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Effects of Pomegranate on Drug Metabolizing Cytochrome P450 Enzymes Expressions in Rats

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Abstract: Drugs clearance depends on cytochromes P450 enzymes activities, their inhibition can lead to overexposure and toxicity while there induction can increase drug elimination and attenuate its pharmacological effect. Currently there is a wide trend to drinking of pomegranate juice as a nutrient, therapy for some disease or even adjuvant with some treatment in the Middle East. This study aimed to investigate the possible modulator effects of pomegranate juice on drug metabolism to avoid drugs toxicity or subclinical dosing. Wistar rats were supplemented with pomegranate juice for 21 days before the administration of different CYPP450 inducers. The results showed that drinking of pomegranate juice led to attenuation of rats' hepatic CYP3A2, CYP2B1 and CYP1A1\2 and upregulation of CYP2C11 mRNA either their basal or Phenobarbital-induced, or S.III-induced expression levels. This may increase or decrease the bioavailability of these enzymes substrates drugs which may be an evidence of the potential of pomegranate juice to be a perpetrator in drug–drug interactions. This also may in part explain the pomegranate juice-chemo preventive effect against cancer. It also may affect the clearance of these drugs that are substrates of these enzymes that should be put in its right clinical considerations.

Key words: Pomegranate · Rats · Hepatic · CYP1A · CYP2B · CYP3A

INTRODUCTION

Most of the endogenous and exogenous compounds are metabolized by Cytochrome P450 enzymes (CYPs) [1]. These enzymes constitute the major enzyme family capable of catalyzing the oxidative biotransformation of most drugs and other lipophilic xenobiotics and are therefore of particular relevance for clinical pharmacology [2]. Cytochromes P450 have been shown to be involved in metabolism of drugs and toxic chemicals [3,4]. Drugs clearance depends on CYP enzymes activities therefore, their inhibition can lead to overexposure and toxicity [5]. While there induction can increase drug elimination and attenuate its pharmacological effect [6]. Induction or inhibition of cytochrome P450 enzymes is probably the most common cause for documented drug interactions [6]. A large number of factors including physiological factors (hormones, development and disease); and environmental elements can affect drug metabolizing enzymes [7]. It has been shown that hepatic CYP activities were affected by the component of feed as malnutrition in rats caused CYP [8].

Pomegranates has a high content of compounds as polyphenols in particular, ellagitannins, condensed tannins and anthocyanins[9]substances able to inactivate the products of the oxidative catabolism that trigger cell

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disorders and aging [10]. Moreover, Pomegranates contains other phytochemicals, organic and phenolic acids, sterols and triterpenoids, fatty acids, triglycerides and alkaloids [11]. Pomegranates extract has been shown to be effective against breast, lung, colon and skin cancer [12]. The polyphenols content of Pomegranate juice, especially anthocyanins and tannins are also capable to quench the effects of ultraviolet (UV) rays, contributing to minimize the primary risk factor of skin cancer [13]. It was revealed that pomegranate juice suppresses cancer activity through the combined antioxidant and anti-inflammatory effects by modulating the inflammatory cell signaling in colon cancer cells [14]. Pomegranate polyphenols, ellagitannin rich extract (PE) made from fruit skins standardized to ellagitannins, can restraint prostate cancer cell caused by chronic inflammation via suppressing NF-κB pathway, which is well-established signaling pathway mediating the inflammation relevant to cancer [15].

The protective effect of pomegranate juice consumption on various diseases is receiving considerable attention at present in all medical fields [16] Pomegranate fruit has long been cultivated and consumed as a fresh fruit or in beverage form especially in the Mediterranean region[17]. Pomegranate juice consumption has grown tremendously recalling its proposed chemopreventive, chemotherapeutic, antiatherosclerotic and anti-inflammatory effect [16]. There is a wide practiced drinking of pomegranate juice as a nutrient, therapy for some disease or even adjuvant with some treatment in the Middle East especially in the Arab country. This make it mandatory to investigate the possible modulator effects of pomegranate juice on drug metabolism through studying its effect on drug metabolizing enzymes CYPs450 to enable physicians to predict the dosing and the kinetics of the prescribed drugs for patients to avoid drug toxicity or subclinical dosing . In this study we are aiming to investigate the effect of raw pomegranate juice on the expression of cytochrome P450 enzymes in rats.

MATERIALS AND METHODS

Sudan III and Phinobarbital, from Sigma Chemical Co., (St. Louis, Mo, USA). For preparation of pomegranate juice, Fresh Taif red pomegranate was purchased from local market in Taif City. Fresh fruits, were peeled and the edible portion was squeezed. The juice was filtered through Whatman No. 1 filter paper and the filtrate was divided into 10 mL aliquots in 15 mL Falcon tubes and stored at -80 °C until used.

Experimental Animals: Male Wistar rats (N=48) were purchased from the Animal House, King Abdel Aziz Univ., Jeddah, KSA. A total of 48 adult male Wistar rats weighing about 200-250 g were used in the present study. Animals were kept under observation for about 4 days before the onset of the experiment. They were maintained in stainless steel cages at normal atmospheric temperature of 25 ± 2 °C and good ventilation.

Treatment:

Group I: (8 Rats) served as control (receive only water).

Group II: (8 Rats) received raw pomegranate juice by drinking 30 ml/kg daily for 25 days

Group III: (8) received water daily for 21 days and at the 22nd, 23rd and 24th days rats were orally administered Sudan III in a dose of (80 mg/kg BW) dissolved 50% in olive oil .

Group IV: (8 Rats) received pomegranate juice by drinking 30 ml /kg daily for 21 days then at the 22nd, 23rd and 24th days pomegranate juice plus Sudan III in a dose of 80mg/kg

Group V: (8 Rats) received water daily for 21 days then at the 22nd and 24th days intraperitoneal injection of Phenobarbital in a dose of 80 mg/kg dissolved in DW.

Group VI: (8 Rats) received raw pomegranate juice in a dose of 30 ml/kg daily for 21 days then at the 22nd and 24th days pomegranate juice plus Phenobarbital in a dose of 80 mg/kg. The dose of POM juice was chosen based on a previous study (12). POM juice was administered to the rats in the morning, 2 h after water deprivation and the rats consumed the given dose within 2 h, in order to assure no changes due to environmental conditions. The dose of S.III was chosen according to a previous study [17].

Sampling: At the 25th day, rats were sacrificed through cervical dislocation under light ether anesthesia and the tissues specimen of all groups from liver, were taken and allocated in Eppendorf tubes and snap frozen in liquid nitrogen and kept at -80°C until used for RT-PCR studies

Analysis of Genes Expression: RNA extraction: Total RNA extracted from the collected liver samples using Qiazol Reagent according to the manufacturer's instructions. Briefly, 100 mg of each tissue sample were homogenized in 1ml QIAzol (QIAGEN Inc., Valencia, CA) then 0.3 ml chloroform were added to the homogenate.

Table 1: Sequences and conditions of polymerase chain reaction primers.

Name	sense 5'..... 3	anti-sense 5'..... 3´	Ann. Tm.	BP
â-actin	ATGTACGTAGCCATCCAGGC	TCCACACAGAGTACTTGCGC	56°C	628
CYP1A1	CCATGACCAGGAACTATGGG	TCTGGTGAGCATCCAGGACA	56°C	341
CYP1A2	GCAGGTCAACCATGATGAGAA	CGGCCGATGTCTCGGCCATCT	56°C	334
CYP2C11	TGCCCCTTTTTACGAGGCT	GGAACAGATGACTCTGAATTCT	55 °C	368
CYP3A2	TTGATCCGTTGTTCTTGTCA	GGCCAGGAAATACAAGCAA	52°C	342
CYP2B1	TCTCACTCAACACTACGTTC	CTGGGAAAGGATCCAAGCCTGGG	58°C	450

Then, the mixtures were shaken for 30s followed by centrifugation at 4°C and 12,500 rpm for 20 min. The supernatant layer have been transferred into a new set of tubes and an equal volume of isopropanol were added to the samples, shaken for 15 seconds and were centrifuged at 4°C and 12500 rpm for 15 min. The RNA pellets were washed with 70% ethanol, briefly dried up then dissolved in Diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically (GelDoc-It imaging system (UVP, LLC Upland, CA 91786, USA) at 260 and 280 nm. The 260/280 optical density ratio of all RNA samples was 1.7-1.9. A total of 2 µg RNA was reverse transcribed using oligo-dT primers and Moloney murine leukaemia virus reverse transcriptase (SibEnzyme Ltd., Novosibirsk, Russia).

cDNA Synthesis: For synthesis of cDNA, mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µl sterilized DEPC-water were incubated in the PeX 0.5 thermal Cycler (Thermo Electronic Corporation, Milford, Ma) at 70°C for 10 min for denaturing. Then, 4 µl of 5X RT-buffer, 2 µl of 10 mM dNTPs and 100 U RevetAid Premium reverse transcriptase (Fermentas Canada Inc. Harrington Court, Burlington Ontario) were added and the total volume was completed up to 20 µl by DEPC water. The mixture then was re-incubated in the thermal Cycler at 30°C for 10 min, at 42°C for 1 h and at 90°C for 10 min then, preserved at -20°C until used..

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). RNA from hepatic tissues was analyzed by semi-quantitative RT-PCR using corresponding specific primers for the indicated genes (Table 1). The primers were designed using Oligo-4 computer program according to the nucleotide sequences in Gen Bank (http://www.ncbi.nlm.nih.gov/genbank/;) and were synthesized by Macrogen Korea (Seoul, South Korea). PCR was conducted in a final volume of 25 µl consisting of 1 µl cDNA, 1 µl (10 picomoles) of each primer (forward and reverse) and 12.5 µl PCR Master Mix (Promega Corporation, Madison, WI, USA). The final volume was brought to 25 µl using sterilized, nuclease-free deionized water. PCR was carried out using a PeX 0.5 Thermal Cycler (Thermo Hybaid PXE 0.2 Thermal Cycler (Thermo Electron Corp., Madison, WI 53711, USA) and the following cycling conditions were used: Denaturation at 94°C for 5 min for one cycle, followed by 28-35 cycles of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer (Table 1) and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min. As an internal reference, glyceraldehyde 3-phosphate dehydrogenase mRNA expression was detected using specific primers (Table 1). PCR products subsequently underwent 1.5% agarose gel electrophoresis (Bio Basic Inc., Markham, ON, Canada) in Tris-Borate-ethylenediaminetetraacetic acid buffer at 100 V for 30 min with ethidium bromide staining. PCR products were visualized under ultraviolet (UV) light and images were captured using a gel documentation system, (GelDoc-It imaging system (UVP, LLC Upland, CA 91786, USA). Band intensities from the various rats from each group were quantified densito-metrically using ImageJ software version 1.47 (http://imagej.en.softonic.com/).

RESULTS

Effect of Pomegranate juice on CYP3A2 mRNA Expression: To examine the possible modulator effect of Pomegranate juice on hepatic CYP3A2 expression, the mRNA expressions of CYP3A2 was measured by semi-quantitative RT-PCR. CYP3A2 mRNA expression was down regulated by Pomegranate juice drinking for 25 days. Administration of Phenobarbital (PB) up regulated CYP3A2 mRNA expression than normal levels. Meanwhile when administrated with PB, Pomegranate juice decreased the PB-induction of CYP3A2 mRNA. Sudan III administration to rats showed suppressive effect on CYP3A2 mRNA expression. This suppressive effect of S.III was augmented with Pomegranate juice supplementation. (Fig. 1).

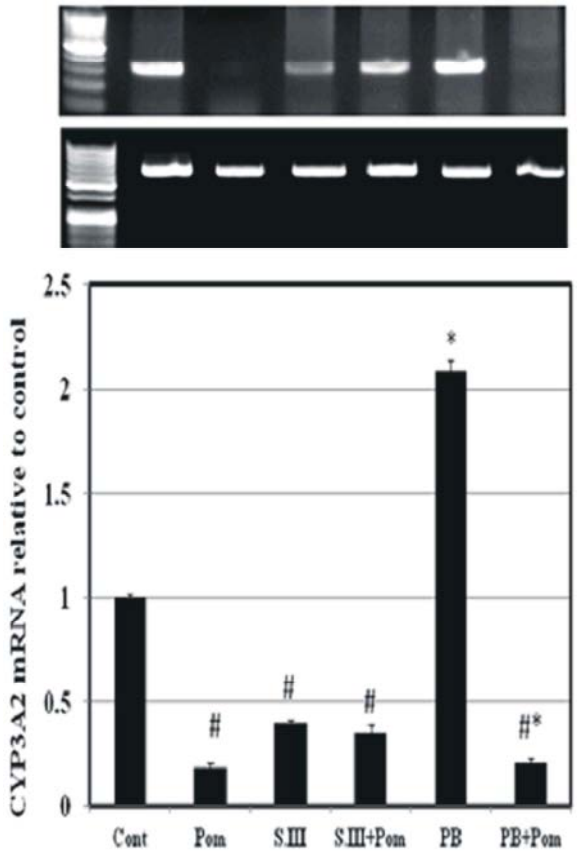

Fig. 1: Effect of Pomegranate juice on CYP3A2 mRNA expression:
Total RNA was prepared from liver tissues and the expression levels of CYP3A2 mRNA were analyzed by semi?quantitative RT-PCR. Values are presented as the mean ± standard error of 8 rats. *P<0.05 higher than cont group. # P<0.05 lower than control group #* lower than PB-treated group. Cont, control; Pom, Pomegranate-supplement group; S.III, Sudan III treated group; S.III+Pom, Sudan III+ Pomegranate-supplement group; PB, Phenobarbital treated group and PB+Pom, PB + Pomegranate-supplement group.

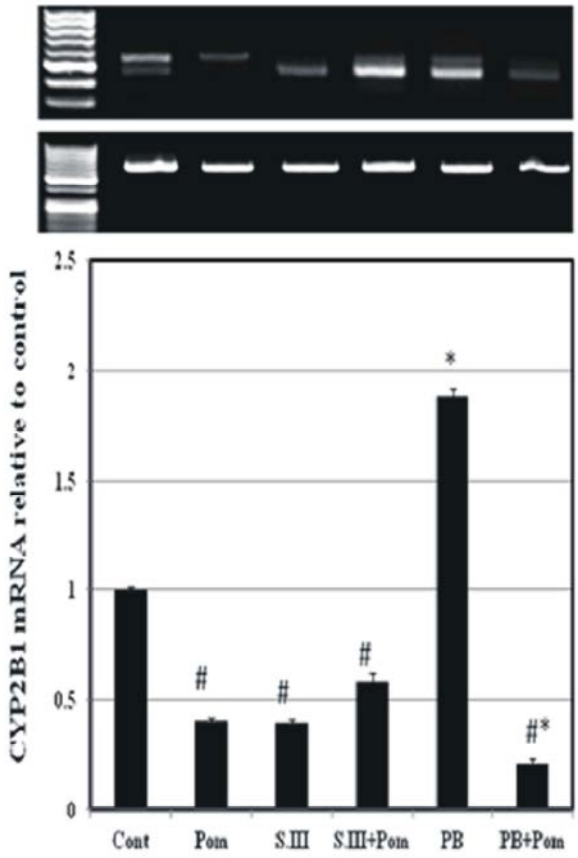

Fig. 2: Effect of Pomegranate juice on CYP2B1 mRNA expression:
Total RNA was prepared from liver tissues and the expression levels of CYP2B1 mRNA were analyzed by semi?quantitative RT-PCR. Values are presented as the mean ± standard error of 8 rats. *P<0.05 higher than cont group. # P<0.05 lower than control group #* P<0.05 lower than PB-treated group. Cont, control; Pom, Pomegranate-supplement group; S.III, Sudan III treated group; S.III+Pom, Sudan III+ Pomegranate-supplement group; PB, Phenobarbital treated group and PB+Pom, PB + Pomegranate-supplement group.

Effect of Pomegranate Juice on CYP2B1 and CAR mRNA Expressions: To examine the possible modulator effect of Pomegranate juice on hepatic CYP2B1 expression, the mRNA expressions of CYP2B1 was measured by semi-quantitative RT-PCR. CYP2B1 mRNA expression was downregulated by Pomegranate juice drinking for 25 days. Administration of Phenobarbital up regulated CYP2B1 mRNA expression compared to control. Meanwhile, when administrated with PB, Pomegranate juice prevented the PB-induction of CYP2B1 mRNA. Sudan III administration to rats for 3 days showed suppressive effect on CYP2B1 mRNA expression. This S.III-suppressive effect was augmented in the rats group supplemented with Pomegranate juice (Fig. 2).

Effect of Pomegranate Juice on CYP1A1/CYP1A2 mRNA Expressions: To examine the possible modulatory effect of Pomegranate juice on hepatic CYP1A1/CYP1A2 mRNA expressions, the mRNA expressions of hepatic CYP1A1 and CYP1A2 mRNA expressions were measured

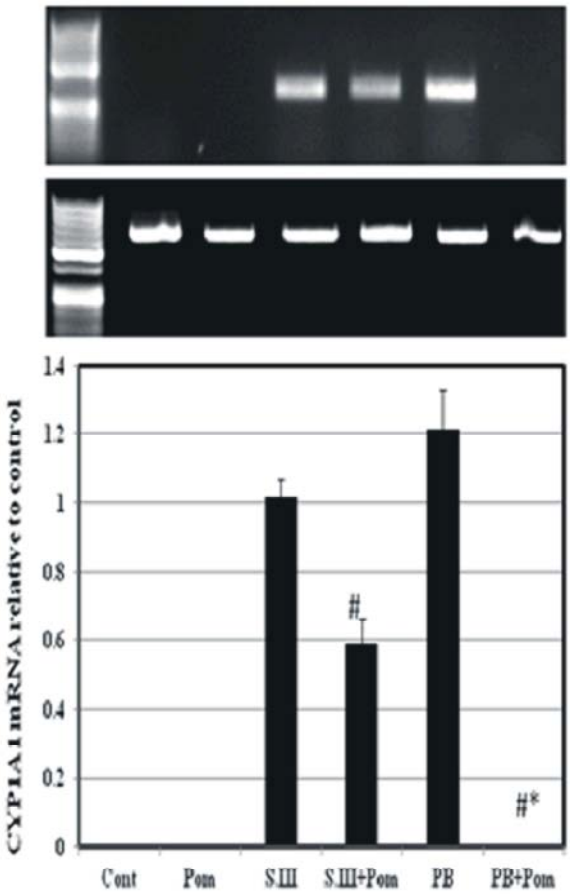

Fig. 3: Effect of Pomegranate juice on CYP1A1 mRNA expression:
Total RNA was prepared from liver tissues and the expression levels of CYP1A1 mRNA were analyzed by semi?quantitative RT-PCR. Values are presented as the mean ± standard error of 8 rats. # P<0.05 vs cont group. #* P<0.05 lower than S.III-treated group. Cont, control; Pom, Pomegranate-supplement group; S.III, Sudan III treated group; S.III+Pom, Sudan III+ Pomegranate-supplement group; PB, Phenobarbital treated group and PB+Pom, PB + Pomegranate-supplement group.

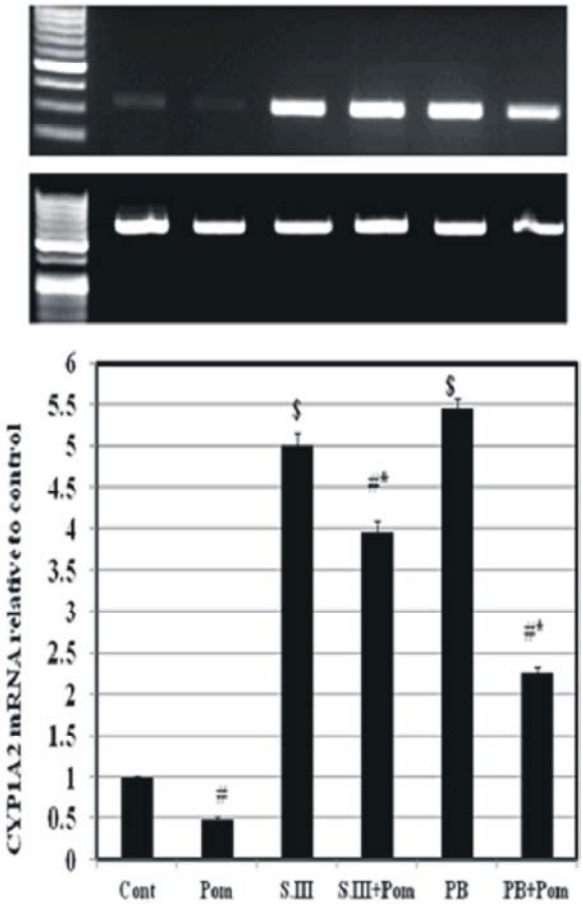

Fig. 4: Effect of Pomegranate juice on CYP1A2 mRNA expression:
Total RNA was prepared from liver tissues and the expression levels of CYP1A2 mRNA were analyzed by semi-quantitative RT-PCR. Values are presented as the mean ± standard error of 8 rats. # P<0.05 vs cont group. $ P<0.05 higher than control group. #* lower than S.III-treated or PB treated group. Cont, control; Pom, Pomegranate-supplement group; S.III, Sudan III treated group; S.III+Pom, Sudan III+ Pomegranate-supplement group; PB, Phenobarbital treated group and PB+Pom, PB + Pomegranate-supplement group.

by semi-quantitative RT-PCR. CYP1A1 mRNA expression which was not expressed in the normal state, was clearly induced with S.III administration for 3 days. Pomegranate juice supplementation did not affect the S.III-induced CYP1A1 mRNA expression. Administration of Phenobarbital (PB) upregulated CYP1A1mRNA expression than normal levels meanwhile when administrated with PB, Pomegranate juice decreased the PB-induction of CYP1A1mRNA expression (Fig 3). Pomegranate juice supplementation clearly inhibited CYP1A2 mRNA expression than normal level. Sudan III or PB administration for 3 days caused induction of CYP1A2 mRNA expression. Pomegranate juice supplementation downregulated the induced CYP1A2 mRNA expression either with Sudan III or PB administration (Fig 4).

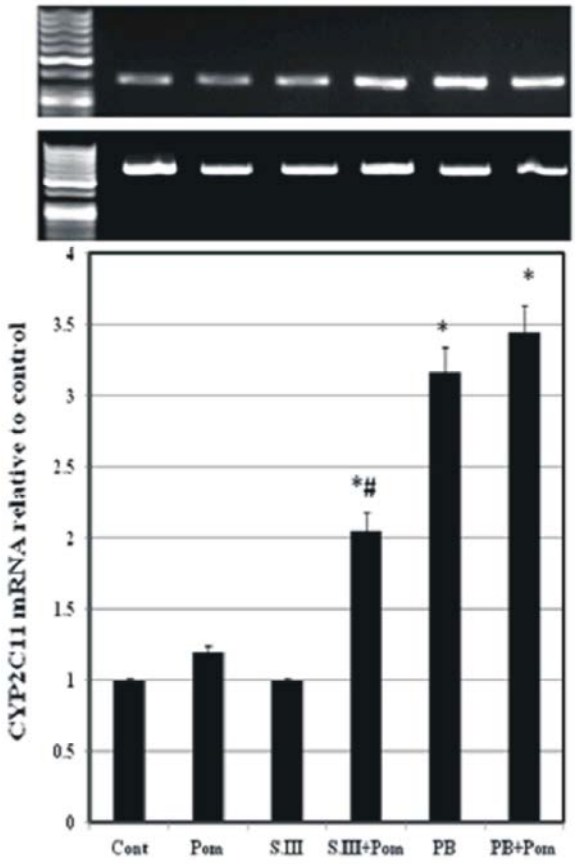

Fig. 5: Effect of Pomegranate juice on CYP2C11 mRNA expression:
Total RNA was prepared from liver tissues and the expression levels of CYP1A2 mRNA were analyzed by semi-quantitative RT-PCR. Values are presented as the mean ± standard error of 8 rats. * P<0.05 vs cont group. #* lower than S.III-treated or PB treated group. Cont, control; Pom, Pomegranate-supplement group; S.III, Sudan III treated group; S.III+Pom, Sudan III+ Pomegranate-supplement group; PB, Phenobarbital treated group and PB+Pom, PB + Pomegranate-supplement group.

Effect of Pomegranate Juice on CYP2C11 mRNA Expressions: The constitutively expressed CYP2C11 was not affected by either Pomegranate juice or S.III administration. However, pomegranate juice administration with S.III upregulated CYP2C11 mRNA expression compared to control levels. Phenobarbital administration caused induction of CYP2C11 mRNA expression compared to control levels. The PB-induced CYP2C11 mRNA expression was further upregulated with Pomegranate juice supplementation to rats (Fig 5).

DISCUSSION

Cytochrome P450 enzymes induction can increase drug elimination and decreases its plasma concentration and therefore attenuate its pharmacological effects [6], while their inhibition can lead to overexposure and toxicity [5]. Induction or inhibition of CYP3A4 expression is considered a major clinical concern for drug-drug interactions in patients receiving multiple CYP3A4-metabolizing drugs [18]. In this study the inhibition of CYP3A2 in rats may indicates the possible inhibition of human hepatic CYP3A4 by pomegranate juice drinking. Human CYP3A4 exhibits a 73% homology of the amino acid sequences and some substrate preference with Rat CYP3A2 [19]. Human CYP3A4 and rat CYP3A2 are involved in the metabolism of erythromycin, nifedipine, lidocaine, testosterone, aflatoxin B1 and benzo (a) pyrene [19, 20]. CYP3A4 metabolizes not only xenobiotics including majority of drugs and carcinogens [21, 22] but also many endogenous compounds such as cholesterol, bile acids, fatty acids, prostaglandins, leukotrienes, retinoids and biogenic amines [21,23]. The inhibition of rat hepatic CYP3A2 by pomegranate juice in this study is in consistence with a previous report showed the inhibitory effect of pomegranate juice on the metabolism of rat CYP3A2 and human CYP3A4 substrate (carbamazepine) [24]. This suppressive effect of pomegranate juice on CYP3A2 may be attributed to its high content of catechin [25] that was reported to be a potent inhibitor of CYP3A2 [26]. This inhibitor effect of Pomegranate juice on CYP3A2 may explain the increase in bioavailability of buspirone after pre-treatment with pomegranate juice [27]. This may add a further evidence of the potential of pomegranate juice to be a perpetrator in drug–drug interactions mediated by CYP3A4 that was recently suggested [28]. The inhibition of CYP2B1 enzyme expression by Pomegranate juice presented in this study could be attributed to the pomegranate major phenolic compound ellagic acid [29] that was reported to confer a potent inhibitory effect of Epilobium hirsutum extract on CYP2B [30]. Rat's CYP2B1 has about 75% identity with the human CYP2B6 [31]. Rat and human CYP2B activate natural and synthetic procarcinogens, as aflatoxin B1, 6-aminochrysene, benzo[a] pyrene, 7,12-dimethylbenz [a] anthracene and dibenz[a, h]anthracene [32]. Therefore the suppressive effect of pomegranate juice on CYP2B1 expression may explain in part the chemopreventive effect of Pomegranate juice against cancer [33, 34]. Moreover, rat CYP2B1 and human CYP2B6 have the ability to hydroxylate testosterone and lidocaine [35].

The inhibition of CYP2B1 expression in this study may in part explain keeping testosterone levels high in rats intoxicated with CCL4 and supplemented with Pomegranate juice [36]. More than 90% of known chemical carcinogens, including aromatic amines and polycyclic aromatic hydrocarbons (PAH)s, are substrates of CYP1A1 and CYP1A2 [37-39] and their metabolism by these two enzymes often results in the formation of active carcinogenic metabolites [40-41]. It has been reported that Sudan dyes could potently induce CYP1A1 and 1A2 mRNA and protein expression in rats [17]. CYP1A2 was shown to be induced also by Phenobarbital through CAR activation (42). The suppressive effect of the basal CYP1A2 and the S.III-induced CYP1A1 and CYP1A2 is a new finding that may explain in part the potential chemopreventive effect of pomegranate juice for prostate cancer [43]. Moreover, Hepatic CYP1A2 is one of the key enzymes having an important role in the metabolic clearance of 5% of currently marketed drugs [44]. Of its substrates are drugs, such as theophylline, caffeine, phenacetin and propranolol [4]. The suppressive effect of CYP1A2 by pomegranate juice may affect the clearance of these drugs and therefore increase their half life time and pharmacological effects. CYP2C11 is the predominant cytochrome P450 enzyme expressed constitutively in the liver of adult male rats comprises approximately 50% of the total hepatic CYP in the adult male rat [45, 46]. The upregulating effect of pomegranate juice on CYP2C11 demonstrated in this study, may be attributed to its anti-inflammatory properties (487) as CYP2C11 expression was demonstrated to be inhibited by inflammatory cytokines especially interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF) [48]. CYP2C11 is involved in the metabolism of benzphetamine, aminopyrine, benzo(a)pyrene, antipiryne, aflatoxin B1, R-mephenytoin and S-warfarin [19, 49]. CYP2C11 mediates tstosterone androstenedione and vitamin D hydroxylation (50-52). Rat's CYP2C11 exhibits a 77% homology of the amino acid sequence, some substrate preference and a functional analogy with human CYP2C9, which catalyzes the metabolism of such clinically important drugs as S-warfarin, phenytoin, ibuprofen, diclofenac, tolbutamide and antidepressant drugs, as well as steroids and arachidonic acid [53, 54, 51, 55]. The induction of rats' hepatic CYP2C11 in this study, may indicates the ability of pomegranate juice drinking to accelerate the human metabolism of such drugs that are substrate of CYP2C9 therefore shorten their half life time in the body and reduce their effectiveness.

CONCLUSION

In this study, drinking of pomegranate juice led to attenuation of rats' hepatic CYP3A2, CYP2B1 and CYP1A1\2 and upregulation of CYP2C11 mRNA either their basal or Phenobarbital-induced, or S.III-induced expression levels. This may increase or decrease the bioavailability of these enzymes substrates drugs which may be an evidence of the potential of pomegranate juice to be a perpetrator in drug–drug interactions. This may explain the pomegranate juice-chemopreventive effect against cancer. Moreover, this suggested effect on the clearance of the drugs that are substrates of these enzymes should be put in its right clinical considerations to adjust the dosing protocol of drugs in patients who drink pomegranate juice.

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