Impact of Herbal Medicines like Nigella sativa, Trigonella foenum-graecum, and Ferula asafoetida, on Cytochrome P450 2C11 Gene Expression in Rat Liver

Authors
H. M. Korashy1, F. I. Al-Jenoobi2, M. Raish1, A. Ahad1, A. M. Al-Mohizea2, M. A. Alam2, K. M. Alkharfy1,4, S. A. Al-Suwayeh2

Affiliations
1 Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
2 Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
3 Department of Clinical Pharmacy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
4 Biomarkers Research Program, King Saud University, Riyadh, Saudi Arabia

Abstract

Aim: Combined use of herbs and drugs may result in clinically important herb-drug interactions. The majorities of these interactions are thought to be metabolism-based and involve induction or inhibition of cytochrome P450 (CYP). The current study was designed to investigate the effect of some commonly used herbs on rat CYP2C11 gene expression and metabolic activity.

Methods: Wistar rats were treated for 7 days with increasing doses of 3 herbs; Nigella sativa, Trigonella foenum-graecum, and Ferula asafoetida. Thereafter, CYP2C11 mRNA and protein levels were determined by real-time polymerase chain reaction (RT-PCR) and western blot analyses, respectively. In vitro metabolic activity of CYP2C11 was performed on rat hepatic microsomes using tolbutamide as specific substrate.

Results: Our results showed that all the 3 herbs significantly inhibited the mRNA and protein expression levels of CYP2C11 in a dose-dependent manner. Furthermore, the in vitro enzyme metabolic activity study showed a significant decrease in the formation of 4-hyroxy-tolbutamide, a tolbutamide metabolite, at the higher doses. The inhibitory effects of the investigated herbs on rat CYP2C11 was in the order: Nigella Sativa > Trigonella foenum-graecum > Ferula asafoetida.

Conclusions: The 3 herbs are strong inhibitor of CYP2C11 expression, which can lead to an undesirable pharmacological effect of clinically used CYP2C11 substrate drugs with a low therapeutic index.

Introduction

Over the past decades, herbal medicines are increasingly used as they are usually assumed to be safe and harmless. The World Health Organization (WHO) has recently reported that approximately 30–50% of total medicinal consumption in China is herbal preparation, whereas between 50–70% of North Americans and Europeans have used herbal medicine at least once in their lives [1,2]. Herbal drug supplements have traditionally been used alone or in combination with prescribed drugs for the treatment of several medical illnesses [3,4]. Such combinations could be associated with alterations in the pharmacokinetics and/or pharmacodynamics of the drugs. The majorities of these interactions are thought to be metabolism-based and involves induction or inhibition of the cytochrome P450 (CYP) system in the liver and other important organs. The CYP enzymes are a super family of haem containing enzymes. In humans, CYP enzymes are important in the production of compounds such as cholesterol, corticosteroids and fatty acids. The most important feature of the CYP enzymes is its unique role in the intra-cellular metabolism by incorporation of an oxygen atom stereo-specifically into inert chemical bonds [3,5]. Among CYPs, CYP2C subfamily represents an important group of isozymes for drug metabolism in human, as these constitute about 16% of the total hepatic CYP complement, and is known to be responsible for the metabolism of roughly 15% of drug oxidations. Human CYP2C9 is a major CYP enzyme involved in the metabolism of a wide range of therapeutic agents, including non-steroidal anti-inflammatory drugs, oral anticoagulants and oral hypoglycemic agents [5]. CYP2C9 also contributes to the metabolism of fatty acids, prostanoids and steroid hormones. The rat forms of the human CYP2C9 equivalent CYP2C isoforms include CYP2C6 and CYP2C11, with CYP2C11 being a 77% homologue of the human CYP2C9 and the more abundantly expressed CYP in rat [6].
The majority of serious cases of drug interactions are as a result of the interference of the metabolic clearance of one drug by yet another co-administered drug, food or natural product. Gaining mechanistic knowledge towards such interactions has been accepted as an approach for avoiding adverse reactions. To date, researches regarding drug-herb interactions are limited mostly to case reports and a few systematic reviews [7–9]. Herbal drug supplements in the world have traditionally been used alone or in combination with prescribed drugs for the treatment of several medical illnesses [4]. A number of herbal remedy-drug interactions have been reported [10]. For example, it has been reported that herbal medicine and supplements have differentially modulated the induction of experimental as well as human CYP [11]. Some of the recent investigations have suggested modulations of CYP-mediated drug elimination as a major mechanism responsible for such interactions [12]. As a consequence, concomitant administration of these herbal medicines and clinically used drugs very likely raises the potential for herbal-drug interactions via altering the activity of certain CYP enzymes which may lead to serious clinical consequences.

Several herbs are commonly used in many Asian countries for a variety of health-related problems. For example, black seed, the dried ripe seed of *Nigella sativa* Linn. (Family; Ranunculaceae), is used as a folk medicine for the treatment of various ailments such as hypertension, diabetes, inflammation, asthma and dyslipidemia [1, 13]. The important chemical constituents in the herb include fixed oil (30–33%) and a volatile oil (0.43–0.72%) wt/wt of the seeds weight which contains thymoquinone (18.4–24%), p-cymene (31.7%), and α-pinene (9.3%) [13, 14]. Fenugreek herb is the dried ripe seed of *Trigonella foenum-graecum* Linn. (Family Fabaceae), which is used in treating kidney diseases, cellulites, diabetes, and tuberculosis in addition to its hypolipidemic and anticoagulant activities [15, 16]. The chemical constituents of fenugreek are alkaloids containing trigonelline (0.13%), choline (0.05%), gentianne, carpine and saponine [17]. Asafoetida, the gum resin obtained from *Ferula asafoetida* Linn. (Family Umbelliferae), is commonly used as spices and condiments as well as treatment of various ailments such as anti spasmodic, carminative, laxative, expectorant and vermifuge [18]. An analysis of asafoetida shows it consists of 40–64% resinos materials composed of ferulic acid, umbel-lifone, asaresitannols, and far-nesiforols A, B, and C, in addition to volatile oils (3–17%) [17–19]. However, the in vivo effects of these herbal medicine to potentially modulate the expression of CYP2C11 level have not been investigated yet. Therefore, the objectives of the current study was to investigate the potential effects of 3 commonly used local herbal medicines, *Nigella sativa*, *Trigonella foenum-graecum*, and *Ferula asafoetida*, on the expression of CYP2C11 gene at the mRNA, protein, and metabolic activity levels in rat liver tissues.

**Materials and Methods**

**Materials**

Protease inhibitor cocktail, tobutamide (TB), and 4-hydroxytolbutamid (4-OH-TB) were purchased from Sigma-Aldrich (St. Louis, MO). TRizol reagent was purchased from Invitrogen Co. (Grand Island, NY). The High-Capacity cDNA reverse transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems® (Foster City, CA). Rat forward and reverse primers were obtained from Integrated DNA Technologies (IDT) (Coralville, IA). Chemiluminescence Western blotting detection reagents was obtained from GE Healthcare Life Sciences (Piscataway, NJ). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, CA). CYP2C11 and β-actin polyclonal primary antibodies, and IgG peroxidase secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-Nicotinamide adenine dinucleotide 2’-phosphate reduced tetratsodium salt hydrate (β-NADPH) was purchased from Bio Basic Inc. (Markham, ON, Canada). Potassium dihydrogen phosphate and HPLC-grade acetonitrile were obtained from Fisher Scientific (Leicesteshire, UK) and Winlab (Leicesteshire, UK) respectively. Nitrazapam was of BP reference standard. All chemicals used were of the highest available commercial purity. All the herbs were purchased in dry form from Saudi market. HPLC grade solvents were used for HPLC determinations. All other chemicals were purchased from Fisher Scientific Co. (Toronto, ON).

**Herbal medicine preparation**

Crude herbs *Nigella sativa*, *Trigonella foenum-graecum* and *Ferula asafoetida* were purchased from a well-known herbal medicine supplier (Bin-Menkash, Riyadh) and were authenticated by taxonomist, Department of Pharmacognosy College of Pharmacy, King Saud University. A voucher specimen was deposited at the Herbarium of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The shade dried herbs were powdered and stored in dried sterile containers until use. The reported human doses for the selected herbs *Nigella sativa*, *Trigonella foenum-graecum* and *Ferula asafoetida* were approximately 3, 5, and 3 g/day respectively [19–21]. The equivalent rat doses for investigated herbs were determined by using following equation [22]:

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\text{Rat equivalent dose (mg/kg)} = \left( \frac{\text{human dose (mg/kg)}}{\text{human body weight (kg)}} \times \text{body weight factor} \right) \times \text{Rat body weight factor} \times \frac{\text{Rat body weight (kg)}}{\text{human body weight (kg)}}
\]

Where, *Km* for rat is 6 while *Km* for human is 37. The equivalent rat doses of *Nigella sativa*, *Trigonella foenum-graecum* and *Ferula asafoetida* herbs were found to be around 300, 500, and 300 mg/kg, respectively. Accordingly, the dose ranges for each herb were as follows: *Nigella sativa* (50–400 mg/kg), *Trigonella foenum-graecum* (25–600) and *Ferula asafoetida* (50–450). The equivalent rat doses were selected on the basis of calculated human dose to rule out the percentage influence of active constituent within the herbs. The ranges of the above doses were selected in the current study on the basis of their effectiveness in modulating CYP2C11 activity in rats and are within the usual dose range used by humans.

**Animal**

Adult male Wistar albino rats, weighing 230–250g, were maintained under standard laboratory conditions of a 12-h light/dark cycle at 25°C±2°C. The animals had free access to pulverized standard rat pellet diet with water *ad libitum* and fasted overnight prior to the experiments. The study was approved by the Experimental Animal Care Committee, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. All animals were maintained in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80–23; 1996).
Experimental design and treatments

Wistar albino rats, randomly assigned into 6 groups of 6 rats each for each herb, were treated by oral administration by gavage every day for 7 consecutive days with increasing doses of 3 herbs in normal saline as follows: *Nigella sativa* seed suspension (0, 50, 100, 200, 300, and 400 mg/kg), *Trigonella foenum-graecum* seed suspension (0, 75, 150, 300, and 750 mg/kg), and *Ferula asafoetida* oleogum resin suspension (0, 50, 75, 150, 300, 450 mg/kg). These animal doses have been selected based on the equivalent human doses using specific equation [22]. All rats were sacrificed on the day 8th and liver tissues were dissected for the preparation of liver microsomes (used for the determination of CYP2C11 activity and protein expression) and homogenates (used for the determination of CYP2C11 mRNA expression levels).

RNA extraction and cDNA synthesis

The total cellular RNA from the liver tissues was isolated using TRIzol reagent (Invitrogen®) according to the manufacturer’s instructions and quantified by measuring the 260/280 ratio (>1.8). Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems®), according to the manufacturer’s instructions as described previously [23]. Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μl of 10 × reverse transcriptase buffer, 0.8 μl of 25 × dNTP mix (100 mM), 2.0 μl of 10 × reverse transcriptase random primers, 1.0 μl of MultiScribe reverse transcriptase, and 3.2 μl of nuclease-free water. The final reaction mixture was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 min, and finally cooled to 4 °C.

Quantification of mRNA expression by Real-Time Polymerase Chain Reaction (RT-PCR)

Quantitative analysis of CYP2C11 mRNA expression was performed by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems®) [23]. The 25-μl reaction mixture contained 0.1 μl of 10 μM forward primer and 0.1 μl of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μl of SYBR Green Universal Master mix, 11.05 μl of nuclease-free water, and 1.25 μl of cDNA sample. Rat primers for CYP2C11 (forward: CACCACATCTACGTGAGTTGG and reverse: GTCTGCCCTTTTGACACCGAA) and β-actin (forward: CGACATCATGTGGTGAGACCTTCA and reverse: GTGGTACGACACAGGCTACA) were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The fold change in the level of these genes between treated and untreated rates were corrected by the levels of β-actin. The RT-PCR data were analyzed using the relative gene expression (i.e., ∆∆Ct) method, as described and explained previously [24]. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene β-actin and relative to a calibrator. The fold change in the level of CYP2C11 between treated and untreated cells, corrected by the level of β-actin, was determined using the following equation: fold change = 2−ΔΔCt , where ∆Ct = Ctgt(target)−Ctgt(β-actin) and ΔΔCt=ΔCttreated−ΔCtuntreated.

Microsomal incubation conditions

Incubation conditions were selected based on previous study [27]. TB (5.0 μl of 15 mM to give a final concentration of 0.15 mM) was added to the tubes before the addition of rat liver microsomes (0.25 mg protein/ml final concentration) and potassium phosphate buffer (0.1 M, pH 7.4), then mixed gently, and pre-incubated in a shaker water bath at 37 °C for 30 min. The reaction was initiated by the addition of 25 μl of 20 mM NADPH (1.0 mM final concentration) to complete a final volume of 0.5 ml, and incubated for a further 30 min. Moreover, negative microsomal incubations were performed without adding NADPH or without microsomes. The reaction was terminated by the addition of cold methanol (250 μl) with vigorous shaking for 2.0 min. Nitrazepam (25 μl) from a stock solution of 1.0 μg/ml was added as an internal standard to each tube. The mixture was centrifuged at 9000 × g for 10 min; a 500 μl of the supernatant was transferred to HPLC autosampler vial and 50 μl of that was injected for analysis. Formation of the metabolite was found in the preliminary experiments to be linear with respect to time and protein concentration under the described conditions.

Analysis of 4-OH-TB in rat liver microsomes

The formation of 4-OH-TB in liver microsomes was analyzed using previously published HPLC methods with slight modification [27,28]. A symmetry column (150 × 3.9 mm ID, 5 μm) purchased from Waters® (Milford, MA) was used for this assay.

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Mobile phase consisted of acetonitrile: 0.02 M potassium dihydrogen orthophosphate (25:75; pH adjusted to 3.4 with orthophosphoric acid). The flow rate was 1.5 ml/min. The detector was operated at fixed wave length at 230 nm. Stock solutions of TB, and 4-OH-TB were prepared in acetonitrile and methanol; respectively. Calibration curve for 4-OH-TB was constructed using a concentration range of 0.05–2.5 μM. Each calibration curve was prepared to contain a fixed concentration of TB (0.15 mM), microsomes and buffer but without the addition of NADPH.

### Statistical analysis

The comparative analysis of the results from various experimental groups with their corresponding controls was performed using Sigma Stat® for Windows (Systat Software, Inc, CA). One-way analysis of variance (ANOVA) followed by Dunnett’s test was carried out to assess which treatment groups showed a significant difference from the control group. The differences were considered significant when p<0.05.

### Results

#### Effect of herbs on the mRNA expression of CYP2C11

RT-PCR analysis demonstrated that all tested herbs significantly down-regulated the mRNA expression of CYP2C11. For example, *Nigella sativa* at doses 100–400 mg/kg markedly and significantly inhibited CYP2C11 mRNA levels by approximately 83% (Fig. 1a). On the other hand, only higher doses of *Trigonella* (300 and 600 mg/kg) significantly decreased the expression of CYP2C11, by approximately 35% (Fig. 1b). Similar to *Nigella sativa*, treatment of rats with *Ferula asafoetida* significantly decrease (60%) CYP2C mRNA at all doses, with the exception of dose 50 mg/kg which did not significantly alter the mRNA expression of CYP2C11 (Fig. 1c). Taken together, these results clearly suggest a transcriptional regulation of CYP2C11 by the investigated herbs.

#### Effect of herbs on the protein expression of CYP2C11

Western blot analysis showed that the CYP2C11 protein levels were significantly down-regulated by all tested doses of *Nigella sativa* and *Trigonella foenum-graecum* herbs in a dose-dependent fashion, and only by the higher doses of *Ferula asafoetida*, in a manner similar to the RNA results. For example, the highest doses of *Nigella sativa*, *Trigonella foenum-graecum*, and *Ferula asafoetida* inhibited CYP2C11 protein levels by approximately 90%, 85%, and 40%, respectively (Fig. 2).

#### Effect of herbs on the metabolic activity of CYP2C11

Effects of the herbs were evaluated based on the formation of CYP2C11-mediated 4-OH-TB from TB in rat liver microsomes. Our results showed that *Nigella sativa*, *Trigonella foenum-graecum* and *Ferula asafoetida* inhibited CYP2C11-mediated 4-OH-TB formation in a dose-dependent manner. *Nigella sativa* exhibited the maximum inhibitory effect on the CYP2C activity by approximately 53% at the highest dose, 400 mg/kg (Fig. 3a). Whereas, *Trigonella foenum-graecum* and *Ferula asafoetida* decreased CYP2C11 metabolic activity by approximately 43% and 39%, respectively at their highest doses (Fig. 3b, c).

### Discussion and Conclusions

Traditional medicine has received great attention as an alternative clinical therapy because its therapeutic efficacy is mild and broad, and the incidence of adverse reactions is relatively low in comparison with synthetic drugs. However, combined use of herbs and drugs may increase or reduce the effects of either component, which may result in clinically important herb-drug interactions [29].

Many herbs and natural compounds isolated from herbs have been identified as inhibitors of CYP2C enzyme, and hence may interfere with drug metabolism causing drug- and food-drug in interactions and toxicities. CYP2C9 is one of the most abundant CYP enzymes in the human liver (20% of hepatic total CYP content), where it metabolizes approximately 15% clinical drugs (~4 100 drugs), including a number of drugs with narrow thera-

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**Fig. 1** Effect of a *Nigella sativa*, b *Trigonella*, and c *Ferula asafoetida* on CYP2C11 mRNA levels in rat liver. CYP2C11 mRNA were quantified using RT-PCR and normalized to β-ACTIN housekeeping gene as described in the Materials and Methods. Duplicate reactions were performed for each experiment and the values represent mean of fold change ± SEM, n = 6. *p*<0.05 compared to control.

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The induction or inhibition on activity of CYP2C9 may lead to some undesirable effects. For example, it has been suggested that the use Danshen, (*Salvia miltiorrhiza*), a popular traditional Chinese medicinal herb that is used for the treatment of cardiovascular disease, with drugs that are particularly metabolized by CYP2C, such as warfarin, should be monitored [31]. In addition, polysaccharide peptides from *Coriolus versicolor* are known to inhibit TB 4-hydroxylation in the rat in vitro and in vivo [6]. Additionally, several studies have demonstrated pharmacokinetic interactions of herbs specifically *Ferula asafoetida*, *Trigonella foenum-graecum*, and *Nigella sativa* with drugs in animal models [32–36]. Unfortunately, none of these studies have investigated the molecular mechanisms involved. Therefore, in the current study we have initially addressed the question of whether herbs are able to modulate the CYP2C11 at the gene expression. For this purpose, rats were pretreated with wide-range doses of investigated herbs were analyzed hepatic CYP2C11 mRNA levels. Our results show that all tested herbs significantly down-regulated the mRNA expression of CYP2C11. Importantly, we have showed that the inhibitory effects of herbal medicine on CYP2C11 mRNA in herbs-treated rats is translated into a decrease in the protein function at the translational levels, we measured the CYP2C protein levels using Western blot analysis.

![Fig. 2](image1.png) **Fig. 2** Effect of a Nigella sativa, b Trigonella, and c Ferula asafoetida on CYP2C11 protein levels in rat liver. CYP2C11 protein levels were determined by Western blot analysis and then detected using the enhanced chemiluminescence method as described in the Materials and Methods. One of the 3 representative experiments is shown. Values are presented as mean ± SEM, n = 3. + p < 0.05 compared to control.

![Fig. 3](image2.png) **Fig. 3** Effect of a Nigella sativa, b Trigonella, and c Ferula asafoetida on the formation of 4-OH-tolbutamide rat liver microsome. Liver microsomes were incubated with tolbutamide, CYP2C11 substrate, and the formation 4-OH-tolbutamide metabolite was determined by HLPC as described in the Materials and Methods. Values are presented as mean ± SEM, n = 3. + p < 0.05 compared to control.
were significantly down-regulated by all investigated herbs, *Nigella sativa* (90% at 400 mg/kg), *Trigonella foenum-graecum* (80% at 600 mg/kg), and *Ferula asafoetida* (40% at 450 mg/kg) showed significant inhibition of hepatic CYP2C11 protein expression in similar manner to the mRNA results. These results are in agreement with a previous study showed that *Nigella sativa* significantly inhibited CYP2C11 mRNA and protein levels in rats [2]. To further substantiate the inhibitory effect of the investigated herbs, we examined the effect on the CYP2C11 metabolic activity in rats liver microsomes. The most widely used substrate probes for determining CYP2C9 activity in liver microsomes are (S)-warfarin (7-hydroxylation), tolbutamide (methylhydroxylation), and diclofenac (4-hydroxylation). Among these substrates, CYP2C9 is essentially solely responsible for TB hydroxylation and thus TB is accepted widely as a prototypic substrate for the assessment of hepatic CYP2C9 activity, both in vitro and in vivo [30]. Therefore, we measured the catalytic activity of CYP2C11 by measuring the ability of herbs to decrease the hepatic microsomal formation of 4-OH-TB. Fig. 3 shows that incubation of liver microsomes obtained from *Nigella sativa* treated rats with TB significantly decreased the formation of 4-OH-TB by all tested doses to a maximum inhibition of approximately 50% at the highest doses. Likewise, *Trigonella foenum-graecum* and *Ferula asafoetida* inhibited the metabolite formation significantly.

The results of the current study clearly explore the molecular (transcriptional and translational) mechanism of inhibition of CYP2C11 gene by the herbs. These findings are in agreement with several previous studies demonstrated that *Nigella sativa*'s main constituents thymoquinone inhibited CYP2C11 enzyme [2]. In addition, some flavonoids in *Trigonella foenum-graecum*, such as vitexin, tricin, naringenin, querceatin and tricin-7-O-beta-D-glucopyranoside are known CYP inhibitors [37–39]. Moreover, terpenoidal and ferulic acid compounds in *Ferula asafoetida* are CYP2C modulators [40, 41]. Taken together, it could be postulated that herbs constituents act as competitive or non-competitive inhibitors of CYP2C11, depending on whether they bind to the substrate binding site or an allosteric binding site of the enzyme and further studies are needed [42].

In conclusion, the present work provided the first evidence that 3 commonly used herb remedies *Nigella sativa*, *Trigonella foenum-graecum* and *Ferula asafoetida* significantly inhibited the expression of CYP2C11 gene at the mRNA, protein, and metabolic activity levels in rats liver and hence may increase the risk of toxicity from co-administered drugs that are CYP2C11 substrates with narrow therapeutic indices such as warfarin, TB, and phenytoin [39].

**Acknowledgement**

This project was supported by NSTIP strategic technologies programs, number (BIO1046-02-10) in the Kingdom of Saudi Arabia

**Conflict of Interest**

There are no financial or other interests with regard to this manuscript that might be construed as a conflict of interest. All of the authors are aware of and agree to the content of the manuscript and their being listed as an author on the manuscript.

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