Ct = \Delta Ct\ (1) - \Delta Ct\ (2)$

$\text{Relative expression} = 2 - \Delta\Delta Ct$

where, ΔCt (1) = delta Ct of unknown sample, ΔCt (2) = delta Ct of control, CYP1A2 or CYP3A1 = target gene, and GAPDH = housekeeping gene.

Statistical analysis

Data are presented as median (interquartile range) values. Comparison of quantitative variables between the treatment group and control group was performed using independent Mann-Whitney U test for data not conforming to the normal distribution (SPSS version 13). Statistical significance level was at $\alpha < 0.05$.

Results and discussion

Inhibitory effects of gallic acid and ellagic acid on CYP450 enzymes *in vitro*

The potential inhibitory effects (indicated by IC50 values) of gallic acid and ellagic acid on each CYP450 enzyme, including selective CYP450 isoform inhibitors, are summarized in **Table 2**. The potency of the inhibitory activity was classified into 3 levels: Potent (IC50 < 20 µg/mL), moderate (IC50=20-100 µg/mL) and weak (IC50 > 100 µg/mL) [37]. Gallic acid produced potent inhibitory activity on CYP1A2, CYP2C9 and CYP2C19 and weak inhibitory activity on CYP3A4 and CYP2D6. Ellagic acid, on the other hand, produced moderate inhibitory activity on all CYP450 enzymes, except CYP3A4 (weak activity).

The mechanisms of the inhibitory effects of gallic acid on CYP1A2, CYP2C9 and CYP2C19 analyzed by Lineweaver-Burk, Dixon, and secondary plots are presented in **Figures 2** - **4**. Results showed that the Vmax for all CYP450 enzymes decreased with increasing concentrations of gallic acid, while the Km remained unchanged. This suggested the non-competitive nature of the inhibitory effect of gallic acid on these CYP450 enzymes. The Ki values for the inhibitory potency of gallic acid on the CYP1A2, CYP2C9 and CYP2C19 enzymes were 255, 123 and 36 µM, respectively.

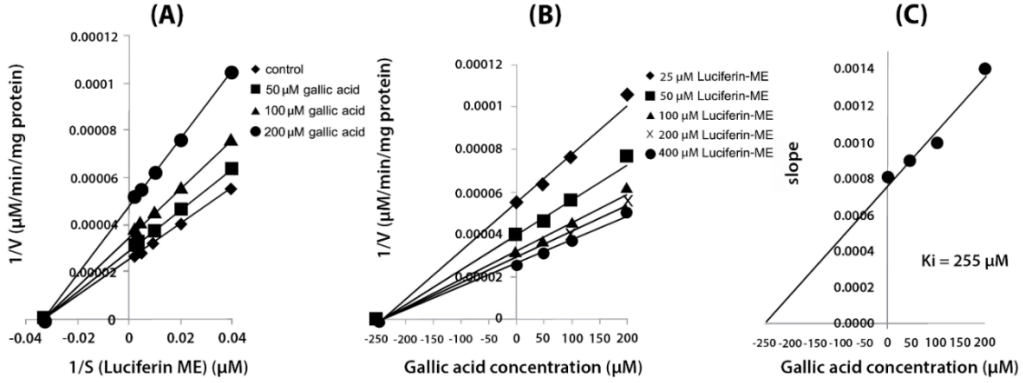

Figure 2 Inhibitory effects of gallic acid on CYP1A2 presented by representative (A) Lineweaver-Burk plot, (B) Dixon plot and (C) secondary plot.

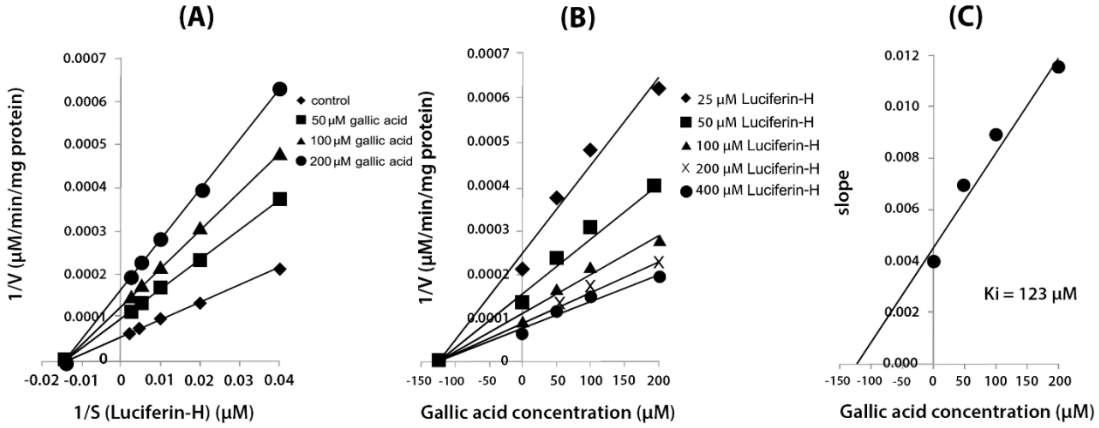

Figure 3 Inhibitory effect of gallic acid on CYP2C9 presented by representative (A) Lineweaver-Burk plot, (B) Dixon plot and (C) secondary plot.

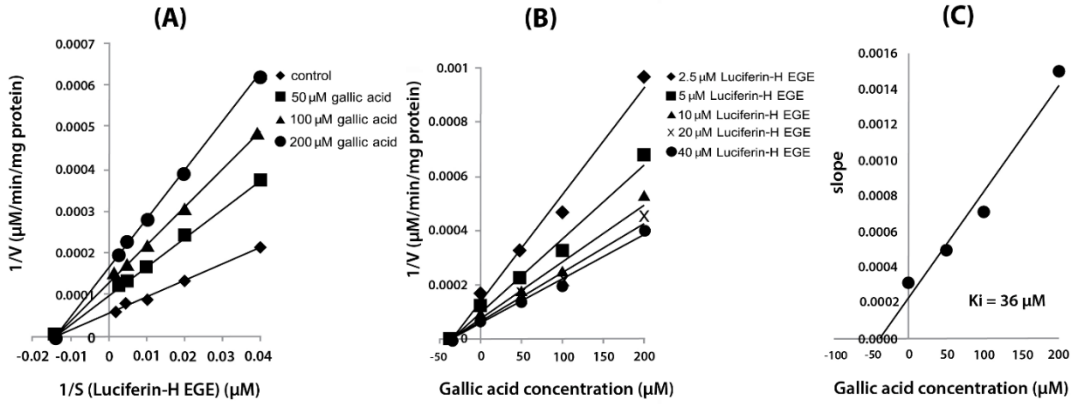

Figure 4 Inhibitory effect of gallic acid on CYP2C19 presented by representative (A) Lineweaver-Burk plot, (B) Dixon plot and (C) secondary plot.

Table 2 Potency of inhibitory activity (expressed as IC_{50} values) of gallic acid and ellagic acid on CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 enzymes. Data are presented as median (interquartile range) values of 3 independent assays for each CYP enzyme.

Enzymes	IC_{50}		
	Gallic acid	**Ellagic acid**	**Selective inhibitors**
CYP1A2	47.0 (45.65 - 47.10) μM (8.0 μg/mL)	185.3 (181.90 - 189.15) μM (56.0 μg/mL)	0.18 (0.16 - 0.19) μM (∝-Naphthoflavone)
CYP2C9	75.69 (75.20 - 75.71) μM (12.88 μg/mL)	282.4 (281.97 - 288.70) μM (85.34 μg/mL)	0.24 (0.23 - 0.25) μM (Sulfaphenazole)
CYP2C19	62.55 (61.99 - 64.72) μM (10.64 μg/mL)	179.48 (161.62 - 183.10) μM (54.24 μg/mL)	2.07 (2.02 - 2.20) μM (Troglitazone) μM
CYP3A4	592.80 (589.78 - 605.88) μM (100.85 μg/mL)	694.53 (669.86 - 703.08) μM (209.88 μg/mL)	0.10 (0.10 - 0.10) μM (Ketoconazole)
CYP2D6	772.92 (754.30 - 807.72) μM (131.49 μg/mL)	139.76 (130.20 - 145.79) μM (42.24 μg/mL)	0.01 (0.01 - 0.02) μM (Quinidine)

Inducing effect of Triphala formulation on CYP1A2 and CYP3A1 mRNA expression

The inducing activity of Triphala formulation on CYP1A2 and CYP3A1 at mRNA level was evaluated by using RT-PCR. Triphala formulation given at the daily doses of 1,000, 3,000 and 5,000 mg/kg body weight for 28 days did not upregulate the expression of CYP3A but CYP1A2 mRNA level. CYP1A2 mRNA expression was significantly upregulated by 2- and 3-fold in rat liver samples treated with Triphala formulation at dose levels of 1,000 and 3,000 mg/kg body weight, respectively, compared with the untreated control group (p-value < 0.05). At the highest dose level of 5,000 mg/kg body weight, mRNA expression was comparable to control (**Figure 5**).

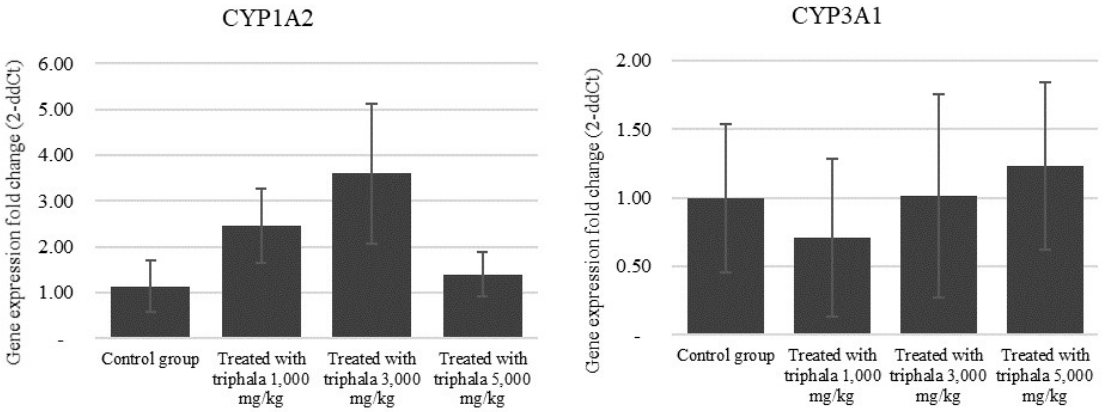

Figure 5 CYP1A2 and 3A1mRNA expression levels in rat livers treated with 1,000, 3,000 and 5,000 mg/kg body weight Triphala formulation for 28 days and control groups. GAPDH was used as an internal control. CYP1A2 and 3A11RNA expression levels were quantified use RT-PCR analysis in relation to GAPDH. Data are expressed as median (interquartile range*)* values from triplicated experiments (n = 10).

Discussion

The potentials of gallic acid and ellagic acid, the main phenolic constituents of the Triphala formulation to inhibit the activity of major CYP450 enzymes were investigated *in vitro* using a bioluminescent-based CYP450 assay with human rCYP1A2, rCYP2C9, rCYP2C19, rCYP2D6 and rCYP3A4 enzymes. Luminogenic CYP450 substrates which are pro-substrates for firefly luciferase (proluciferins) were used in the assay [39]. This approach is rapid and eliminates the requirement for sample preparation before detection by methods such as mass-spectrometry or high-performance liquid chromatography. Most importantly, the assay is substantially more sensitive than most fluorescent-based CYP450 assays and obviates any interference from fluorescent analytes [40]. Herbs containing phenols, alkaloids and terpenoids with fluorescence properties interfere with experimental analysis when fluorescence-based assays are applied [41,42]. The current assay has successfully been applied for the evaluation of the inhibitory potential of the extracts of 57 widely used plants from traditional Chinese medicine (TCM) on CYP3A4 [43].

The results of the present study showed that gallic acid potently inhibited the activity of CYP1A2, CYP2C9 and CYP2C19 but weakly inhibited CYP2D6 and CYP3A4 activity. The potencies of the inhibitory effects of all CYP450 selective inhibitors (α-naphthoflavone, sulfaphenazole, troglitazone, ketoconazole and quinidine) were comparable with those reported in previous studies using different assay methods [32,33]. The inhibitory potencies of gallic acid on all investigated CYP450 enzymes were markedly low compared with the selective CYP450 inhibitors used in the experiments (**Table 2**). The weak inhibitory effects of both compounds on CYP2D6 and CYP3A4 observed in this study were consistent with previous reports using rat liver microsomes and fluorometric assays [32,33]. In the study using rat liver microsomes [32], the mean IC50 values of gallic acid extracted by ethanol for CYP2D6 and CYP3A4 were 540.97 μM (92.03 μg/mL) and 512.81 μM (87.24 μg/mL), respectively. The corresponding IC50 values for the standardized Triphala formulation dissolved in ethanol were 105.03 and 119.65 μg/mL, respectively. In another study using a fluorometric assay [33], the mean IC50 values of gallic acid extracted by ethanol for both enzymes were 83.84 μg/mL (492.83 μM) and 72.13 μg/mL (423.99 μM), respectively. The corresponding IC_{50} values of ellagic acid extracted by ethanol were 102.69 μg/mL (339.81 μM) and 74.32 μg/mL (245.93 μM), respectively. Similar potencies were observed

for both compounds extracted with DMSO [32,33]. It was noted that the potency of the inhibition of gallic acid on CYP2D6 was comparable to, while that of ellagic acid was relatively more potent (about 2-fold) than that of the standardized extract of the Triphala formulation and each plant component (*E.officinalis, T.bellirica and T.chebula)* [32]. For CYP3A4, the inhibitory potencies of both gallic acid and ellagic acid were relatively weaker than Triphala formulation and each plant component. The IC50 values of the Triphala formulation, *E. officinalis, T. bellirica and T.chebula* for CYP2D6 were 105.03, 109.96, 90.20 and 102.35 µg/mL, respectively. The corresponding IC50 values for CYP3A4 were 119.65, 152.11, 77.94 and 95.52 µg/mL, respectively [32]. The inhibitory potency of ellagic acid on all CYP450 enzymes, except CYP3A4 (weak inhibition) was moderate. This contradicts with the results from a previous study showing potent inhibitory effect of ellagic acid on several CYPs isoforms including CYP2C19, CYP3A4, CYP 1A2 and CYP 2C9. The mean IC_{50} values of ellagic acid were 1.95, 4.80, 5.14 and 7.14 µg/mL, respectively. In addition, ellagic acid did not appear to inhibit on CYP2D6 [44]. In the *in vitro* study using rat hepatocytes, ellagic acid was shown to significantly inhibit CYP2C9, CYP3A and P-glycoprotein (P-gp)-mediated efflux in the intestine [45]. The results may suggest the contribution of other chemical constituents in the Triphala formulation apart from gallic acid and ellagic acid, on CYP2D6 and CYP3A4 activity. CYP3A4 is the most critical human CYP450 isozyme because it is involved in the metabolism of more than 50 % of all prescription drugs and accounts for 30 % of the total CYP450 protein content in the liver [46,47]. CYP2D6, second to CYP3A4, is responsible for the metabolism of more than 30 clinically important drugs, such as metoprolol and several other β-blockers, antiarrhythmics, antidepressants, neuroleptics and morphine-related drugs [48-50]. Many of these drugs exhibit narrow therapeutic indexes, particularly when coupled with the polymorphic nature of the CYP2D6 gene, which could result in an increased risk of drug interactions [50]. From a beneficial therapeutic point of view, gallic acid and ellagic acid have been reported to significantly enhance the systemic bioavailability of drugs such as diltiazem, metoprolol and nifedipine in animal models [51,52].The mechanisms involved are possibly through inhibition of CYP3A- and/or CYP2D6-mediated metabolism as well as P-gp in the intestine and/or liver.

Gallic acid potently inhibited CYP1A2, CYP2C9 and CYP2C19 activities, while ellagic acid moderately inhibited these CYP enzymes **(Table 2)**. The mechanism of inhibition by gallic acid was non-competitive type for all three CYP450 enzymes by binding to the non-active site of the enzymes (**Figures 2 - 4**). CYP2C9 makes up approximately 18 - 20 % of the liver CYP450 proteins. It is involved in the metabolism of drugs with narrow therapeutic index values, such as warfarin and phenytoin [53]. CYP2C19 is involved in the metabolism of approximately 10 % of drugs in current clinical use, most notably the antiplatelet drug clopidogrel, antigastric ulcer drug omeprazole, antiseizure drug mephenytoin, antimalarial proguanil and anxiolytic diazepam [54].The inhibitory effects of gallic acid on CYP2C9 and CYP2C19 are unlikely to produce clinically relevant interactions since the Ki values were much higher than the expected maximum plasma concentration of gallic acid following the intake of an Acidum gallicum tablet (containing 50 mg gallic acid), red wine (containing 4 mg gallic acid) or Assam black tea (containing 50 mg gallic acid) [55-57].

Results from the *ex vivo* study of liver samples from rats suggested that chronic dosing of Triphala formulation at low (1,000 mg/kg body weight) and moderate (3,000 mg/kg body weight) dose levels significantly induced CYP1A2 and mRNA expression. High dose level (5,000 mg/kg body weight) and all dose levels, respectively, did not produce a significant modulation of the expression of CYP1A2 or CYP3A1 (CYP3A human homolog in rat). Consistent with previous results, the CYP1A2 but not CYP3A1 was reported in the liver of rats feeding with green or black tea (2 % drinking water, 6 weeks) which also contains gallic acid [58,59]. The CYP1A subfamily (CYP1A1 and CYP1A2) plays a vital role in the metabolism of 2 important classes of environmental carcinogens, *i.e.,* polycyclic aromatic hydrocarbons and arylamines, and some drugs, such as caffeine, theophylline, verapamil and clozapine. It was interesting to note for both inducing and inhibitory properties of Triphala formulation on CYP1A2. The inhibitory effect on CYP1A2 was observed with gallic and ellagic acids, while the inducing effect was found with the plant extract (Triphala formulation). CYP1A2-mediated inhibition of the activation of pre-carcinogens to carcinogens should be beneficial for cancer chemoprevention, while CYP1A2-mediated induction would enhance the activation of pre-carcinogens to carcinogens. It is noted however for the antimutagenic and anticarcinogenic effects of gallic acid, which have been associated with its effects on various carcinogen metabolizing enzymes, particularly CYP1A [60].

Conclusions

The results of the in vitro study suggest the propensity of gallic and ellagic acid to inhibit hepatic metabolism of the co-administered herbal products or drugs by human CYP450 enzymes, particularly CYP1A2, CYP2C9 and CYP2C19. The induction study in rats showed the potential inducing effect of Triphala formulation on CYP1A2. Until the correlation between plasma concentrations of gallic acid/ellagic acid and clinical relevance drug interaction has been confirmed, caution should be exercised when administering Triphala formulation with drugs with narrow therapeutic index values such as warfarin, cyclosporine and digoxin, as well as in patients with impaired hepatic function.

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