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Potential inhibitory effect of herbal medicines on rat hepatic cytochrome P450 2D gene expression and metabolic activity

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The aim of current study was to investigate the effect of some commonly used medicinal herbs on the regulation of rat CYP2D gene expression and its metabolic activity. Wistar albino rats were treated for seven consecutive days with selected doses of five commonly used herbs (*Trigonella foenum-graecum, Ferula asafoetida, Nigella sativa, Commiphora myrrha and Lepidium sativum*). Thereafter, rat livers were harvested and CYP2D mRNA levels were determined by RT-PCR. The metabolic activity of CYP2D was performed on rat hepatic microsomes using dextromethorphan as specific substrate. All investigated herbs produced inhibition of CYP2D mRNA expression and metabolic activity. The inhibitory potential of investigated herbs on rat CYP2D mRNA was in the following order: *Commiphora myrrha* > *Nigella sativa* > *Lepidium sativum* > *Trigonella foenum-graecum* > *Ferula asafoetida*. Whereas, the inhibitory potential of investigated herbs on CYP2D mediated enzyme metabolic activity was found in following order: *Nigella sativa* ≥ *Lepidium sativum* > *Trigonella foenum-graecum* > *Commiphora myrrha* > *Ferula asafoetida*. The current study shows that only used herbs reduce CYP2D activity in rat liver microsomes at the transcriptional levels. Such effects could lead to undesirable pharmacological effects of clinically used low therapeutic index CYP2D substrate drugs.

1. Introduction

A number of studies have reported many herb drug interactions (Huang and Lesko 2004; Ioannides 2002) some of them (St. John's wort, common valerian, common sage, *Ginkgo biloba*, *Echinacea purpurea* and horse chestnut) inhibit CYP2D metabolic activity in cultured primary human hepatocytes (Hellum and Nilsen 2007). Zhao et al. (2011) found *Peganum harmala* to act as a substrate and inhibitor for CYP2D and suggested that it may alter the pharmacokinetics of co-administered CYP2D substrate drugs. Whereas alcoholic extracts of curcuma and aniseed significantly altered the CYP2C9 metabolic activity in human liver microsomes (Al-Jenoobi 2010). Other examples of herb-drug interactions, were reposted in typical studies on St. John's wort (Rengelshausen et al. 2005), grapefruit juice (Paine et al. 2005) and garlic (Van den Bout-van den Beukel et al. 2006). Some of the investigations have suggested the modulation of cytochrome P450 (CYP) enzymes mediated drug elimination as a major mechanism responsible for such types of interactions (Gurley et al. 2002).

Amongst the six human CYP enzymes, CYP2D ranks second to CYP3A4 in terms of the number of drugs whose biotransformation it facilitates. Classes of drugs whose pharmacokinetic profiles are dictated by CYP2D include antidepressants, antipsychotics, β-receptor antagonists, analgesics, and antiarrhythmic agents (Ingelman-Sundberg 2005). Inhibition of drug-metabolizing enzymes may lead to increased plasma levels of drugs administered concomitantly, to prolong the pharmacological effects, and to increase drug toxicity (Silverman 1988). To the best of our knowledge no information is available regarding the effects of the herbs *Trigonella foenum-graecum, Ferula asafoetida, Nigella sativa, Commiphora myrrha* and *Lepidium sativum* on the mRNA expression and metabolic activity of the CYP2D gene.

2. Investigations and results

In this study, Wistar albino rats were treated with these herbal medicines. Then, rat livers were harvested and CYP2D mRNA levels were determined using RT-PCR. The effect of the investigated herbs on metabolic activity of CYP2D was also determined on rat hepatic microsomes using dextromethorphan as specific substrate. It was observed that all tested herbs were able to significantly inhibit the mRNA expression levels of CYP2D. In particular, *Commiphora myrrha, Nigella sativa, Trigonella foenum-graecum* and *Ferula asafoetida* caused dose-dependent inhibition. Among the investigated herbs, *Commiphora myrrha* showed the most potent inhibition (83%) on CYP2D mRNA expression at doses ≥ 200 mg/kg (Fig. 1A) followed by *Nigella sativa* (78%) (Fig. 1B), whereas the least inhibition was observed with *Ferula asafoetida* (66%) (Fig. 1E).

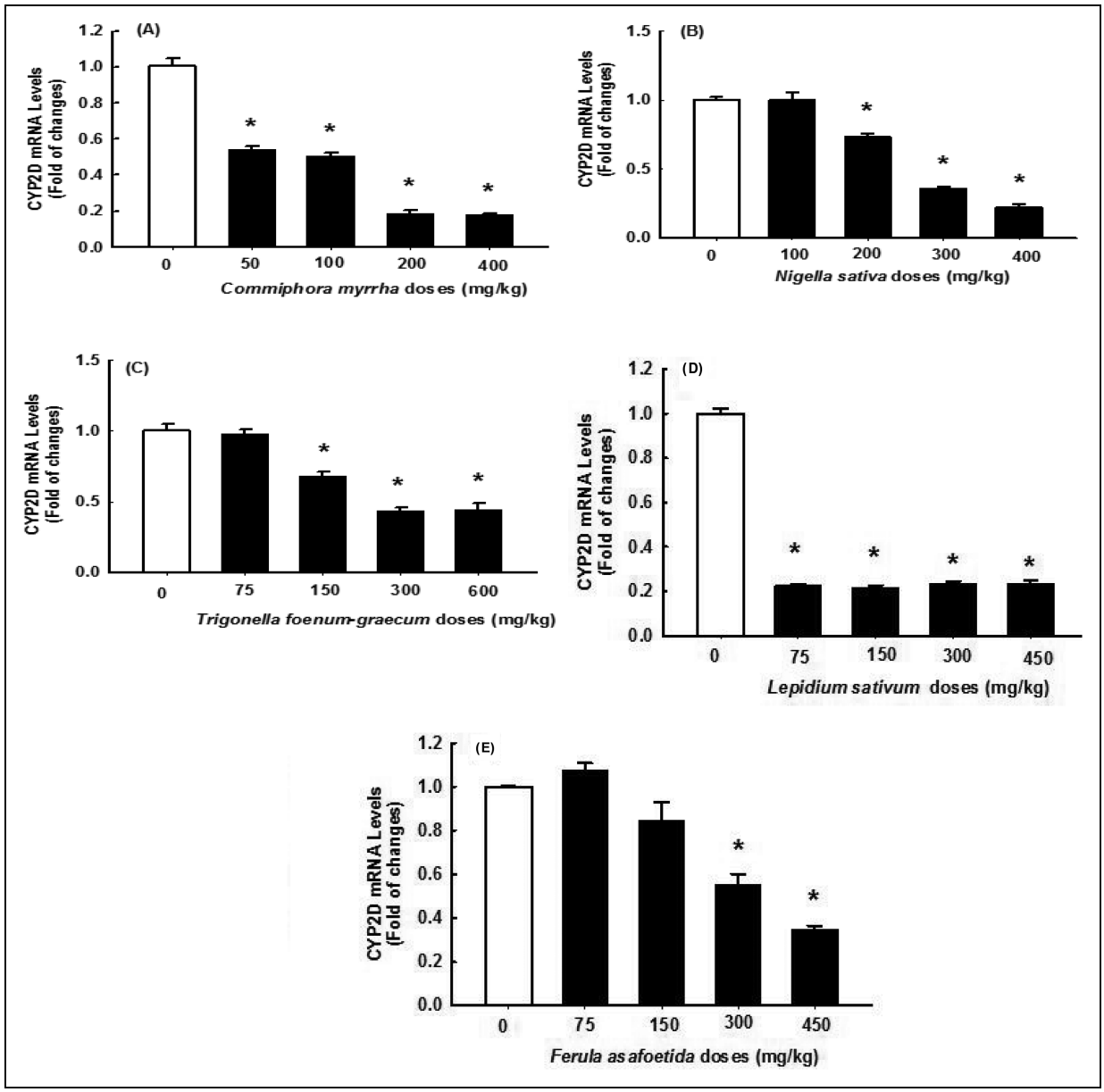

Fig. 1: Effect of (A) *Commiphora myrrha*, (B) *Nigella sativa,* (C) *Trigonella foenum-graecum*, (D) *Lepidium sativum,* (E) *Ferula asafoetida* on CYP2D mRNA expression in rat liver (mean ± SEM, $*p < 0.05$).

We further investigated these herbs for their metabolic activity using DEX as a probe drug in rat liver microsomes (Fig. 2).

DEX is extensively metabolized in the liver by N and O-demethylations. O-Demethylation is primarily metabolized by CYP2D to form DOR. It was observed that *Commiphora myrrha* inhibits CYP2D activity in rat liver microsomes as compared with control. *Commiphora myrrha* produced a dose dependent inhibition on the CYP2D metabolic activity. The maximum ($p < 0.01$) decrease in the formation of DOR (32%) was observed at the higher dose of *Commiphora myrrha* i.e 400 mg/kg (Fig. 2A). Amongst the investigated herbs, *Nigella sativa* and *Lepidium sativum* produced a maximum inhibitory effect on CYP2D-mediated DOR formation. *Nigella sativa* produced significant inhibition of CYP2D activity and a decrease in the formation of DOR was observed (Fig. 2B). The maximum ($p < 0.01$) inhibition (about 36%) of metabolite formation was observed at its highest doses (400 mg/kg) (Fig. 2B). It was observed that *Trigonella foenum-graecum* also inhibited CYP2D activity. It significantly ($p < 0.001$) decreased the formation of DOR at higher doses and the maximum decrease in metabolite formation (about 33%) was observed at the dose of 300 mg/kg of *Trigonella foenum-graecum* (Fig. 2C). While in case of *Lepidium sativum,* maximum inhibition ($p < 0.001$) was produced by 75 mg/kg of the herb (36%) (Fig. 2D). On the other hand metabolic activity of CYP2D was not modulated by *Ferula asafoetida* at all tested doses (Fig. 2E).

The combined use of herbs and drugs may increase or decrease the effects of either component, which may result in clinically important herb–drug interactions (Venkataramanan et al. 2006). Many authors have reported that medicinal herbs induce/inhibit CYP-mediated metabolism of therapeutics drugs. As a well-known example, St John's wort has been reported to decrease the blood concentrations of drugs by inducing hepatic CYP3A4 and CYP2E1 activities (Gurley et al. 2002; Izzo 2004). Grapefruit juice is an inhibitor of the CYP3A4 enzyme and interacts with a variety of medications, such as cyclosporin and some 1,4-dihydropyridine calcium antagonists, leading to elevated plasma concentrations (Dahan and Altman 2004). Many patients frequently take both herbal medicine and conventional medicines without noticing any drug interactions. However, for the safe

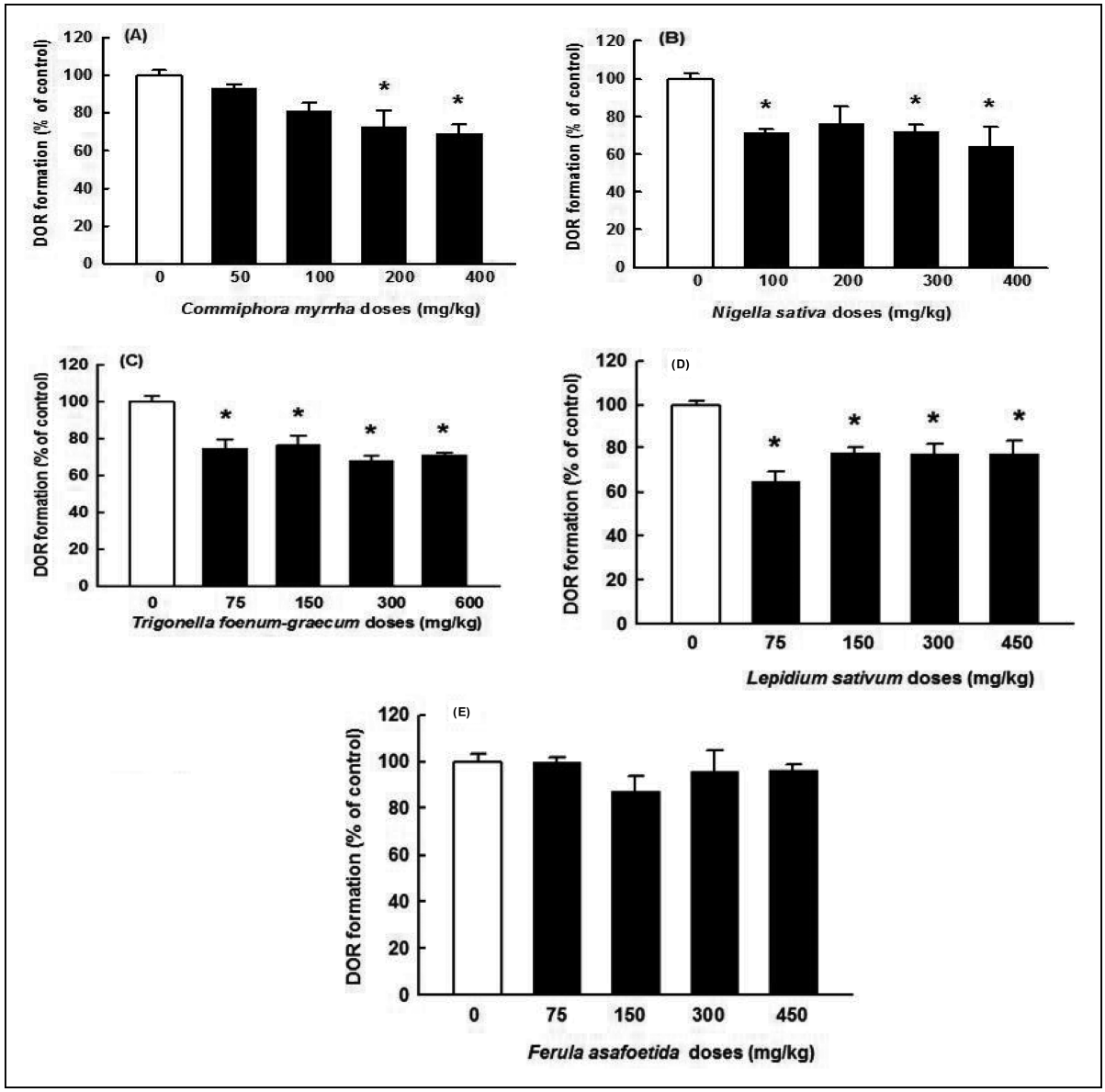

Fig. 2: Effect of (A) *Commiphora myrrha*, (B) *Nigella sativa,* (C) *Trigonella foenum-graecum,* (D) *Lepidium sativum*, (E) *Ferula asafoetida* on CYP2D-dependent DOR formation in rat liver microsomes (mean ± SEM, $*p < 0.05$).

use of herbal medicine, it is important to indicate any possibility of CYP-mediated interactions between herbal medicine and concomitantly administered drugs.

To address this issue, the effects of *Trigonella foenum-graecum*, *Ferula asafoetida*, *Nigella sativa*, *Commiphora myrrha*, and *Lepidium sativum* on CYP2D expression levels and on CYP2D mediated metabolic activity were determined.

Among the investigated herbs, *Commiphora myrrha* and *Nigella Sativa* showed potent inhibition on CYP2D mRNA expression. These results are in agreement with the previous reports that *Nigella sativa* significantly inhibited the metabolic activity of CYP2D enzyme (Al-Jenoobi et al. 2010, 2013). There are many reports that CYP2D is inhibited by herbs such as St. John's wort, Ginseng, Curcuma,Vinca and *Angelica dahurica* (Radix) (Obach 2000; Asano et al. 2001; Chang et al. 2002; Shinozuka et al. 2002).

To further substantiate our mRNA results, the inhibitory potential of selected herbs on microsomal metabolic activity was evaluated. The inhibitory potential of studied herbs on CYP2D was in following order *Nigella sativa* (36%) ≥ *Lepidium sativum* (36%) > *Trigonella foenum-graecum* (33%) > *Commiphora myrrha* (32%) > *Ferula asafoetida* (13%) using DEX as a probe drug in rat liver microsomes. These results further confirm that CYP2D is inhibited by studied herbs on transcriptional as well as translational level.

In conclusion, the present study indicated that *Commiphora myrrha*, *Nigella sativa*, *Lepidium sativum, Trigonella foenum-graecum* showed significant inhibition of CYP2D mRNA expression and metabolic activity, whereas the least inhibition of CYP2D was observed with *Ferula asafoetida.* These herbs could potentially inhibit the metabolism of co-administered drugs primarily metabolited by CYP enzymes.

3. Experimental

3.1. Materials

β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH) was purchased from Bio Basic Inc. (Ontario, Canada). Dextromethorphan hydrobromide (DEX) and Dextrorphan-D-Tartrate (DOR) were purchased from ICN Biomedicals, Inc. (Eschwege, Germany). TRIzol reagent was purchased from Invitrogen Co. (Grand Island, New York). The High-Capacity cDNA reverse transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Foster City, USA). Rat CYP2D forward and reverse primers were obtained from the integrated DNA Technologies (Coralville, USA). Diethyl

pyrocarbonate (DEPC) was purchased from Sigma Chemical Co. (St. Louis, USA). Potassium dihydrogen phosphate and HPLC-grade acetonitrile were obtained from Fisher Scientific (Leicestershine, UK) and Winlab (Leicestershine, UK) respectively. Codeine was of BP reference standard. HPLC grade solvents were used for HPLC determinations. All other materials are of analytical grade.

3.2. Herbal medicines and doses

The selected herbs were purchased from a local market, and authenticated by a medicinal plant expert at the Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The animal doses of these herbs were determined on the basis of equivalent human dose using the following equation (Reagan-Shaw et al. 2008). The aqueous saline suspension of each finely powdered herb was administered orally.

$$Human\ equivalent\ dose\ (mg/kg) = Animal\ dose\ (mg/Kg) \times \frac{Animal\ Km}{Human\ Km}$$

Accordingly, the rat doses that have been selected for each herbs were as follows: *Commiphora myrrha* (0, 50, 100, 200, 400 mg/kg), *Nigella sativa* (0, 100, 200, 300, 400 mg/kg), *Trigonella foenum-graecum* (0, 75, 150, 300, 600 mg/kg), *Lepidium sativum* (0, 75, 150, 300, 450 mg/kg) and *Ferula asafoetida* (0, 75, 150, 300, 450 mg/kg).

3.3. Experimental design and animals treatments

Wistar albino rats weighing approximately 200 ± 20 g, were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were maintained in accordance with the recommendations of the 'Guide for the Care and Use of Laboratory Animals approved by the center (NIH publications no. 80-23; 1996). All animals were maintained under standard laboratory conditions of a 12 h light/dark cycle at 25 ± 2 °C. The animals were given pellet diet with water *ad libitum*.

A total of 125 wistar albino rats were equally assigned to five herbs of 25 rats each. For each herb, rats were randomly divided into five groups of 5 animals each. Rats were treated with the aforementioned selected different doses from each herb for 7 consecutive days. On 8th day, the overnight fasted animals were treated and sacrificed. The liver was harvested and stored at − 80 °C, until utilized. The liver homogenates were prepared to determine CYP2D mRNA expression levels and the liver microsomes were prepared for determination of CYP2D metabolic activity.

3.4. RNA extraction and cDNA synthesis

Total cellular RNA was isolated from hepatic tissues using TRIzol reagent according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (> 1.8). Thereafter, first strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit, according to the manufacturer's instructions as described previously (Korashy et al. 2012). Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μL of 10x reverse transcriptase buffer, 0.8 μL of 25x dNTP mix (100 mM), 2.0 μL of 10x 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.

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

Quantitative analysis of specific 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, Foster city, USA) (Korashy et al. 2012). 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 CYP2D (forward: TGGACCTCAGTAACATGCCA and reverse: GATGCAAGGATCACACCTTG) and β-ACTIN (forward: CCAGATCATGTTTGAGACCTTCAA and reverse: GTGGTACGACCAGAGGCATACA) were synthesized. The fold change in the level of CYP2D between treated and untreated groups were corrected by the levels of β-ACTIN. The RT-PCR data were analyzed using the relative gene expression (i.e.,((CT) method (Livak and Schmittgen 2001). 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 target genes between treated and untreated groups, corrected by the level of β-ACTIN, was determined using the following equation: fold change $= 2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct_{(target)} - Ct_{(\beta\text{-actin})}$ and $\Delta(\Delta Ct) = \Delta Ct_{(treated)} - \Delta Ct_{(untreated)}$.

3.6. Microsomal incubation conditions

Rat hepatic microsomes were prepared by differential centrifugation method (Iba et al. 1977). Total microsomal protein content was determined at 660 nm, according to the procedure of Lowry, using bovine serum albumin as a standard (Lowry et al. 1951).

DEX solution in methanol, at a final concentration of 25 μM per incubation mixture, was transferred into clean tubes before the addition of microsomes (0.5 mg protein/mL) 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 3 min. The metabolic reaction was initiated by the addition of 1 mM NADPH and 6 mM $MgCl_2$, in a final volume of 0.5 mL, and incubated for a further 30 min. The reaction was terminated by addition of 10 μL perchloric acid (70%) with vigorous shaking for 2 min. Codeine (15 μL) from a stock solution of 100 μM/mL in methanol was added as an internal standard to each tube. The mixture was centrifuged at 13000 g for 15 min, supernatant was transferred into HPLC vials and injected for analysis of the metabolite (Bendriss et al. 2001; Al-Jenoobi et al. 2010).

3.7. 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. The differences were considered significant when $p < 0.05$.

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