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ORIGINAL ARTICLE

Modulation of CYP2D6 and CYP3A4 metabolic activities by *Ferula asafetida* resin

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Abstract Present study investigated the potential effects of *Ferula asafetida* resin on metabolic activities of human drug metabolizing enzymes: CYP2D6 and CYP3A4. Dextromethorphan (DEX) was used as a marker to assess metabolic activities of these enzymes, based on its CYP2D6 and CYP3A4 mediated metabolism to dextrorphan (DOR) and 3-methoxymorphinan (3-MM), respectively. *In vitro* study was conducted by incubating DEX with human liver microsomes and NADPH in the presence or absence of Asafetida alcoholic extract. For clinical study, healthy human volunteers received a single dose of DEX alone (phase-I) and repeated the same dose after a washout period and four-day Asafetida treatment (phase-II). Asafetida showed a concentration dependent inhibition on DOR formation (*in vitro*) and a 33% increase in DEX/DOR urinary metabolic ratio in clinical study. For CYP3A4, formation of 3-MM in microsomes was increased at low Asafetida concentrations (10, 25 and 50 μg/ml) but slightly inhibited at the concentration of 100 μg/ml. On the other hand, *in vivo* observations revealed that Asafetida significantly increased DEX/3-MM urinary metabolic ratio. The findings of this study suggest that Asafetida may have a significant effect on CYP3A4 metabolic activity. Therefore, using *Ferula asafetida* with CYP3A4

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drug substrates should be cautioned especially those with narrow therapeutic index such as cyclosporine, tacrolimus and carbamazepine.

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1. Introduction

Numerous studies have reported that traditional medicines may modulate the metabolic activity of cytochrome P-450 enzymes (CYP). Since, the use of traditional medicines is widespread in developing countries, so the extent of modulation of metabolizing enzymes needs to be assessed (Bhattaram et al., 2002; Mukherjee and Wahile, 2006; Müller and Kanfer, 2011). CYP modulating herbs may also influence the metabolism of co-administered CYP drug substrates. Such incidences may lead to altered bioavailability and pharmacokinetics of co-administered substrates (Müller and Kanfer, 2011; Izzo and Ernst, 2001). Further, the consequences will be more serious with narrow therapeutic index drugs (Barone et al., 2001). Several incidences of herb-drug interactions based on modulation of CYP enzymes are reported (Fang et al., 2011; Ho et al., 2011; Han et al., 2011; Boullata, 2005; Doehmer and Eisenbraun, 2012). In humans most of the drugs are metabolized by CYP3A4, CYP2D6, CYP2C19, CYP2C9, CYP2E1 and CYP1A2 (Zhou, 2008; Lee et al., 2013a,b). Geum chiloense (“hierba del clavo”) increases the blood concentration of cyclosporine, while ginger has the opposite effect (Duclos and Goecke, 2001; Chiang et al., 2006). Myricetin inhibited the drug metabolizing enzymes CYP3A4 and CYP2C9 (Choi et al., 2010). St John’s wort induces CYP3A4 isoenzyme and P-glycoprotein; and has been reported to reduce the blood level of digoxin, cyclosporine, tacrolimus, amitriptyline, midazolam, warfarin, indinavir, phenprocoumon and theophylline (Barone et al., 2001; Hu et al., 2005; Izzo, 2005). Septilin significantly decreased the $T_{1/2a}$, $T_{1/2e}$, $AUC_{0\text{-inf}}$, C_{max} and AUC_{0-24} of carbamazepine (Garg et al., 1998). Sho-seiryu-to extract powder delays the absorption and accelerates carbamazepine metabolism (Ohnishi et al., 1999). Ginkgo flavonoids increased the activity of CYP3A4 (Diamond et al., 2000).

Ferula asafetida L. belongs to family apiaceae. Its oleo-gum resin is obtained by incising or cutting the living roots and rhizomes. The resin is solid or semisolid with alliaceous odor and a bitter acrid taste. The resinous material comprises ferulic acid, umbelliferone, asaresinotannols, umbelliferone ethers, gums and volatile oils. The sulfides of volatile oils are responsible for the characteristic flavor of Asafetida (Sadraei et al., 2003; Kajimoto et al., 1989). Asafetida has been used as a folk medication for various ailments including flatulence, cough, asthma, bronchitis, hysteria, and convulsions (Eigner and Scholz, 1999).

Present study assesses the potential effects of *F. asafetida* on the metabolic activities of hepatic metabolizing enzymes CYP3A4 and CYP2D6 using dextromethorphan (DEX) as a probe. *In vitro* investigations were carried out by using human liver microsomes, while an *in vivo* study was conducted in healthy human subjects. In liver, DEX is mainly metabolized through N- and O-demethylations. The O-demethylation of DEX to dextrorphan (DOR) is predominantly mediated by CYP2D6 (Barnhart, 1980; Schadel et al., 1995). Therefore, DEX is commonly used as a marker for CYP2D6 activity (Kerry et al., 1994; Hu et al., 1998; Takashima et al., 2005; Wojtczak et al., 2007). The N-demethylation of DEX to 3-methoxymorphinan (3-MM) is mediated by CYP3A4 (Gorski et al., 1994). So, DEX has been used as a common probe for both CYP3A and CYP2D6 activities (Spanakis et al., 2009; Yu and Haining, 2001), as well as *in vivo* in several studies (Ducharme et al., 1996; Funck-Brentano et al., 2005; Jones et al., 1996).

2. Materials and methods

Nicotinamide adenine dinucleotide phosphate (NADPH) and β-glucuronidase (76,800 U/ml) were purchased from Helix Pomatia, ICN Biomedicals Inc., Costa Mesa, AC, USA. The syrup of dextromethorphan hydrobromide was obtained from Riyadh Pharma, Medical and Cosmetic products Co. Ltd., Riyadh, Saudi Arabia. Dextromethorphan (DEX) hydrobromide, dextrorphan hydrobromide and 3-methoxymorphinan hydrobromide were purchased from ICN Biomedicals Inc., Warrenale, USA. Human liver microsomes having a protein concentration of 20 mg/ml were purchased from Human Biologics International LLC (HBI, Scottsdale, USA), shipped in small vials with dry ice and stored in a deep freezer at −80 °C. *F. asafetida* dried resin was purchased from the local Saudi Market. The internal standards codeine and betaxolol were of USP reference standard. General-purpose reagents (GPR) were used for extraction processes, while HPLC grade solvents were used for HPLC determinations. All other materials were of analytical grade.

2.1. Extract of plant material

Dried Asafetida resin was purchased from the Saudi market and grounded to a fine powder. This dried resin powder was exhaustively extracted with ethanol for five days by the cold maceration process. The ethanolic extract of the resin was filtered and concentrated under reduced pressure using a rotatory evaporator. The extract was weighed and its serial dilutions were made with ethyl alcohol (96%) to prepare stock solutions of 1.25, 2.5, 5, 25 and 50 mg/ml concentrations. These stock solutions were stored in a refrigerator until used for incubation.

2.2. Microsomal incubation

Methanolic solution of dextromethorphan (25 μM concentration per final incubation mixture) was transferred into eppendorf tubes and dried with the help of nitrogen. The human liver microsomes (comprising 0.25 mg protein/ml) and appropriate volume of potassium phosphate buffer (0.1 M, pH 7.4) were added to the DEX loaded tubes and gently mixed. This mixture of DEX, microsomes and buffer was pre-incubated at 37 °C for 3 min in a shaker water bath. The metabolic reaction was initiated by adding 1 mM NADPH in a final volume of 0.5 ml. Reaction was allowed for 30 min in the absence

(control) or in the presence of 10 µl Asafetida resin extract (at concentrations of 10, 25, 50 or 100 µg/ml). The metabolic reaction was terminated by adding 10 µl of perchloric acid (70%) with 2–3 min of vigorous shaking. The 25 µl of internal standard (codeine 50 µg/ml) was added to each tube and the tubes were centrifuged at 10,000 rpm for 15 min. The supernatant was separated and transferred into a clean vial and analyzed by HPLC.

2.3. Clinical study

A clinical study was conducted in nonsmoking, healthy volunteers (2 males and 4 females) of ages 18–35 years. Study protocol was explained to each volunteer and written informed consent was received from them. Study protocol was approved by the Ethics Committee at the College of Medicine, King Saud University; Riyadh (approval number is 1426037). The subjects were instructed not to take caffeine or products comprising caffeine, for at least 24 h before dosing. Further, none of these subjects was receiving any other traditional or conventional medications or grapefruit comprising products for at least 2 weeks before and during the study. The clinical study was carried out in two phases with a washout period of two weeks. In phase-I, all subjects received a single dose of dextromethorphan (10 ml of DEX hydrobromide syrup (15 mg/5 ml). In phase-II, powder of Asafetida resin was administered orally in a dose of 1.0 g twice a day for four consecutive days. On the last day of dosing, subjects received 30 mg DEX (10 ml of 15 mg/5 ml syrup) and Asafetida resin, simultaneously. The subjects abstained from food for two hours before and after DEX dosing and were instructed to empty their bladder predosing. Urine samples were collected and kept at −20 °C until analyzed by HPLC. The urinary metabolic ratios of DEX/3-MM and DEX/DOR were used as indices to metabolic activities of CYP3A4 and CYP2D6 enzymes, respectively.

2.4. Preparation of urine samples

The collected urine samples were hydrolyzed by β-glucuronidase (19,200 U/ml, incubated for 18 h) to obtain unconjugated DEX and its metabolites. 1 ml of hydrolyzed urine was vigorously mixed with 5 ml of extracting solvent mixture (diethyl ether: chloroform: propranolol, 20:9:1 v/v/v) in a clean tube. The tube was centrifuged at 14,500*g* for 10 min. The organic layer was separated and vigorously mixed with 300 µl of 0.1 N hydrochloric acid and centrifuged at 14,500*g* for 10 min. The aqueous layer was separated and betaxolol (50 µl of 50 µg/ml) was added as internal standard to each sample. Samples were analyzed by using HPLC.

2.5. Determination of DEX metabolites in human liver microsomes and urine

DEX and its metabolites were analyzed by Shimadzu Class-VPV 5.02 HPLC instrument using two slightly different analytical methods (Bendriss et al., 2001; Min et al., 1999). The human liver microsomal analytes were eluted on a Nova-Pak® phenyl column (5 µm, 150 × 3.9 mm) using a mobile phase that was composed of an aqueous mixture (1.5% glacial acetic acid and 0.1% triethylamine) and acetonitrile (75:25 v/v), and flowing at a rate of 1 ml/min. Urine samples were eluted on a Zorbax SB-CN column (5 µm, 250 × 4.6 mm). The mobile phase for analysis of urine samples was composed of the aqueous mixture (1.5% glacial acetic acid and 0.1% triethylamine) and acetonitrile (87.5:12.5 v/v). The pH of the mobile phase was adjusted to 3 by orthophosphoric acid. Analytes were monitored using a fluorescence detector at excitation and emission wavelengths of 280 and 330 nm, respectively. Concentrations of DEX and its metabolites in samples were estimated based on the calibration curves of the analytes.

2.6. Statistical analysis

The *in vitro* formation of DEX metabolites in the presence of Asafetida was compared with the control by using a one-way analysis of variance (ANOVA), and a Post hoc Scheffe's multiple comparison test with a significant P value $\leqslant 0.05$. Student's paired t-test was used for statistical analysis of results obtained from clinical study. Statistical analysis was performed by using Graph-Pad Prism version 3.0 for Windows (San Diego, CA, USA).

3. Results

In the *in vitro* study, Asafetida resin alcoholic extract inhibited the formation of DOR from DEX in a concentration dependent manner (Fig. 1). Formation of 3-MM was potentiated at lower concentrations of the extract, while at the highest Asafetida concentration it did not show any significant effect on 3-MM (Fig. 2). The maximum inhibition (about 80% of control) of DOR formation was observed at the highest concentration (100 µg/ml) of Asafetida extract. Order of inhibition of DOR formation was observed as 100 > 50 > 25 ⩾ 10 µg/ml Asafetida extract. Asafetida resin extract at lower concentrations of 10, 25 and 50 µg/ml activates the formation of 3-MM. The activation was highest at 25 µg/ml concentration. The highest concentration (100 µg/ml) of Asafetida extract did not produce any remarkable effect on the formation of 3-MM (Fig. 2).

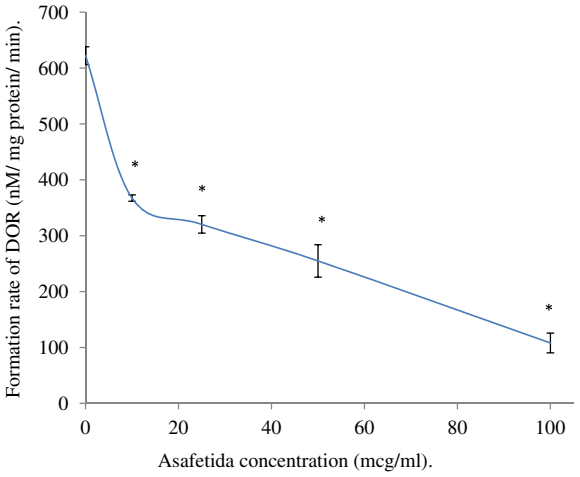

Figure 1 Effect of Asafetida on the formation of DOR from DEX in human liver microsomes (n = 3, mean ± SD). Formation of the metabolite is expressed as nM/mg protein/min. $^{*}P \leqslant 0.05$.

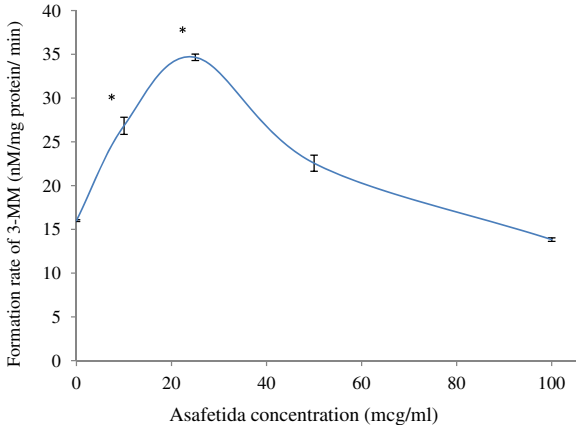

Figure 2 Effect of Asafetida on the formation of 3-MM from DEX in human liver microsomes ($n = 3$, mean ± SD). Formation of the metabolite is expressed as nM/mg protein/min. $^{*}P \leqslant 0.05$.

Clinical study also showed that Asafetida resin powder produced a potent inhibitory effect on the formation of 3-MM and DOR. Table 1 summarizes the metabolic ratios of DEX/DOR and DEX/3-MM, before and after administration of Asafetida resin. The DEX/DOR was moderately increased by Asafetida; while the increase in DEX/3-MM was significant. Almost 75% of DOR was excreted in urine when compared with the control. The percent amounts of DOR and 3-MM excreted in the urine in the presence of Asafetida (compared to control) were about 75% and 50%, respectively.

4. Discussion

The *in vitro* as well as *in vivo* investigations were carried out to study the effect of Asafetida resin (ethanolic extract and powder) on the metabolic activities of CYP2D6 and CYP3A4 hepatic enzymes. Human liver microsomes were used for *in vitro* investigation. The *in vivo* study was conducted on healthy human volunteers. Dextromethorphan and its metabolite were measured in urine samples. *In vitro* microsomal model was used as a rapid screening tool for interaction studies. However, *in vitro* observation does not guarantee whether the same effect will occur in clinical practice, because the contribution of some physiological factors remains unaccounted. Therefore, clinical significance of an *in vitro* study cannot be adopted without *in vivo* confirmation. Thus the formation of DEX metabolites under *in vitro* and *in vivo* conditions was used to assess the metabolic activities of CYP2D6 and CYP3A4. DEX has been used as a probe for CYP2D6 and CYP3A4 enzymes. It is a non-narcotic antitussive agent, safe, effective and non-invasive marker. It is metabolized by N- and O-demethylations catalyzed by CYP2D6 and CYP3A4 to form metabolites such as DOR and 3-MM, respectively. Asafetida significantly inhibited the formation of DOR in liver microsomes. This *in vitro* inhibition was observed as concentration dependent and suggested that Asafetida may have the potential to alter the pharmacokinetics of CYP2D6. But on contrary Asafetida potentiates the *in vitro* activity of CYP3A4 at lower concentrations, but did not produce any remarkable effect at the highest concentration.

Although activation of CYP-mediated reactions has been reported in several previous *in vitro* studies (Atkins et al., 2001; Hutzler et al., 2003; Korzekwa et al., 1998; Tracy, 2006), its *in vivo* relevance is still unclear. Unlike induction, activation is not associated with an increase in the protein level of the activated enzyme but only with its activity. One possible mechanism of CYP3A4 activation, which has been previously suggested by several investigators, is the presence of multiple binding sites at the enzyme active site (Atkins et al., 2001; Korzekwa et al., 1998; Tracy, 2006). This explanation is supported by the fact that several CYP3A4 activators are also known to be metabolized by this enzyme. Based on that, the activation by Asafetida observed in this *in vitro* study could be due to the ability of at least one of its constituents to bind to the CYP3A4 binding site leading to an increase in the rate of 3-MM formation possibly by inducing conformational changes in the enzyme. Activation of CYP3A4 by Asafetida has not been reported before and further *in vitro* studies that investigate the potential effects of individual Asafetida constituents would be helpful in understanding the mechanism of the observed activation, which is out of the scope of this study. Although *in vitro* metabolic activation has not been confirmed *in vivo* before, the opposite significant inhibitory effect on 3-MM observed in the clinical study is difficult to explain. However, such opposite effects suggest that this interaction may be time and concentration dependent. Dose of Asafetida and duration of treatment may influence the outcome of the interaction. Another possible explanation for the significant *in vivo* inhibition that was not seen *in vitro* could be due to the ability of Asafetida to affect CYP3A4 protein expression, which does not occur in the microsomal *in vitro* model and is considered to be one of its major limitations.

Numerous clinical studies have reported the effects of some natural products on the metabolism of conventional drugs.

Table 1 Urinary metabolic ratio (MR) of DEX with its metabolites in human subjects ($n = 6$).

Subjects	Urinary metabolic ratio DEX /DOR		Urinary metabolic ratio DEX/3-MM	
	MR, control	MR, phase-II	MR, control	MR, phase-II
1	0.214	0.264	7.522	12.034
2	0.015	0.026	2.012	3.949
3	0.020	0.063	4.641	16.070
4	0.054	0.072	5.530	7.948
5	0.055	0.094	1.532	3.673
6	0.076	0.057	1.314	1.301
Mean	0.072	0.096	3.759	7.496
SD	0.073	0.085	2.532	5.655
P value	0.037		0.036	

Therefore, the clinical study was conducted to confirm the *in vitro* results. *In vivo* modulation of metabolic activity of CYP2D6 and CYP3A4 in the presence of Asafetida was assessed by determining the urinary metabolic ratio of DEX/DOR and DEX/3-MM. Clinical study findings suggest that Asafetida has a significant inhibitory effect on human CYP3A4 and a marginal effect on CYP2D6. These findings were consistent with animal studies conducted on rats, wherein oral administration of *F. asafetida* for one week resulted in the reduction of mRNA and protein of CYP3A, in rat liver (Data unpublished).

5. Conclusion

In conclusion, the significant Asafetida inhibitory effect on CYP3A4 metabolic activity was an important finding of this study. Based on that, taking Asafetida with CYP3A4 drug substrates should be cautioned, especially those known to have narrow therapeutic index such as cyclosporine, tacrolimus, everolimus, carbamazepine and quinidine.

Declaration of interest

The author(s) declare that they do not have any conflict of interests and approved the final version of this article.

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