Effect of Red Clover on CYP Expression: An Investigation of Herb-Drug Interaction at Molecular Level

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Hormone replacement therapy and selective estrogen receptor modulator are the most common therapy for women going through menopause. These therapies though popular fail to relieve withdrawal symptoms such as hot flashes, fatigue, leg cramps and nausea. This scenario necessitates to herbal preparations as alternative which may lead to simultaneous intake of herbal preparations, containing flavonoids, as well as Selective estrogen receptor modulator hence creating a phenomenon of herb drug interaction. Here we investigate the effect of red clover on steady state mRNA levels of rat cytochrome P 450 enzymes. Further, red clover's effect on cytochrome P 450's expression has been investigated when co-administered with tamoxifen and raloxifene. Exposure to red clover resulted in significant down regulation of all the cytochrome P 450 isoform mRNA except cytochrome P 450 2C13 and cytochrome P 450 3A2. When red clover is given in combination with tamoxifen or raloxifene altered level of cytochrome P 450 enzyme mRNA is observed. Present results suggest that herbal medical preparations such red clover has potential for herb drug interaction.

Key words: Cytochrome P 450 expression, herb-drug interaction, raloxifene, red clover, tamoxifen

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Red clover (RC, *Trifolium pratense* L.) is a perennial plant that grows wild in America, Europe, Australia, Asia, and northern Africa. The flower head, which ranges from pink to purple or red, is the part of the plant used in herbal remedies. It contains isoflavones (a major class of phytoestrogen) such as genistein, daidzein, biochanin A (BCA) and formononetin (FMN)[1]. Phytoestrogens have a property to bind with estrogen receptors, they show a higher binding affinity to estrogen receptor β (ER-β) than to estrogen receptor α (ER-α) and recruit regulators needed for transcription of target genes[2]. They show positive estrogenic effects mainly in tissues expressing ERβ, such as bone or cardiovascular system[3,4]. RC extracts are sold as dietary supplements in the form of tablets (Promensil), capsules, tea, liquid preparations and are available in supermarkets and health food stores in both industrialized and developing countries. They are believed to relieve postmenopausal symptoms such as hot flushes, preventing bone loss and for aid in maintaining men's prostate health[5]. Increasing use of herbal medications has led women suffering from menopausal disorders to seek an alternative to conventional hormone replacement therapy (HRT), because of concerns regarding benefits and risks[6]. Market nowadays is flooded with lots of different preparations intended to substitute HRT, often without medical prescription. Such preparations are sold as nutritional supplements and are made from various plant extracts, mostly from soy (*Glycine max*) and RC[7].

Herbal products including herbal medicines, nutraceuticals and dietary supplements have become popular alternative to conventional medicine worldwide. Interaction between herbal products and conventional drugs is one of the major safety concerns these days. Major herb drug interactions are metabolism-mediated and involve either induction or inhibition of cytochrome P 450 (CYP) enzymes which may affect the fate of conventional drugs in body. Apart from enzymatic inhibition these interactions can also affect CYP activity by induction or suppression.

There are many studies showing the effect of herbal medications on CYP expression. One of the most common example is St. John's wort (SJW), which is a natural remedy used for a number of conditions such as insomnia, anxiety and depression[8]. It has been reported to induce CYP3A4 expression in human hepatocytes and activate steroid X receptor (SXR). Hyperforin, one of the constituents of SJW is the likely receptor ligand[8,9]. *Ginkgo biloba* extract (GBE) used in Europe to alleviate symptoms associated with numerous cognitive disorders has been reported to markedly induce levels of CYP2B1/2, CYP3A1 and CYP3A2 mRNA in the liver[10]. Other examples include *Sho-saiko-to*, which has been reported to up-regulate the mRNA expression of CYP2B, CYP3A1, CYP2E1 and CYP4A1 in rats when administered orally up to 2 weeks[11].

Tamoxifen citrate (TAM), an antiestrogen, (non-steroidal derivative of triphenylethylene) is the most commonly used selective estrogen receptor modulator (SERM) in the treatment of postmenopausal, hormone-sensitive, advanced breast cancer[12]. On the other hand raloxifene (RAL), a benzothiophene derivative, is generally used in the prevention of osteoporosis in postmenopausal women[13]. It binds to estrogen receptor and inhibits bone resorption without stimulating endometrium cells[14]. A beneficial effect of the treatment with RAL is that it also halves the risk of breast cancer[15]. In addition, RAL has a protective effect on the cardiovascular system by cutting the levels of low-density lipoprotein-cholesterol and homocysteine[16]. Biotransformation of both TAM and RAL is mainly catalysed by CYP3A4[17,18]. Of the 57 human P450 isoforms, CYP3A4, 1A2, 2B6, 2C9, 2C19 and 2D6 are primarily involved in formation of 4-hydroxy tamoxifen from TAM[19].

SERM therapies have been stated to have various adverse effects for as TAM therapy has been reported to increase the incidence of hot flashes. Besides this, in a trial of metastatic breast cancer higher dosage of RAL resulted in increased incidence of hot flashes, fatigue, leg cramps and nausea[20]. Since, RC preparations relieve women from hot flashes, most common menopausal symptoms, patients or survivors of breast cancer therapy may opt for these preparations[21].

Since, there is high probability of co-administration of RC supplements with TAM and RAL, in the present study; we evaluated the effect of RC on CYP isoforms expression in rats. In this study rat CYP450 analogues of human CYP450 were monitored for any change in their steady state level of mRNA these include CYP1A2, 2B1, 2B2, 2C6, 2C11, 2C13, 2E1, 3A1, 3A2, 3A18.

TAM and RAL were purchased from Sigma Aldrich Ltd (St. Louis, MO, USA). RC capsules were purchased from Nature's Way (Vancouver, Canada). Each capsule contains 400 mg of RC blossoms and herb (*Trifolium pratense*). Trizol reagent and Pure Link RNA Mini Kit were from Invitrogen life technologies (Carlsbad, CA, USA), High capacity RNA to cDNA Master Mix was purchased from Applied Bio systems (Foster city, CA, USA), SYBR Premix Ex Taq was purchased from TaKaRa bio (Shiga, Japan), Primers were designed online using http://www.roche-applied-science.com and supplied by Eurofins genomics (Bangalore, India) all the primers were high purity salt free (HPSF) purified (Table 1).

Young, male Sprague Dawley rats, weighing 200-220 g, were procured from the National Laboratory Animal Centre, CDRI (Lucknow, India). Rats were housed in well ventilated cages at room temperature (24±2°) and 40-60% relative humidity while on a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of one week prior to the experiment.

The rats were fasted overnight (14-16 h) prior to the experiment but given free access to water. Rats were divided randomly into four groups (n=5, each); one control group and three treatment groups. In the first group vehicle (0.2% sodium CMC) was administered for 7 days. In the remaining three groups RC capsule contents 65 mg/kg/day (suspension in 0.2% sodium CMC) were administered orally for 7 days. 30 min after administration of RC on 8th day RAL at 5 mg/kg (dissolved in a 0.9% NaCl-injectable solution and Tween 80, 9:1, v/v)[22,23], TAM at 10 mg/kg (dissolved in a 0.9% NaCl-injectable solution and Tween 80, 9:1, v/v)[24,25] and vehicle (0.9% NaCl-injectable solution and Tween 80, 9:1, v/v), respectively were administered orally. After 48 h post dosing rats were euthanized by excess of anaesthesia and the liver tissues were collected and stored at –80° until further use.

TABLE 1: SEQUENCE OF QPCR PRIMERS FOR RAT CYP ENZYMES AND GAPDH

Target	Sense 5'-3'	Antisense 3'-5'
CYP1A2	CTACAACTCTGCCAGTCTCCAG	CCTCTCAACACCCAGAACACT
CYP2B1	CGGACCTTTTCCCTCCTAAG	GGAACCCAGAGAAGAACTCAAA
CYP2B2	CATCTCATGCTGAGTCACTTCC	GACCATGGAGGGCTGATAAGT
CYP2C6	GAGCTGTGCTTCATT CCTCTCTA	AGTTATTTGAAGGGAG AAAATACAACA
CYP2C11	GGAGACAGAGCTTTGGGAGA	CAATGATTGGGAGAGGTGTTG
CYP2C13	AGTAAGCTCAACAATGA GATTTGC	AGCTGTGACGTGTGGGTACTT
CYP2E1	TGAGACCACCAGCACAACTC	CAATTTCTGGGTATTTCATGAGG
CYP3A1	GGACCTGCTTTCAGCTCTCA	AAATCCGTAGAGGAGCACCA
CYP3A2	CCACTCACCCTGATA TTCAGAAG	AGGTAGGAGGTGCCTTACTCG
CYP3A18	CCAATGTGCAAAAGAAACTCC	AGAGCATCATAGGTGACAGGTG
GAPDH	TGGGAAGCTGGTCATCAAC	GCATCACCCCATTTGATGTT

Primers were designed online using http://www.roche-applied-science.com

Total RNA was extracted from 0.4-0.6 g of rat liver using TRIzolPlus RNA purification Kit as per the protocol provided with kit. The purity and quantity of the mRNA was determined by 260/280 nm UV spectra. Total mRNA was stored at −80° until used. Total mRNA was reverse transcribed using high capacity RNA to cDNA Master Mix as per the protocol supplied with the kit. The master mix was used for a 20 µl reaction mixture under the following conditions: 4 µl master mix; 2 µl RNA sample; 14 µl nuclease free water. Following was the program for reverse transcriptase PCR: 25° for 5 mins; 42° for 30 mins; 85° for 5 mins; hold at 4°. The product obtained was diluted 4x for real time PCR.

Real Time PCR was performed on Light Cycler 480 II (Roche Diagnostics) with Sybr Green fluorescent label. Samples (10 µl final volume) contained the following: 1x SYBR Premix Ex Taq0.5/0.5µl of each primer; 3 µl of cDNA sample and 1 µl of Nuclease free water. Samples were run in triplicate in 96 well plate provided by Roche Diagnostics. Cycling parameters were as follows:- denaturation 1 cycle 95° for 2 min; amplification 45 cycles 94° for 15 sec, 60° for 20 sec, 72° for 30 sec; melting curve 1 cycle 95° for 5 sec, 65° for 1.05 sec, 97° continuous acquisition mode; cooling 1 cycle 40° for 30 sec. Threshold values (Ct) were calculated automatically by the software. The $\Delta\Delta CT$ method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments.

Approval from the Institutional Animal Ethics Committee was sought and the study protocols were approved before the commencement of the studies. Data was expressed as the mean±SEM. Curve fitting was accomplished in the program Microsoft Office Excel. Statistical comparisons were made using student t-test. Statistical significance was concluded at $p<0.05$. The purity and quantity of the mRNA was

determined by 260/280 nm UV spectra and it was found to be almost 1.8 for all the samples.

To determine if RC can change the steady-state mRNA levels of CYP isoforms, we exposed rats to RC for a period of 7 days. Following treatment, RNA was extracted from liver tissue of dissected rats and the mRNA levels of CYP isoform were quantified by real time RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control (reference gene). Melt-curve analysis confirmed the absence of non-specific amplification products (data now shown). The results of the real time PCR analysis are shown in Table 2.

Exposure to RC resulted in significant down regulation of all the CYP isoform mRNA compared to control except CYP 2C13 and CYP 3A2, CYP 1A2, 2B1/2, 2C11 and 2E1 have a 1.12, 3.27, 2.08, 3.64 and 1.31 fold decrease in mRNA steady state level, respectively. In case of simultaneous treatment of RC (RC) and TAM the expression of CYP1A2, 2B2, 2C11, 2E1, 3A1, 3A18 enzymes was up-regulated compared to RC alone. Out of these CYP 3A1, 2E1 and 1A2 are levelled to control and in case of CYP 2C11 the mRNA expression is increased significantly in comparison of control. CYP 2B2 and 3A18 are though up regulated but the steady state level of mRNA remains significantly low in comparison to control. It indicates significant interaction between RC and TAM.

In addition to this, combination of RC and RAL has also up-regulated the expression CYP 2B1/2, 2C11, 2E1, 3A18, 2C13 compared to RC alone. RC with RAL has no significant effect on CYP3A2 but in case of CYP2C13 the value of mRNA expression is significantly increased in comparison to control. Rest CYP's (i.e. 2B2, 2C11, 3A18) though having an increase in expression but their mRNA levels remains significantly less than control. It indicates significant interaction between RC and RAL.

SERM's, commonly used by women during menopause or post menopause, though effective does not relieves women from many of the menopausal symptoms. Isoflavones derived from RC on the other hand has been reported not only to relieve from frequent hot flushes due to SERM's but also appears to a better option because of their selective estrogen receptor modulator like activity[7]. Along with this, isoflavones have also been reported to have cardio protective effect[26]. Further daidzein one of the phytoestrogen present in RC extracts have been found to inhibit osteoclast differentiation and activity in the same degree as 17β estradiol[27]. Therefore women undergoing TAM and RAL therapy may opt for herbal preparations such as RC, black cohosh, etc as a nutritional supplement which may lead to a great possibility of herb-drug interaction. This study investigates the effect of herb drug interaction at molecular level and for this RC was co-administered with TAM and RAL in separate groups and further mRNA levels were evaluated to unveil the influence of interaction between herbal medications, a popular alternative to HRT, and conventional drugs used for the treatment of breast cancer and osteoporosis, major ailments faced by women going through menopause.

Cytochrome P450s being the major enzymes involved in drug metabolism, accounting for about 75% of metabolic reactions[28]. For this, effect of RC constituents on the steady state mRNA levels of rat CYP isoforms viz. CYP 1A2, 2B1, 2B2, 2C6, 2C11, 2C13, 2E1, 3A1, 3A2 and 3A18 were investigated.

Results depict statistically significant down regulation of steady state mRNA levels of all the CYP's by RC

TABLE 2: COMPARATIVE EXPRESSION PROFILE OF RAT CYP450

CYPs	Control	Red clover	Tamoxifen	Red clover+Tamoxifen	Raloxifene	Red clover+Raloxifene
CYP1A2	188.6±29.6	88.9±14.7	44.4±3.9	189.4±34.1	212.1±35.5	70.2±14.4
CYP2B1	147.9±6.0	34.6±3.6	49.8±8.1	64.9±21.7	171.9±4.5	66.8±12.5
CYP2B2	159.4±29.0	51.6±0.6	84.7±6.8	100.4±11.5	198.2±14.1	92.6±4.1
CYP2C6	136.9±19.7	44.6±9.5	37.5±4.5	30.0±3.3	65.5±9.0	47.2±8.1
CYP2C11	107.6±8.7	23.2±9.0	117.6±11.5	166.8±19.3	64.7±8.4	81.3±1.5
CYP2C13	171.2±26.8	166.7±36.3	134.1±9.8	134.5±17.7	194.5±8.1	236.9±7.2
CYP2E1	135.2±11.0	58.4±14.3	222.7±16.7	148.9±16.8	107.3±15.5	118.2±5.0
CYP3A1	133.8±7.8	82.0±5.4	34.8±3.5	137.1±6.8	115.7±13.1	91.5±2.8
CYP3A2	93.3±5.2	108.0±17.0	111.2±18.4	128.2±7.4	147.0±5.2	129.6±5.3
CYP3A18	131.8±41.9	17.1±2.5	109.8±8.0	52.9±12.1	131.8±9.5	38.2±1.6

mRNA abundance normalized to GAPDH

with an exception of CYP 2C13 and 3A2. Out of the two, CYP3A2 being analogous to human CYP3A4, a major drug metabolizing CYP isoform[29,30], was significantly up regulated suggesting a possibility of induction of the enzyme, this situation may lead to higher metabolism rate which may decrease the duration and intensity of the drug action. Rat analogues of human CYP enzymes, important for the metabolism of TAM and RAL, i.e. CYP 1A2, 2B1/2, 2C11, 2E1[28,31,32] have significant decrease in mRNA level when treated with RC. Asserting the fact that flavonoids interfere with CYP enzymes and hence have great deal of influence on drug metabolism i.e. TAM, RAL.

When exploring the situation for co-administration of TAM+RC, up-regulation of certain CYP's was observed which can be explained by fact that TAM has been previously reported to increase the mRNA levels of certain CYP's in both acute and chronic treatments[33]. But this restoration is not up to the steady state levels in all cases hence indicating to the possibility of effect of flavonoids present in RC on the metabolism of these drugs. CYP 1A2, 2E1, 3A1 were levelled back to control during co-administration but for rest CYP's mRNA levels either remained suppressed or was induced. For CYP 3A18, 2B1, 2B2, 2C13 the mRNA levels remained lowered by the effect of RC and in the case of CYP2C6 the amalgamation even lowered the expression. Induction of expression was observed in CYP2C11 and CYP3A2.

The second group for co-administration had the combination of RAL+RC. Here the down regulation of CYP's by RC was antagonised by co-administration of RAL+RC significantly except CYP1A2, 2C6, 3A1, 3A2. For CYP 2C13 and 3A2 there is induction leading to increased level of mRNA and for the remaining the mRNA levels are lower than control.

From the above stated result it is clearly visible that RC preparations has the potential of interfering with steady state mRNA levels of major drug metabolizing enzymes. This interference could be either induction as in the case of CYP 3A2 or inhibition/suppression as in the case of CYP 1A2, 2B1/2, 2C11, 2E1. Both the situation may lead to altered metabolism of co-administered drugs.

Our present results suggest that herbal medical preparations such RC has potential for herb-drug interaction which is a major safety concern. Since these medications fall into the category of dietary supplements or alternative medicines and, as such, are not regulated by the Food and Drug Administration (FDA) raising concerns regarding their safety and efficacy. Without regulatory standards the actual amount of active ingredients present and to that stated on the label is questionable[34]. Further investigation is required to assess the safety regarding the common use of herbal preparations.

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