Comparative inhibitory potential of selected dietary bioactive polyphenols, phytosterols on CYP3A4 and CYP2D6 with fluorometric high-throughput screening

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Abstract Cytochrome P450 (CYP450) inhibition by the bioactive molecules of dietary supplements or herbal products leading to greater potential for toxicity of co-administered drugs. The present study was aimed to compare the inhibitory potential of selected common dietary bioactive molecules (Gallic acid, Ellagic acid, β -Sitosterol, Stigmasterol, Quercetin and Rutin) on CYP3A4 and CYP2D6 to assess safety through its inhibitory potency and to predict interaction potential with co-administered drugs. CYP450-CO complex assay was carried out for all the selected dietary bioactive molecules in isolated rat microsomes. CYP450 concentration of the rat liver microsome was found to be 0.474 nmol/mg protein, quercetin in DMSO has shown maximum inhibition on CYP450 (51.02 ± 1.24 %) but less when compared with positive control (79.02 ± 1.61 %). In high throughput fluorometric assay, IC_{50} value of quercetin (49.08 ± 1.02 – 54.36 ± 0.85 μ g/ml) and gallic acid (78.46 ± 1.32 – 83.84 ± 1.06 μ g/ml) was lower than other bioactive compounds on CYP3A4 and CYP2D6 respectively but it was higher than positive controls (06.28 ± 1.76 – 07.74 ± 1.32 μ g/ml). In comparison of in vitro inhibitory potential on CYP3A4 and CYP2D6, consumption

of food or herbal or dietary supplements containing quercetin and gallic acid without any limitation should be carefully considered when narrow therapeutic drugs are administered together.

Keywords Cytochrome P450 · Food-drug interaction · CYP450-CO complex assay · Interaction potential · CYP3A4 & CYP2D6

Introduction

Quercetin (QU) and rutin (RU), are secondary metabolites of plants which are regularly consumed by humans (Deschner et al. 1993). Depending on dietary habits or countries, the daily intake has been estimated between 3 and 70 mg, essentially quercetin (Manach et al. 1997). QU and RU are also ingredients in large number of multivitamin and herbal remedies because of its medicinal properties (Erlund et al. 2000). Gallic acid (GA) is a water soluble phenolic acid present in grapes and in the leaves of many plants. It has a wide range of health benefits, with several reports indicating that the compound exerted anti-cancer, anti-inflammatory, cardioprotective and anti-diabetic properties (Mansouri et al. 2014). Ellagic acid (EA) is a natural dietary polyphenol whose benefits in a variety of diseases shown in epidemiological and experimental studies involve anti-inflammation, anti-proliferation, anti-angiogenesis, anti-carcinogenesis and anti-oxidation properties (Kim et al. 2013). β -sitosterol (BS) and stigmasterol (SS), phytosterols found in plant foods especially plant oils, seeds and nuts, cereals and legumes (Muti et al. 2003) that resemble cholesterol both in structure and biological function (Bradford and Awad 2007). Phytosterols have been added to food matrices other than fat spread including low-fat milk, bakery products, orange juices, cereal bars, low

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fat beverages and chocolate bars (Jones and AbuMweis 2009). Apart from beneficial role, dietary supplements and foods, including fruits, vegetables, herbs, spices and teas, which contain complex mixtures of phytochemicals have the greatest potential to induce or inhibit the expression and activity of drug-metabolizing enzymes. Moreover, in recent days, the consumption and use of dietary supplements and food preparations containing concentrated phytochemicals increased dramatically. Polyphenols and phytosterols present in foods (fruits, vegetables, herbs, beverages) and dietary supplements have the greatest potential to modulate activity of xenobiotic-metabolizing enzymes (Hodek et al. 2002).

The cytochrome P450 superfamily (CYPs) has been implicated as one of the most important among drug metabolizing enzymes because it metabolizes wide range of pharmaceuticals (Ilango et al. 2013). In the CYP family, the enzyme CYP3A4 and CYP2D6 are regarded as the most important in drug metabolism, involved in the phase I biotransformation of more than 50 % of clinically used drugs (Subehan et al. 2006). Inhibition of CYP3A4 and/or CYP2D6 enzymes by bioactive molecules of dietary supplements may lead to increased plasma levels of concomitantly administered drug which may prolong the therapeutic effects or increase incidents of drug-induced toxicity (Schroder-Aasen et al. 2012; Usia et al. 2005). Hence an attempt of systematic study has been taken to compare the inhibitory potential of selected dietary polyphenols and phytosterols on CYP3A4 and CYP2D6 through CYP450 inhibition in rat microsomes and high throughput fluorescence assay to predict the interaction capacity with co-administered drugs.

Materials and methods

Chemicals

All the chemicals and solvents for the preparation of rat liver microsomes and CYP450-CO complex assay were of analytical grade and were purchased locally. QU, RU, GA, EA, BS and SS were purchased from Sigma Chemical Co, St Louis, MO, USA. Vivid® CYP450 Screening Kit and Vivid® Substrates were purchased from Invitrogen Drug Discovery Solutions, USA. Vivid® CYP3A4 Red (Cat. no. P2856) and Vivid® CYP2D6 Blue (Cat. no. P2972) screening kit included baculosome (respective isozymes and NADPH-P450 reductase); regeneration system (glucose-6-phosphate, glucose-6-phosphate dehydrogenase) and NADP⁺ were used for the study. 96 well black flat bottom Polystyrene Not Treated Microplate was obtained from Corning (Costar #3915, USA). Ketoconazole and Quinidine was obtained as a gift sample from M/s Micro Labs Pvt. Ltd, Hosur, Tamil Nadu, India and from M/s Trigenesis Life Sciences Pvt. Ltd, Bangalore, Karnataka, India.

Sample preparation

Stock solutions (1 mg/ml) of the Test samples (TS) [GA, EA, BS, SS, QU and RU] were prepared by dissolving in Dimethyl sulphoxide (DMSO) and Ethanol. TS in the range of 1.5–25 µg/ml were prepared from the stock by serial dilution method for concentration dependent inhibition of CYP3A4 and CYP2D6.

CYP450-Carbon monoxide (CO) complex assay using isolated rat liver microsomes

Liver microsomes were isolated from male wistar rats (weight 200–250 kg) based on the previous method described by Tang et al. (Tang et al. 2009). The study was approved by the Institutional Animal Ethical Committee (IAEC151/2012), SRM College of Pharmacy, SRM University. Protein concentration was determined by modified biuret method (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific, Waltham, MA, USA) using bovine serum albumin as standard. Screening of inhibitory activity of TS was performed with pooled rat liver microsomes in 96 well microplate, based on described method (Ponnusankar et al. 2011; Pandit et al. 2011). Briefly in this method, the microsome preparation was diluted with a phospho glycerol buffer (PG) (10 mM potassium phosphate, pH 7.4, 20 % glycerol) and incubated with prepared extract (dissolved in ethanol and DMSO), final volume was adjusted to 200 µL using PG buffer in 96-well micro-well plates. The reaction of the extract and P450 was initiated by the addition of a NADPH generating system (4.20 mg/mL of NADP⁺ in solution of 100 mM glucose-6-phosphate, 100 mM MgCl₂ and 100 U/mL glucose-6-phosphate dehydrogenase) One plate (P) was sealed with tape and kept at room temperature, while another plate (PC) was incubated in the CO chamber for 15 min. 0.5 M sodium hydrosulfite was used to reduce the sample. After addition of sodium hydrosulfite solution (SHS) the P plate remained colorless and the PC sample turned yellow. The absorbance was taken using a Microplate reader (Multiskan™ GO, Thermo Scientific, Waltham, MA, USA) at 450 nm and 490 nm and the absorbance difference was calculated. Ketoconazole was used as a positive control. Appropriate solvent controls were used for the study. The concentration of CYP450 was calculated using the formula;

$$P450 (mM) = \frac{\Delta A_{PC} - \Delta A_P}{91}$$

Where,

ΔA_{PC} is the absorbance difference of the PC sample, and ΔA_P is the absorbance difference of the P sample.

The percentage inhibition was calculated using the following formula.

$$\% \text{ inhibition} = \frac{\text{Blank} - \text{Test}}{\text{Blank}} \times 100$$

High throughput fluorometric assay of CYP3A4 and CYP2D6

High throughput screening (HTS) assay was performed in black 96-well microplates. Fluorescence readings were obtained on BioTek FLx 800 fluorescence microplate reader (Bio Tek, US) using appropriate excitation/emission (λ) wavelength. The assay was performed according to a protocol provided by Invitrogen Drug Discovery Solutions, USA. TS were analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP iso-enzymes and specific CYP Substrates. For determination of IC_{50} value two-fold serial dilution of TS was prepared and the plates were incubated for 20 min at 37 °C. The enzymatic reaction was initiated by the addition of a mixture of $NADP^+$ and the appropriate substrate. Plates were incubated for 10 min at 37 °C and then reaction was stopped by the addition of 0.5 M tris base. Appropriate solvent control was used for the study. Respective positive controls were used for CYP3A4 and CYP2D6. All measurements were performed in triplicate. Product formation from the fluorogenic probes were determined from the fluorescence data at seven different concentrations of the inhibitors and TS. IC_{50} values were determined by using the following formula.

$$\% \text{ inhibition} = \left(1 - \frac{\text{RFU in presence of test compound}}{\text{RFU in absence of test compound}} \right) \times 100$$

Where, RFU is Relative fluorescence unit

Statistical analysis

All data are presented as mean values standard error mean (SEM). The results were subjected to one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was performed by fixing the significance level at $P < 0.05$ and above. IC_{50} values were obtained using mean enzyme activity versus inhibitor concentration curves using GraphPad prism Version 5.01 (GraphPad Prism Software Inc., USA).

Results and discussion

Determination of CYP450 concentration and percentage inhibition through CYP450-CO Complex assay

CYP contains a heme iron (Fe^{2+}) that gives a characteristic absorption spectra at 450 nm when complexed with CO.

Based on this principle, CYP450–CO complex method was used to assess the inhibitory potential of selected bioactive compounds. The chemical structures of bioactive compounds were shown in Fig. 1. Concentration-dependent inhibition of CYP450 has been shown that the TS had less potential to inhibit the CYP enzymes when compared to positive control. The concentration of protein in isolated rat liver microsome was found to be 7.9 mg/mL. Ethanol and DMSO solution of GA, EA, BS, SS, QU and RU showed a concentration dependent inhibition of CYP450 (Fig. 2a and b). The CYP450 concentration of the diluted microsome sample was calculated to be about 0.474 nmol/mg protein. Two different solvents were used to assess the CYP450 inhibition, the results have shown minor variation; this confirms that the solvent effect was minimized. From Fig. 2c, QU in DMSO has shown maximum inhibition on CYP450 (51.02 ± 1.24 %) followed by GA (44.88 ± 0.87 %), RU (39.44 ± 0.76 %), EA (40.42 ± 1.01 %), SS (38.21 ± 1.16 %) and BS (37.88 ± 0.65 %). However, to confirm the possible inhibition by the TS, appropriate solvent controls were also used in the study and the percentage inhibition was calculated after neutralizing the solvent effect. So it was ensured that the DMSO and ethanol concentrations used in this study did not interfere in the CYP450 interactions. Flavonoids have the potential to modulate the activity of CYP450. The interaction between grapefruit juice and drugs has been potentially ascribed to a number of constituents. QU is one the important flavonoid in grapefruit juice responsible for the interaction (Huang et al. 2004). A few authors investigated the bioavailability of QU after several days or weeks of supplementation and stated that the elimination of QU metabolites is quite slow, with reported half-lives ranging from 11 to 28 hrs. This could favor accumulation in plasma with repeated intakes and leads to interaction with conventional drugs (Noroozi et al. 2005; Erlund et al. 2002; Conquer et al. 1998). GA and its esters has ability to increase the bioavailability of co-administered drugs by inhibiting the CYP450 mediated drug metabolism (Benet and Wachter 2000).

Determination of IC_{50} by high throughput fluorometric assay

Fluorometric assays are most commonly used to test the compounds as CYP inhibitors in early drug discovery due to its sensitivity, speed, low cost and easy to use (Stresser et al. 2000). Fluorometric assay was our next approach to ensure the drug interaction potential of TS through the important CYP isoforms (CYP3A4, CYP2D6). Inhibitor potency was quantified by determination of IC_{50} values. IC_{50} determinations are typically performed at a fixed substrate concentration and the relationship between inhibitor concentrations (Gubler et al. 2013). Selected dietary bioactive constituents were assayed between concentrations ranging from 1.5 to 25 $\mu\text{g/ml}$. All samples were assayed in triplicate, the endpoint mode was selected and IC_{50} values were calculated (Table 1).

Fig. 1 Structures of selected dietary bioactive polyphenols and phytosterols

Concentration dependent percentage inhibitions of the test compound on both of the isozymes were observed (Fig. 3).

This concentration ranges showed good behavior of concentration dependent percentage inhibition on CYP3A4 and

Fig. 2 **a** and **b**. Concentration dependent inhibition of QU, GA, RU, EA, BS & SS in DMSO & Ethanol. **c**. Percentage inhibition of QU, GA, RU, EA, BS & SS in DMSO & Ethanol versus positive control, Ketoconazole. Values are expressed in mean $\pm$ SEM; $n=3$; ANOVA followed by Dunnett's multiple comparison Test. Level of significance * $p<0.05$, ** $p<0.01$ and *** $p<0.001$

Table 1 IC₅₀ (μg/ml) value of QU, GA, RU, EA, BS and SS on CYP3A4 and CYP2D6

Test sample	Solvent	IC ₅₀ (μg/ml) (CYP3A4)	IC ₅₀ (μg/ml) (CYP2D6)
QU	DMSO	49.08±1.02 ^a	81.68±1.38 ^b
	Ethanol	54.36±0.85 ^a	87.16±2.15 ^b
GA	DMSO	66.54±1.04 ^a	78.46±1.32 ^b
	Ethanol	72.13±2.67 ^a	83.84±1.06 ^b
RU	DMSO	66.98±1.64 ^a	98.06±1.02 ^b
	Ethanol	74.02±0.42 ^a	104.32±0.72 ^b
EA	DMSO	69.47±1.18 ^a	97.34±1.55 ^b
	Ethanol	74.32±2.08 ^a	102.69±2.87 ^b
BS	DMSO	174.54±0.54 ^a	189.42±2.12 ^b
	Ethanol	188.42±2.15 ^a	202.87±1.42 ^b
SS	DMSO	86.24±2.42 ^a	98.72±1.62 ^b
	Ethanol	102.65±1.06 ^a	124.76±2.54 ^b
Ketoconazole	DMSO	07.20±0.56	-
	Ethanol	07.74±1.32	-
Quinidine	DMSO	-	05.84±0.68
	Ethanol	-	06.28±1.76

Values are expressed in mean±SEM, ^a $P<0.001$, ^b $P<0.001$ versus positive control ketoconazole and quinidine respectively. Abbreviations: DMSO, dimethyl sulfoxide; QU, Quercetin; RU, Rutin; GA, gallic acid; EA, ellagic acid; BS, β-Sitosterol; SS, Stigmasterol

CYP2D6. The study findings revealed that the dietary bioactive compounds had less inhibition potential on the tested isozymes compared to their respective positive inhibitors.

IC₅₀ values for positive controls were in good agreement to literature reports (Zou et al. 2002). IC₅₀ value of QU (49.08±

1.02–54.36±0.85 μg/ml) and GA (78.46±1.32–83.84±1.06 μg/ml) was lower than other bioactive compounds on CYP3A4 and CYP2D6 respectively so it leads to high interaction potential with co-administered drugs when compared to other constituents. There was no significant difference in the

Fig. 3 Concentration dependent inhibitory effect of QU, GA, RU, EA, BS & SS on CYP3A4 in DMSO (a) & Ethanol (b) versus Ketoconazole, CYP2D6 in DMSO (c) & Ethanol (d) versus Quinidine

IC₅₀ of GA, QU and RU from our study between already reported studies (Ponnusankar et al. 2011; Ho et al. 2001). On the basis of in vitro results, the order of inhibitory potential of TS on CYP3A4 and CYP2D6 were identified as QU>RU>GA>EA>SS>BS and GA>QU>EA>RU>SS>BS respectively. Even though IC₅₀ values of QU and GA was higher than the positive control, due to poor elimination, concurrent use of QU and/or GA with therapeutic drug, increases the oral bioavailability via inhibition of CYP3A subfamily by QU (Choi et al. 2011; Ho et al. 2001). In another study in rats investigating the co-administration of Cyclosporine (CyA) and mulberry-another fruit known to contain the antioxidant flavonoid QU-CyA bioavailability was significantly reduced; investigation of the mechanism showed that mulberry activation of CYP3A and P-gp was implicated (Hsu et al. 2013). Li and Choi 2009 reported that oral bioavailability of etoposide enhanced by QU could mainly be due to inhibition of P-gp-mediated efflux and CYP3A-catalyzed metabolism in the intestine by QU. In view of these findings, foods or herbs rich in QU should be avoided in patients treated with CyA or etoposide to ensure that therapeutic efficacy is maintained.

Conclusion

In comparison of in vitro inhibitory potential, all the selected dietary bioactive molecules shown inhibition on CYP3A4 and CYP2D6. Even though our findings suggests that QU and GA had higher inhibitory potential among studied bioactive molecules, it leads to weak interaction with conventional medicines that are metabolized by CYP3A4 and CYP2D6 since IC₅₀ value of QU and GA was higher than that of positive controls but due to the poor elimination rate of its metabolites, consumption of food or herbal or dietary supplements containing polyphenols, particularly, QU and GA without any limitation should be carefully considered when narrow therapeutic drugs are administered together.

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