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Research article

Inhibitory effects of Triphala on CYP isoforms *in vitro* and its pharmacokinetic interactions with phenacetin and midazolam in rats

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ABSTRACT

Context: Direct evidence of Triphala-drug interactions has not been provided to date.
Objective: This study was aimed to determine the effects of Triphala on cytochrome P450 (CYP) isoforms and P-glycoprotein (P-gp) *in vitro,* and to investigate pharmacokinetic interactions of Triphala with CYP-probes in rats.
Materials and methods: Effects of Triphala on the activities of CYP isoforms and P-gp were examined using human liver microsomes (HLMs) and Caco-2 cells, respectively. Pharmacokinetic interactions between Triphala and CYP-probes (i.e., phenacetin and midazolam) were further examined in rats.
Results: Triphala extract inhibited the activities of CYP isoforms in the order of CYP1A2>3A4>2C9>2D6 with the IC_{50} values of 23.6 ± 9.2, 28.1 ± 9.8, 30.41 ± 16.7 and 93.9 ± 27.5 μg/mL, respectively in HLMs. It exhibited a non-competitive inhibition of CYP1A2 and 2C9 with the K_i values of 23.6 and 30.4 μg/mL, respectively, while its inhibition on CYP3A4 was competitive manner with the *K*i values of 64.9 μg/mL. The inhibitory effects of Triphala on CYP1A2 and 3A4 were not time-dependent. Moreover, Triphala did not affect the P-gp activity in Caco-2 cells. Triphala, after its oral co-administration at 500 mg/kg, increased the bioavailabilities of phenacetin and midazolam by about 61.2% and 40.7%, respectively, in rats.
Discussion and conclusions: Increases observed in the bioavailabilities of phenacetin and midazolam after oral co-administration of Triphala in rats provided a direct line of evidence to show Triphala-drug interactions *via* inhibition of CYP1A and CYP3A activities, respectively. These results, together with the lack of time-dependency of CYP 1A2 and 3A4 inhibition *in vitro*, suggested that the inhibitory effect of Triphala is primarily reversible.

1. Introduction

Triphala has been widely used as an herbal formulation that consists of an equal proportion of *Terminalia bellerica* Roxb., *Terminalia chebula* Retz. and *Emblica officinalis,* Gaertn. Gallic, ellagic, chebulagic and chebulanic acids are the major phytoconstituents in Triphala formulations (Baliga et al., 2012; Prasad and Srivastava 2020). Triphala has been traditionally used as a laxative for the treatment of chronic constipation and gastrointestinal disorders (Kumar et al., 2016). Triphala exhibits diverse biological properties including antioxidant, anti-inflammatory, antipyretic, analgesic, antibacterial, antimutagenic, hypolipidemic, anticancer and antidiabetic activities (Kumar et al., 2016; Phetkate et al., 2020). Moreover, our previous work demonstrated that the water extract of Triphala possesses an antihyperuricemic effect in mice (Sato et al., 2017). Based on the clinical safety (Phetkate et al., 2020) and several pharmacological activities of Triphala, this popular herbal product has been recognized as an alternative medicine which potentially exhibits synergistic effects when combined with drugs (Kumar et al., 2016; Manoraj et al., 2019).

Drug-herb interactions have been detected and paid attention in recent years, typically due to bioavailability changes of drugs when

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combined with some herbs (Rombolà et al., 2020). Despite the popular use of Triphala, bioavailability changes of drugs combined with Triphala usage have not been examined *in vivo*. Furthermore, the effects of Triphala on CYP-mediated drug metabolism in human liver microsomes (HLMs) or on P-glycoprotein transporter (P-gp) have not been determined so far. Although there are species differences in the expression of various CYP isoforms, drug-herb interactions in rat models might be qualitatively extrapolated to human when *in vivo* CYP-probes, the specificity of which are comparatively defined between rats and human, are employed.

Therefore, the objectives of the study were to determine the effects of Triphala on CYP activities in HLMs and on P-gp transport function in Caco-2 cells, and to investigate *in vivo* pharmacokinetic interactions of Triphala extract with CYP-probes (i.e., phenacetin and midazolam) in rats. Moreover, time dependency of the CYP inhibitions by Triphala was investigated using HLMs.

2. Materials and methods

2.1. Chemicals

Gallic acid, chebulagic acid, chebulinic acid, ellagic acid, 4′-hydroxydiclofenac, 6β-hydroxytestosterone, acetaminophen, phenacetin, quinidine, verapamil, and pooled human liver microsomes were purchased from Sigma-Aldrich (St Louis, MO, USA). Caco-2 cells (ATC.HTB-37) were supplied by American Type Culture Collection (Manassas, VA, USA). Testosterone, rhodamine-123 (Rho-123) and midazolam were purchased from Wako Pure Chem (Tokyo, Japan). All other reagents and chemicals were of analytical grade and used without further purification.

2.2. Plant materials

The fresh fruits of *E. officinalis, T. bellerica* and *T. chebula* were collected in Lampang Province, Thailand in December 2019. Taxonomic authentication was identified by the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. Voucher specimens of *E. officinalis, T. bellerica* and *T. chebula* were preserved and deposited under voucher specimen numbers PBM-005668, PBM-005669 and PBM-005670, respectively at their herbarium. Plant materials were cleaned and cut, and the seeds of each individual fruit component of Triphala were removed. Plant samples were then dried at 60 °C for 48 hr under air-drying conditions. Each seedless dried fruit was blended to a fine powder and sieved through no.14 mesh.

2.3. Preparation of Triphala extract

The water extract of Triphala was prepared using a decoction. Each 30 g of fruit powder was boiled with 1 L of distilled water for 15 min and filtered using filter paper. The pooled filtrates were concentrated using a rotary vacuum evaporator and converted to powder by freeze drying (Sivasankar et al., 2015). Then, the percentage of yield extract was calculated. The obtained extract was stored in a light protected and closed container at -20 °C until analysis.

2.4. Quantification of phytochemical compounds in the water extract of Triphala by high-performance liquid chromatography (HPLC)

The phytochemical compounds contained in Triphala were assessed by using HPLC according to previous study (Patel et al., 2010). The standard compounds (gallic, ellagic, chebulagic and chebulinic acids) and Triphala extract were freshly dissolved in methanol (1 mg/mL) and applied to the HPLC system using a reversed-phase C-18 column (250 mm × 4.6 mm, 5 μm). Elution was performed at a flow rate of 0.8 mL/min with acetonitrile as solvent A and *O*-phosphoric acid in water (0.3%) as solvent B using a gradient elution program of 0–5 min with 90–88% B, 5–6 min with 88–86% B, 6–9.5 min with 86–80% B, 9.5–10.5 min with 80–79% B, 10.5–12 min with 79–78% B, 12–22 min with 78–76% B and 22–30 min with 76–90% B. Detection was performed at a UV wavelength of 254 nm. The contents of gallic, ellagic, chebulagic and chebulinic acids in the Triphala extract were quantified by comparison with calibration curves of each standard compounds.

2.5. In vitro inhibitory effects of Triphala extract on CYP450 isoforms in pooled human liver microsomes (HLMs)

2.5.1. Concentration-dependent effect of Triphala extract on CYP1A2 and CYP3A4 activities

The inhibitory effects of Triphala extract on four types of human drug-metabolizing CYP enzymes (CYP1A2, CYP2C9, CYP2D6 and CYP3A4) were investigated using each specific substrate and inhibitor according to previous reports (Table 1) (Hirunpanich et al., 2006; Zhang et al., 2019).

The inhibitory effects of Triphala extract on individual CYP isoforms were evaluated using a previous method with some modifications (Salsali et al., 2004; Varghese et al., 2014; Zhang et al. 2007, 2019). Briefly, a portion of HLMs in the final protein concentration of 0.28 mg/mL in 100 mM potassium phosphate buffer (pH 7.4) was pre-incubated with Triphala extract (12.5–125 μg/mL), gallic acid, ellagic acid or one of the CYP 1A2, 2C9, 2D6 and 3A4 inhibitors (as positive control), i.e., α-naphthoflavone (0.025–0.1 μM), sulfaphenazole (0.01–1 μM),

Table 1. HPLC conditions for the determination of the metabolites catalyzed by CYP isoforms.

CYP isoform	Substrate (concentration)/ metabolite	Mobile phase	Condition of mobile phase	UV detection wavelength (nm)	Ref.
1A2	Phenacetin (150 μM)/ Acetaminophen	Water (A): Acetonitrile (B)	Gradient system of A: B 0–5 min (90:10 to 20:80), 5–10 min (20:80 to 5:95), 10–12 min (20:80 to 90:10), 12–15 min (90:10)	240	(Varghese et al., 2014; Zhang et al., 2007)
2C9	Diclofenac (10 μM)/4′-Hydroxydiclofenac	Acetonitrile (A): 0.3% Phosphoric acid (B)	Isocratic system of A:B:C = 60:40	280	(Salsali et al., 2004)
2D6	Dextromethorphan (100 μM)/ Dextrophan	Acetonitrile (A): 0.1% Phosphoric acid (B)	Isocratic system of A:B:C = 60:40	280	(Liu et al., 2006; Salsali et al., 2004)
3A4	Testosterone (75 μM)/6β-Hydroxytestosterone	Solvent A: DI water: Acetonitrile: Methanol (580:20:400) Solvent B: DI water: Acetonitrile: Methanol (280:320:400)	Gradient system of A:B 0–10 min (100:0 to 63:27), 10–20 min (63:27 to 0:100), 20–25 min (0:100), 25–30 min (0:100 to 100:0) 30–40 min (100:0)	245	(Hirunpanich et al., 2006)

Abbreviations: CYP: cytochrome P450, UV: ultraviolet, Ref: References.

quinidine (0.5–2 μM) and ketoconazole (0.002–0.2 μM), at 37 °C for 5 min. Then, the reaction was initiated by the addition of an NADPH (1.3 mM) in 100 mM potassium phosphate buffer pH 7.4 and each probe substrate for CYP isoform (Kosaka et al., 2020). The mixture was further incubated at 37 °C for various intervals (30, 10, 20 and 15 min for the determination of CYP 1A2, 2C9, 2D6 and 3A4, respectively) and terminated by adding ice-cooled acetonitrile. Reactions were performed in triplicate. The samples were centrifuged at 3,000 rpm for 10 min and an aliquot of the supernatant (100 μL) was transferred into different tubes for further HPLC analysis. The metabolite of each CYP substrate was quantified using an HPLC system, as presented in Table 1 (Hirunpanich et al., 2006; Liu et al., 2006; Salsali et al., 2004; Varghese et al., 2014; Zhang et al., 2007). The yield of the corresponding metabolite was calculated using a calibrated curve of each standard metabolite. Then, the concentration of each metabolite found in the samples were converted the percent inhibition compared to those obtained from the control, which was calculated by Eq. (1) (Zhang et al., 2019) as follows:

$$\textit{Percentage of inhibition}\ (\%) = \left[\frac{1 - C_{sample}}{C_{control}}\right] \times 100 \quad (1)$$

where C_{sample} and $C_{control}$ represent the concentration of metabolites generated in HLMs with the test compound and control, respectively.

The half maximal inhibitory concentration (IC_{50}) of Triphala extract was calculated by a linear regression analysis of the dose-response curve plotting the percentage of inhibitory activity vs log-concentration of Triphala extract.

To further determine the type of enzyme inhibition of Triphala extract on the CYP1A2, CYP2C9 and CYP3A4 in HLMs, Lineweaver-Burk plot analysis was performed according to the method of Sato et al. (2020) using phenacetin, testosterone and diclofenac, respectively. Various concentrations of water extract of Triphala (12.5–50 μg/mL) were incubated with a probe substrate i.e., phenacetin (75–300 μM), testosterone (25–200 μM) or diclofenac (5–20 μM), and the enzyme reaction was performed as described above. Double reciprocal plots ($1/[v]$ vs. $1/[S]$) were prepared to obtain Michaelis-Menten constant (K_m), the inhibition constant (K_i) and maximum metabolic rate (V_{max}) by nonlinear regression analysis using Solver add-in equipped with Microsoft Excel 2010, using the following equation (Eq. 2) (Sato et al., 2020):

$$v = \frac{V_{max} \cdot S}{K_m + S\left[1 + \frac{I}{K_i}\right]} \quad (2)$$

where v is the reaction velocity (mmol/min) and S and I represent the substrate and inhibitor concentrations in the unit of μM and μg/mL, respectively.

2.5.2. Time-dependent effect of Triphala extract on CYP1A2 and 3A4 activities

Triphala extract (0 (control) or 50 μM), α-naphthoflavone (0.05 μM) or ketoconazole (0.1 μM) was pre-incubated for 0, 5, 10, 20 min with HLMs (in the final protein concentration of 0.28 mg/mL in 100 mM potassium phosphate buffer, pH 7.4) (Hirunpanich et al., 2006; Zhou et al., 2005). After the pre-incubation periods, the reaction was initiated by the addition of an NADPH (1.3 mM) in 100 mM potassium phosphate buffer (pH 7.4) and a substrate for CYP1A2 (phenacetin, 150 μM) or 3A4 (testosterone, 75 μM). The reaction mixture was further incubated at 37 °C for 30 and 15 min for the determination of CYP 3A4 and 1A2 reactions, respectively. Then, reaction was terminated by adding ice-cooled acetonitrile. The samples were centrifuged at 3000 rpm for 10 min and an aliquot of the supernatant was employed for further HPLC analysis of the generated metabolites of CYP1A2- and 3A4-catalyzed reactions, i.e., acetaminophen and hydroxytestosterone, respectively. The remaining activities of CYP1A2 and 3A4 were presented as a percentage of the residual activity and the observed activity at pre-incubation at 0 min was arbitrarily set as 100% (Taesotikul et al., 2011).

2.6. Effect of Triphala extract on P-gp activity in Caco-2 cell monolayers

2.6.1. Cell viability of Caco-2 cells

Caco-2 cells (ATC.HTB-37 Caco-2; colon adenocarcinoma; human (*Homo sapiens*)) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in CO_2 incubator, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Medium was changed 2–3 days per week. At 80–90% confluence, the cells were passage using trypsin/EDTA. To determine cell viability, Caco-2 cells were seed in 96-well plate at a density of 2×10^4 cells/well. After overnight incubation, the cells were treated with various concentration of Triphala extract (62.5–1,000 μg/mL) and incubated for 4 hr. Cell viability was determined by MTT assay. For transport studies, Caco-2 cells were seeded in 12-well Transwell-Clear plates at a density of 1.5×10^5 cells cm^{-2}/insert. The integrity of the cell monolayer was measured by transepithelial electrical resistance (TEER) measurements. After 3 weeks, the monolayers that developed a TEER of approximately 300–500 cm^2 were used for the transport studies (Hubatsch et al., 2007).

2.6.2. Effect of Triphala extract on rhodamine-123 permeability across Caco-2 cells

After 21 days of culture, the complete medium was removed, and then Caco-2 cell monolayers were washed three times with pre-warmed Hanks' balanced salt solution (HBSS, pH 7.4) and pre-incubated in HBSS at 37 °C for 30 min. Then, 2.5 μg/mL Rho-123 as the P-gp substrate was added to the apical (0.5 mL) or basolateral side (1.5 mL) with or without the water extract of Triphala (125 μg/mL) or verapamil (5 μg/mL) as a positive control. The receiver chamber contained the corresponding volume of HBSS medium. The plate was incubated for 120 min at 37 °C. Sample aliquots of 200 μL were collected from each receiver chamber at 0, 30, 60, 90, and 120 min, and 200 μL of HBSS was added to the receiver chamber to maintain a constant volume. All collected samples were analyzed by HPLC using a reversed-phase HPLC column (250 mm × 4.6 mm, 5 μm). Detection was performed at excitation and emission wavelengths of 500 and 525 nm, respectively. The mobile phase consisted of a solution of acetonitrile and water at a ratio of 35:65. The flow rate of mobile phase was 0.8 mL/min (Wang et al., 2018). The apparent permeability coefficient (P_{app}) of Rho-123 across Caco-2 cells was calculated according to Eq. (3) (Hubatsch et al., 2007).

$$P_{app} = \frac{dQ}{dT} \times \frac{1}{AC_0} \quad (3)$$

where dQ/dT is the transport rate of Rho-123 appearing in the receiver chamber, A is the surface area of the cell monolayer, and C_0 is the initial concentration of Rho-123 in the donor chamber.

The efflux ratio (ER) of apparent Rho-123 permeability from basolateral to apical side over apical to basolateral side in the cell monolayer was calculated as previously reported using the Eq. (4) (Cvetkovic et al., 1999; Hubatsch et al., 2007; Wang et al., 2018):

$$ER = \frac{P_{app}(basolateral - apical)}{P_{app}(apical - basolateral)} \quad (4)$$

where P_{app} (basolateral – apical) and P_{app} (apical – basolateral) are the P_{app} of Rho-123 from the basolateral to the apical chamber and from the apical to the basolateral chamber, respectively.

2.7. Pharmacokinetic Triphala-drug interactions in rats

According to the results of the *in vitro* on inhibition effect of Triphala on CYP isoforms in HLMs, Triphala exhibited the strongest inhibitory effect on CYP1A2 and CYP3A4. Therefore, phenacetin and midazolam

were selected as the probe substrates of CYP1A2 and CYP3A4, respectively (Gao et al., 2014; Hirunpanich et al., 2008; Namba et al., 2017), to investigate the Triphala-drug interactions in rats. Male Wistar rats weighing 180–200 g were acclimatized to environmentally controlled condition (a temperature-controlled facility with a 12-hr light/dark cycle) for at least 1 week. The rats were fasted for approximately 12 hr with *ad libitum* access to water. The femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling under isoflurane as the anesthesia. Some rats were cannulated at the femoral vein for iv administration. The experiments were performed after obtaining the permission of the Institutional Animal Ethics Committee (approval number; PYT 003/2020).

2.7.1. Pharmacokinetic interactions of Triphala extract with phenacetin in rats

For oral co-administration of Triphala extract with phenacetin, male Wistar rats were divided into 3 groups of different treatments. Group 1 served as the control group and rats in this group were orally administered water followed by oral administration of phenacetin (30 mg/kg). Group 2 and 3 were orally administered with 100 and 500 mg/kg Triphala extract, respectively followed by oral gavage of phenacetin (30 mg/kg). Blood samples (approximately 200 μL) were collected from the cannulated femoral artery at 1, 2.5, 5, 15, 30, 60, 120, 240 and 360 min after phenacetin administration and replaced with an equal volume of normal saline.

In a separate study, 5 mg/kg phenacetin dissolved in a mixture of 25% ethanol, 25% polyethylene glycol 400 and 50% water was intravenously administered through the femoral vein after pre-oral administration of water (group 1 as control group) compared with 100 and 500 mg/kg Triphala extract which were administered to group 2 and 3, respectively. Then blood samples (approximately 200 μL) were collected via the femoral artery at 5, 10, 15, 30, 60 and 90 min after iv administration of phenacetin. Plasma was obtained by centrifuging the blood samples at 4000 rpm for 10 min and immediately frozen at –80 °C until analysis. The quantification of the phenacetin concentration in plasma samples was determined by HPLC according to a previous study (Gao et al., 2014).

2.7.2. Pharmacokinetic interactions of Triphala extract with midazolam in rats

For oral administration of Triphala extract with midazolam, male Wistar rats were divided into 3 groups of different treatments. Group 1 (control group) was orally administered with water, followed by oral administration of midazolam (20 mg/kg). Group 2 and 3 were orally co-administered with 100 and 500 mg/kg of Triphala extract, respectively, with midazolam (20 mg/kg). Blood samples were collected from the cannulated femoral artery at 5, 10, 20, 30, 45, 60, 120 and 180 min after midazolam administration.

For iv administration of midazolam dissolved in a mixture of 25% ethanol, 25% polyethylene glycol 400, and 50% water with co-oral administrations of Triphala extract, rats were divided into 3 groups. Group 1(control), group 2 and 3, were orally pre-treated with water, 100 or 500 mg/kg Triphala extract, respectively. After 1 hr rats were intravenously administered with midazolam (5 mg/kg) through the femoral vein. Blood samples (200 μL) were collected *via* the femoral artery at 5, 10, 30, 60, 90 and 120 min after iv administration of midazolam (Hirunpanich et al., 2008). Plasma was separated by centrifuging blood samples at 8,000 rpm for 8 min and immediately frozen at -80 °C until analysis. The concentration of midazolam in plasma samples was analyzed by HPLC, as previously described (Hirunpanich et al., 2008; Namba et al., 2017).

2.7.3. Pharmacokinetics analyses

After oral and iv administration of phenacetin or midazolam alone as a control and after combination with Triphala extract in rats, pharmacokinetic parameters of phenacetin and midazolam (i.e., AUC_{0-t}, $AUC_{0-\infty}$, $T_{1/2}$, C_{max}, T_{max}, Vd_{ss}/F, CL_{tot}/F and MRT) were calculated as non-compartmental variables using Microsoft Excel. Moreover, oral bioavailability (F) of phenacetin and midazolam was examined using the Eq. (5) (Hirunpanich et al., 2008):

$$F(\%) = \frac{AUC_{oral}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{oral}} \times 100 \quad (5)$$

where AUC_{oral} and AUC_{iv} are the AUC after oral and iv administrations, respectively.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). The difference between groups was analyzed with Student's *t*-test. For more than two groups, significance was assessed using one-way ANOVA followed by Dunnett's test for the individual differences using SPSS program version 25. The difference was statistically significant if the probability value was less than 0.05 ($p < 0.05$).

3. Results

3.1. Plant extraction and HPLC quantification of gallic and ellagic acids in the water extract of Triphala

The water extract of Triphala was obtained as a dry powder with a brownish color. The percentage of yield extract was 16.2%. A typical HPLC chromatogram of gallic and ellagic acids in Triphala extract is shown in Figure 1. The contents of gallic, chebulagic, ellagic, and chebulinic acids in the water extract of Triphala were calculated to be 0.75 ± 0.04, 5.8 ± 0.8, 0.86 ± 0.02 and 0.52 ± 0.03 % w/w, respectively

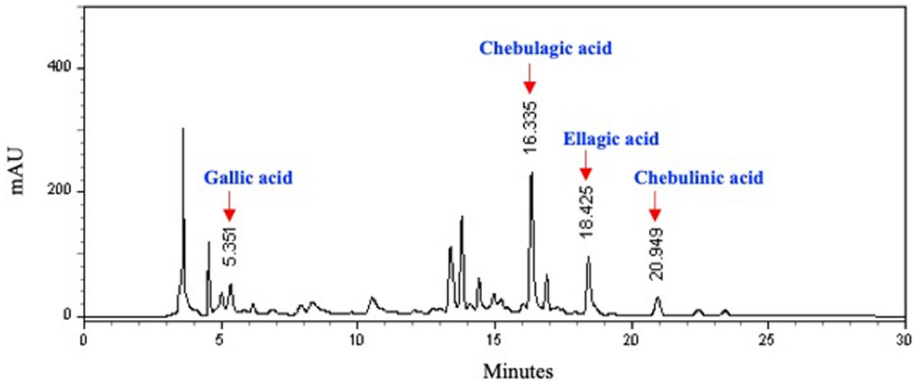

Figure 1. HPLC chromatogram of Triphala water extract with the peaks of gallic, chebulagic, ellagic and chebulinic acids.

3.2. In vitro inhibitory effects of Triphala extract on CYP450 isoforms in human liver microsomes

The *in vitro* inhibitory effects of Triphala extract on CYP1A2, 3A4, 2D6 and 2C9 enzyme activities in HLMs were examined by determining the IC_{50} values for the metabolism of the specific probes compared with specific inhibitors. Triphala extract exhibited stronger inhibitory activity in the order of CYP1A2>3A4>2C9>2D6 with IC_{50} values of 23.6 ± 9.2, 28.1 ± 9.8, 30.4 ± 16.7 and 93.9 ± 27.5 μg/mL, respectively. Triphala extract inhibited CYP activities to a lesser extent than specific CYP inhibitors (Table 2).

To investigate the reversibility of the effect of Triphala extract on CYP 1A2 and 3A activities *in vitro*, the time-dependent inhibition of Triphala on phenacetin and testosterone, as CYP1A2 and 3A4 substrates, metabolism was examined. As shown in Figure 2, ketoconazole (Figure 2A) and α-naphthoflavone (Figure 2B) as a CYP3A and 1A2 inhibitors, respectively, strongly inhibited CYP3A4-mediated 6β-hydroxy testosterone and CYP1A2-mediated acetaminophen in a time-dependent manner with a rate of reaction of 1.5 and 2.8 min^{-1}, respectively. The inhibitory effects of Triphala extract on CYP3A4 and 1A2 were not significantly increased after the elongation of pre-incubation times with the rate of reactions were similar to those of control (0.92 vs 0.72 min^{-1} and 0.9 vs 1.9 min^{-1}, for CYP 3A4 and 1A2, respectively).

Figure 3 shows the Lineweaver-Burk plot of the analysis of Triphala's inhibition of CYP1A2, 3A4 and 2C9 activities in HLMs. These results demonstrated that Triphala inhibited CYP1A2 and 2C9 activities in a non-competitive manner with the K_i values of 23.6 and 30.4 μg/mL, respectively, while its inhibition on CYP3A4 was competitive manner with the K_i value of 64.9 μg/mL.

Table 2. The IC_{50} value of Triphala extract, gallic acid, ellagic acid, and inhibitor of each CYP isoform on CYP3A4, 2D6, 1A2 and 2C9 enzymes in human liver microsomes.

Test extract	IC_{50} value			
	CYP3A4	CYP2D6	CYP1A2	CYP2C9
Triphala extract (μg/mL)	28.1 ± 9.8	93.9 ± 27.5	23.6 ± 9.2	30.4 ± 16.7
Gallic acid (μg/mL)	14.0 ± 13.6	45.1 ± 6.3	15.3 ± 0.9	57.5 ± 53.8
Ellagic acid (μg/mL)	7.0 ± 3.7	12.8 ± 1.8	3.8 ± 1.9	8.2 ± 10.4
Ketoconazole (nM)	10.0 ± 0.1	-	-	-
Quinidine (μM)	-	1.2 ± 0.2	-	-
α-Naphthoflavone (nM)	-	-	43.0 ± 0.1	-
Sulphaphenazole (μM)	-	-	-	1.8 ± 2.4

Data are expressed as mean ± SD (n = 3).

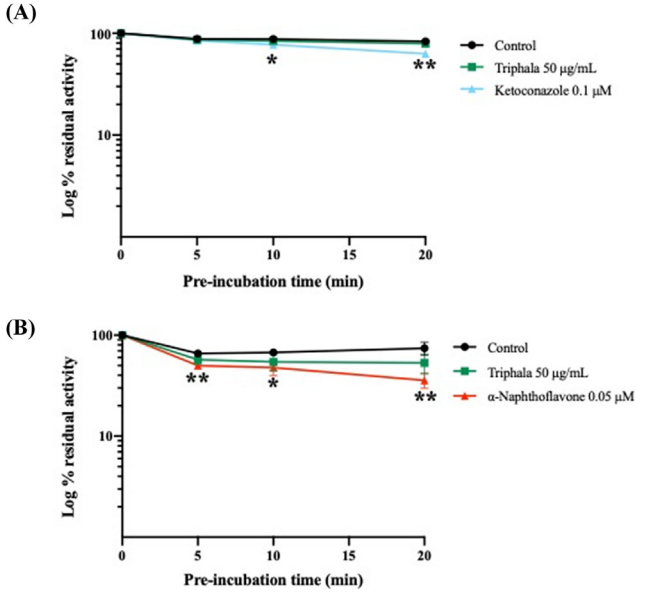

Figure 2. Time-dependent inhibition of CYP3A4-mediated 6β-hydroxy testosterone by Triphala (50 μg/mL) and ketoconazole (0.1 μM) (A) and CYP1A2-mediated acetaminophen by Triphala (50 μg/mL) and α-naphthoflavone (0.05 μM) (B). CYP activity was normalized by the activity observed at 0 min, which arbitrarily set as 100%. Each point represents mean ± SD (n = 3). *,** Significantly different as compared with control at $p < 0.05$ and $p < 0.01$, respectively.

3.3. Effect of Triphala extract on P-gp activity using Caco-2 cell monolayers

Triphala extract at a concentration range of 62.5–1,000 μg/mL showed no cytotoxicity to Caco-2 cells (Figure 4), so that the cultured Caco-2 monolayers were employed to investigate the effect of Triphala on P-gp activity.

3.4. Effect of Triphala extract on Rho-123 permeability across Caco-2 cell line monolayers

As described in Table 3, verapamil (5 μg/mL) significantly increased the P_{app} of rhodamine from apical to basolateral side but decreased the P_{app} (basolateral-apical) of Rho-123 compared with the control group. Furthermore, verapamil significantly decreased the efflux ratio (*ER*) values of Rho-123 compared with the control group ($p < 0.05$) while Triphala extract (125 μg/mL) did not show a significant effect on *ER*.

3.5. Pharmacokinetic Triphala-drug interactions in rats

3.5.1. Pharmacokinetic interactions of Triphala extract with phenacetin in rats

Figures 5(A) and Figure 5(B) represent the mean plasma concentration of phenacetin versus the time profile following the oral and iv administrations of phenacetin with concomitant oral administration of Triphala extract (100 and 500 mg/kg) in rats. The pharmacokinetic parameters are summarized in Table 4. Co-orally administered Triphala extract markedly increased C_{max} and $AUC_{0–6hr}$ of orally administered phenacetin in a dose-dependent manner whereas CL_{tot}/F and Vd_{ss}/F were decreased compared with the control group ($p < 0.05$). In contrast, co-treatment with Triphala did not significantly change the pharmacokinetic parameters of intravenously administered phenacetin. Thus, Triphala extract significantly enhanced the bioavailability (*F*) of phenacetin by approximately 1.2- and 1.6-fold at oral doses of 100 and 500 mg/kg, respectively compared with the control.

3.5.2. Pharmacokinetic interactions of Triphala extract with midazolam in rats

The mean plasma concentration-time profiles of midazolam after oral and iv administration with oral treatment with Triphala extract are presented in Figure 6(A) and Figure 6(B), respectively. As shown in Table 5, Triphala extract at doses of 100 and 500 mg/kg significantly increased $AUC_{0–2.5hr}$ and C_{max} and decreased Vd_{ss}/F and CL_{tot}/F of orally administered midazolam compared with the control group ($p < 0.05$). In contrast, Triphala extract did not significantly change the pharmacokinetic parameters of intravenously administered midazolam. Thus, the oral doses of Triphala extract in rats increased the bioavailability of midazolam by approximately 40.7%.

4. Discussion

Triphala formula contains several major phytoconstituents such as flavonoids (e.g., quercetin), tannins (e.g., gallic acid, ellagic acid, and chebulagic acid), saponins, anthraquinones, amino acids, fatty acids and various carbohydrates, which may account for its numerous

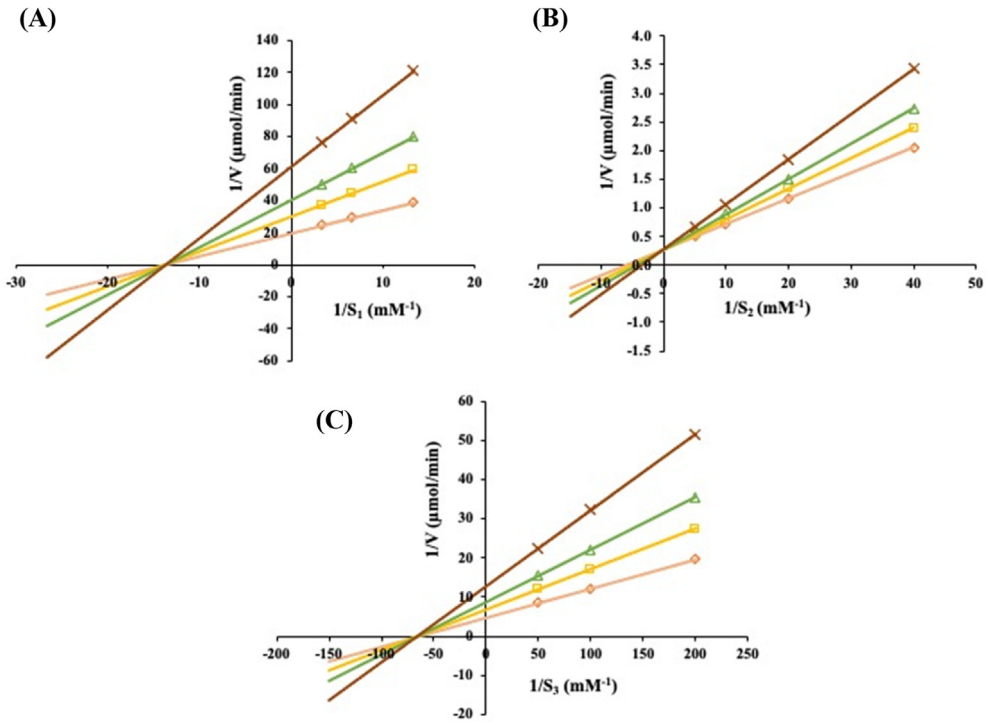

Figure 3. Lineweaver-Burk plots for the inhibition of CYP1A2 (A), CYP3A4 (B) and CYP2C9 (C) by Triphala extract (control (◇), 2.5 (□), 25 (△) and 50 (×) µg/mL) in HLMs. Data represent the mean of triplicate determination. *V* represents velocity and S_1, S_2 and S_3 represent the concentrations of phenacetin, testosterone and diclofenac, respectively.

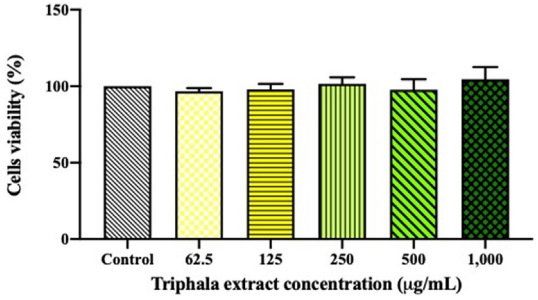

Figure 4. Cytotoxicity of Triphala extract on Caco-2 cells. Values are the mean ± SD (n = 3).

Table 3. The apparent permeability coefficient (P_{app}) and the efflux ratio (*ER*) of rhodamine-123 (2.5 µg/mL) in the absence or presence of Triphala extract.

Compounds	P_{app} (x10^{-5}) (cm/s)		Efflux ratio (ER)
	Apical to Basolateral	Basolateral to Apical	
Rhodamine 123 (control)	6.4 ± 1.0	19.0 ± 0.3	3.2 ± 0.5
Rhodamine 123 + verapamil (5 µg/mL)	11.0 ± 0.8*	16.8 ± 0.8	1.6 ± 0.1*
Rhodamine 123 + Triphala extract (125 µg/mL)	6.9 ± 0.9	14.8 ± 0.3	2.3 ± 0.5

Data are presented as mean ± SEM (n = 3). * represents $p < 0.05$ as compared with control group.

pharmacological activities (Baliga et al., 2012; Kumar et al., 2016; Phetkate et al., 2020). The compositions of active compounds, i.e., gallic, chebulagic, ellagic and chebulinic acids, observed in Triphala extract in the present study are similar to those reported in a previous study (Mahajan and Pai 2011).

Due to the recent utility of Triphala as an alternative medicine, this study was designed to investigate Triphala–drug interactions *via* alterations of CYP and drug transporter activities. Our present study is the first to investigate the effects of Triphala extract on drug-metabolizing activities in HLMs. The comparative inhibitory effects of Triphala extract on various CYP isoforms were in the order of CYP1A2>3A4>2C9>2D6, with the IC_{50} values of 23.6, 28.1, 30.4 and 93.9 µg/mL, respectively. However, Triphala exhibited less inhibitory potential than each specific CYP inhibitor. Moreover, Triphala extract demonstrated inhibitory effects on CYP1A2 and 2C9 in a non-competitive manner while it inhibited CYP3A4 in a competitive manner. The potency of the inhibitory effect of Triphala extract on CYP isoforms is relatively similar to that of its major phytoconstituents, gallic and ellagic acids. These results are comparable with a previous report, which examined the effect of Triphala extract dissolved in ethanol on the CYP450-CO complex system using rat liver microsomes and found that Triphala exhibited inhibitory effects on CYP3A and CYP2D with the IC_{50} of approximately 100 µg/mL (Ponnusankar et al., 2011).

Mechanism based inhibition (MBI) is characterized by an irreversible inhibition of CYP activity resulting from tight binding of a generated metabolite to the enzyme active site, leading to a long-lasting inactivation (Fontana et al., 2005). MBI could irreversibly inhibit CYPs in a concentration- and time-dependent manner. In this study, the inhibitory effect of Triphala was not significantly changed after prolongation of the pre-incubation time in the HLMs as compared with control. The lack of time-dependent inhibition by Triphala on CYP 1A2/3A4 activities suggested that the Triphala extract may not be a mechanism based inhibitor.

Based on the results of *in vitro* inhibitory effects of Triphala on CYP1A2 and CYP3A4, we conducted an *in vivo* study using Triphala extract with phenacetin and midazolam, as typical CYP-probes in rats (Gao et al., 2014; Hirunpanich et al., 2008; Namba et al., 2017). Despite the species differences in the expression of various CYP isoforms, rat models are helpful for understanding the pharmacokinetic drug interactions in humans using CYP-probes *in vivo* (Chen et al., 2020; Elsherbiny et al., 2008; Gao et al., 2014). In particular, phenacetin and

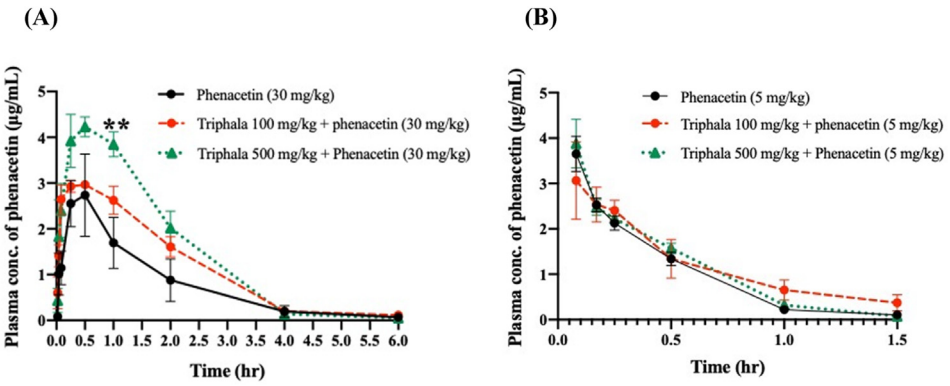

Figure 5. Plasma concentration-time profiles of phenacetin after oral (A) and iv (B) administration with oral treatment with Triphala extract (100 and 500 mg/kg). Data are represented as the mean ± SEM (n = 4–5). *, ** Significantly different compared with the control (phenacetin alone) at $p < 0.05$ and $p < 0.01$, respectively.

Table 4. Pharmacokinetic parameters of phenacetin at its oral and iv doses after oral co-administration of Triphala extract (100 and 500 mg/kg) in rats.

Pharmacokinetic parameters	Oral administration of phenacetin (30 mg/kg)			Intravenous administration of phenacetin (5 mg/kg)		
	Control	Triphala (100 mg/kg)	Triphala (500 mg/kg)	Control	Triphala (100 mg/kg)	Triphala (500 mg/kg)
T_{max} (hr)	0.33 ± 0.10	0.72 ± 0.19	0.50 ± 0.14	-	-	-
C_{max} (μg/mL)	3.31 ± 0.67	3.58 ± 1.06	4.70± 0.20*	3.65 ± 0.39	3.27 ± 0.65	3.88 ± 0.54
Kel (hr^{-1})	0.74 ± 0.11	0.71 ± 0.08	0.99 ± 0.12	2.62 ± 0.19	1.99 ± 0.44	2.80 ± 0.16
$T_{1/2}$ (hr)	0.98 ± 0.12	1.04 ± 0.13	0.74 ± 0.09	0.27 ± 0.02	0.38 ± 0.07	0.25 ± 0.02
$AUC_{0\text{-}t}$ (μg.h/mL)	5.03 ± 1.21	7.11 ± 1.02	9.02 ± 0.93*	1.77 ± 0.19	2.06 ± 0.11	1.96 ± 0.06
CL_{tot}/F, CL_{tot} (L/hr)	7.74 ± 2.63	4.56 ± 0.60	3.46 ± 0.33*	2.90 ± 0.34	2.44 ± 0.13	2.55 ± 0.08
MRT (hr)	1.32 ± 0.20	1.59 ± 0.20	1.29 ± 0.06	0.35 ± 0.02	0.53 ± 0.13	0.36 ± 0.04
Vd_{ss}/F, Vd_{ss} (L/hr)	9.06 ± 1.9	7.34 ± 1.2	4.45 ± 0.4*	1.02 ± 0.1	1.26 ± 0.2	0.93 ± 0.1
Bioavailability (%)	47.5	57.4	76.6	-	-	-

*Significantly different compared with the control (phenacetin alone) at $p < 0.05$, respectively.

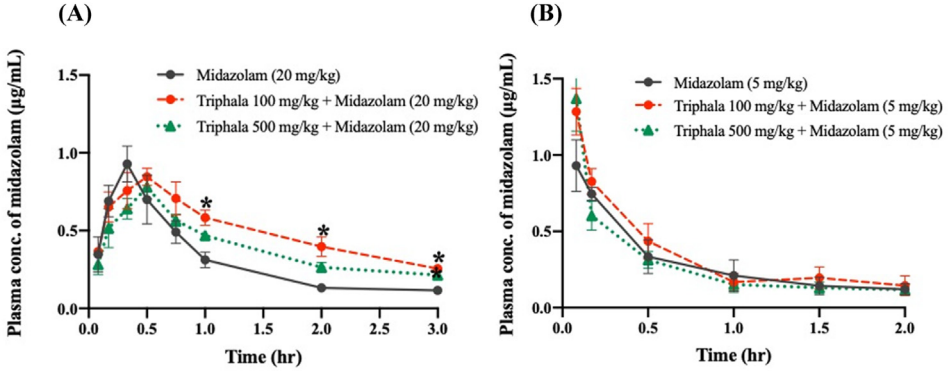

Figure 6. Plasma concentration-time profiles of midazolam after oral (A) and iv (B) administration with oral treatment with Triphala extract (100 and 500 mg/kg). Data are represented as the mean ± SEM (n = 4). * Significantly different compared with the control (midazolam alone) at $p < 0.05$.

midazolam have been utilized as *in vivo* probes for CYP1A and CYP3A isoforms, respectively, in rats (Gao et al., 2014; Namba et al., 2017). In this study, oral co-administration of Triphala extract at the dose of 500 mg/kg significantly enhanced the exposure (AUC) of phenacetin after oral, but not iv, administration. Therefore, it was considered that Triphala extract increased the bioavailability by inhibiting CYP-mediated first-pass metabolism, not by inhibiting the hepatic (systemic) clearances, of phenacetin.

A significant increase in AUC and a decrease in CL_{tot}/F of midazolam were observed when co-administered with Triphala extract at the doses of 100 and 500 mg/kg in rats. Similar to phenacetin, oral treatment with Triphala extract did not change the pharmacokinetic parameters of intravenously administered midazolam, indicating that Triphala extract increased the bioavailability, not the hepatic clearance, of midazolam.

The pharmacokinetic changes of phenacetin and midazolam after Triphala extract indicated that the gallic and ellagic acids absorbed into the portal vein is high enough after oral doses of Triphala to inhibit the first-pass metabolism through the liver.

CYP3A4 and P-gp work together to metabolize their substrate in the small intestine and the inhibition of both CYP3A4 and P-gp could enhance the oral bioavailability of these substrates. To evaluate the effect of Triphala on P-gp activity in Caco-2 cells, the bidirectional transport of Rho-123 in the apparent permeability (P_{app}) from basolateral to apical and apical to basolateral across cultured Caco-2 cell monolayers was determined and used to calculate the efflux ratio (ER)

Table 5. Pharmacokinetic parameters of midazolam at its oral and iv doses after oral co-administration of Triphala extract (100 and 500 mg/kg) in rats.

Pharmacokinetic parameters	Oral administration of midazolam (20 mg/kg)			Intravenous administration of midazolam (5 mg/kg)		
	Control	Triphala (100 mg/kg)	Triphala (500 mg/kg)	Control	Triphala (100 mg/kg)	Triphala (500 mg/kg)
T_{max} (hr)	0.38 ± 0.04	0.53 ± 0.12	0.44 ± 0.06	-	-	-
C_{max} (μg/mL)	0.95 ± 0.13	1.07 ± 0.08	0.81 ± 0.06	0.91 ± 0.13	1.24 ± 0.18	1.28 ± 0.28
Kel (hr^{-1})	0.70 ± 0.10	0.27 ± 0.04	0.27 ± 0.04	0.54 ± 0.14	0.60 ± 0.11	0.56 ± 0.15
$T_{1/2}$ (hr)	1.06 ± 0.19	2.64 ± 0.41	2.65 ± 0.41	0.98 ± 0.39	0.86 ± 0.28	0.89 ± 0.30
AUC (μg.h/mL)	1.07 ± 0.07	3.89 ± 0.34*	2.77 ± 0.31*	0.67 ± 0.27	0.85 ± 0.37	0.73 ± 0.23
CL_{tot}/F, CL_{tot} (L/hr)	18.89 ± 1.08	5.23 ± 0.44*	7.38 ± 0.76*	5.01 ± 1.97	4.44 ± 1.97	4.25 ± 1.50
MRT (hr)	1.48 ± 0.24	3.95 ±± 0.64*	3.86 ± 0.45	1.08 ± 0.46	0.90 ± 0.41	0.82 ± 0.40
Vd_{ss}/F, Vd_{ss} (L/h)	28.05 ± 4.9	20.08 ± 1.5	27.81 ± 1.1	7.44 ± 4.0	4.35 ± 1.6	4.12 ± 1.60
Bioavailability (%)	39.7	59.5	55.9	-	-	-

Data are represented as the mean ± SEM (n = 4).
*Significantly different compared with the control (midazolam alone) at $p < 0.05$.

which reflects the efflux transport ability. Gallic and ellagic acids reportedly inhibit P-gp in Caco-2 cells and enhance the bioavailability of P-gp substrate drugs (Athukuri and Neerati 2017). However, in the present study, no significant change in the efflux ratio of Rho-123 was noted after pre-treatment with Triphala extract compared with the control, suggesting that Triphala extract did not affect the P-gp activity in Caco-2 cells. Therefore, it could be concluded that Triphala extract increases the AUC of orally administered midazolam due to the herb's inhibitory effect on CYP3A in the intestine and/or liver but not on P-gp in the small intestine. The study on the effects of Triphala on the other transporters such as organic anion transporting polypeptide (OATP), organic anion transporting (OAT), multidrug resistant associated protein (MRP) and breast cancer resistance protein (BCRP) which may play a role in pharmacokinetic interaction are suggested to investigate in the next study.

Gallic acid has been reported to inhibit androstenedione 6 β-hydroxylase activity, a CYP3A marker in HMLs (Stupans et al., 2002). The presence of gallic acid in accompany with ellagic acids and other active compounds presented in Triphala extract may be associated with the inhibitory effects of Triphala on both CYP1A and 3A isoforms in rats (Athukuri and Neerati 2017; Barch et al., 1994; Ponnusankar et al., 2011; Stupans et al., 2002). Our preliminary study found that gallic and ellagic acid were observed in plasma in a dose-dependent manner after oral administration of Triphala extract at the doses of 500 and 1,000 mg/kg in rats (supplementary data), which is consistent with recently published studies (Jumpangern et al., 2021). Furthermore, we found that the C_{max} and AUC_{0-12} values of gallic acid were significantly higher than those of ellagic acid, indicating that gallic acid can be absorbed from the intestine to a higher extent than ellagic acid. It is thus suggested that gallic acid plays a major role in Triphala's pharmacological activity *in vivo*.

Taken together with the lack of time-dependent inhibition of Triphala on CYP 1A2 and 3A4 activities *in vitro*, this study demonstrated that the oral bioavailabilities of phenacetin and midazolam were increased after concomitant administration of Triphala extract due to reversible inhibition of CYP1A and 3A activities, respectively, through first-pass transit across the liver. The present study thus provided mechanistic insights into the Triphala-drug interactions.

Since rat CYPs (1A2, 2C11, 2E1, 2D1, 3A1/2 and 2D2) are homologous to human CYPs (1A2, 2C9, 2E1, 2C19, 3A4 and 2D6) (Videau et al., 2012), the present results can be inferred to have clinical application in humans. The Food and Drugs Administration has suggested that the extrapolation of animal dose to human dose is correctly performed through the normalization of the body surface area. Therefore, the calculation of human equivalent dose (HED) was calculated using formular shown in the Eq. (6) (Nair and Jacob 2016):

$$\text{Human equivalent dose (HED)(mg/kg)} = \text{Animal dose (mg/kg)} \times \left(\frac{\text{Animal } k_m}{\text{Human } k_m}\right) \quad (6)$$

where animal dose in this study, k_m for rats and k_m for human are 100 and 500 (mg/kg), 6 and 37, respectively (Nair and Jacob 2016).

This calculation results in a HED of 16 and 81 mg/kg for Triphala extract at the doses of 100 and 500 mg/kg in rats, respectively. Thus, for a 60 kg person, it equates to a 960 and 4,860 mg dose of Triphala extract, respectively. Regard to results from previous clinical studies, daily doses of Triphala are different based on the indications for treatment; 5–10 g/d of Triphala powder or about 2,000 mg/d of Triphala extract for the treatment of type 2 diabetic and hyperlipidemia (Phimarn et al., 2021). Based on the results of our study, therefore, we strongly recommend a caution when Triphala extract is to used simultaneously with phenacetin or midazolam. Further clinical studies are needed to evaluate the Triphala-drug interaction human.

5. Conclusion

This present study is the first to investigate direct *in vitro* and *in vivo* evidence of the pharmacokinetic interactions between Triphala and the CYP-probes of phenacetin and midazolam in rats *via* CYP1A and 3A inhibition. Clinical caution needs to be thus taken when drugs metabolized by CYP1A2 and/or 3A4 enzymes are used in combination with Triphala extract, since adverse events of these drug may be pronounced by Triphala intake.

Declarations

Author contribution statement

Jannarin Nontakham: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Pongpun Siripong: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Hitoshi Sato & Vilasinee Sato: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Savita Chewchinda & Kuntarat Arunrungvichian: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jantana Yahuafai: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Arman Syah Goli: Performed the experiments.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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