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Evaluation of the synergistic effect of *Allium sativum*, *Eugenia jambolana*, *Momordica charantia*, *Ocimum sanctum*, and *Psidium guajava* on hepatic and intestinal drug metabolizing enzymes in rats

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ABSTRACT

Aims/Background: This study was to investigated the synergistic effect of polyherbal formulations (PHF) of *Allium sativum* L., *Eugenia jambolana* Lam., *Momordica charantia* L., *Ocimum sanctum* Linn., and *Psidium guajava* L. in the inhibition/induction of hepatic and intestinal cytochrome P450 (CYPs) and Phase-II conjugated drug metabolizing enzymes (DMEs). Consumption of these herbal remedy has been extensively documented for diabetes treatment in Ayurveda. **Methodology:** PHF of these five herbs was prepared, and different doses were orally administered to Sprague–Dawley rats of different groups except control group. Expression of mRNA and activity of DMEs were examined by real-time polymerase chain reaction and high performance liquid chromatography in isolated liver and intestine microsomes in PHF pretreated rats. **Results:** The activities of hepatic and intestinal Phase-II enzyme levels increased along with mRNA levels except CYP3A mRNA level. PHF administration increases the activity of hepatic and intestinal UDP-glucuronyltransferase and glutathione S-transferase in response to dose and time; however, the activity of hepatic sulfotransferase increased at higher doses. **Conclusions:** CYPs and Phase-II conjugated enzymes levels can be modulated in dose and time dependent manner. Observations suggest that polyherbal formulation might be a possible cause of herb-drug interaction, due to changes in pharmacokinetic of crucial CYPs and Phase-II substrate drug.

KEY WORDS: Cytochrome P450, herb-drug interaction, microsomes, sulfotransferase, UDP-glucuronyltransferase

INTRODUCTION

Diabetes is a chronic endocrine disorder, characterized by absolute or relative insulin deficiency. The peoples of the world especially in India are facing major health problem such as diabetes. There were 366 million people with diabetes in 2011, a number projected to rise to 552 million by 2030 [1]. Herbal remedies remain one of the major approaches as complementary alternatives in the management of diabetes mellitus. This is because their benefits are well documented from a historical perspective in diabetic populated areas [2]. A huge proportion of Indian population relies on the use of traditional medicines for the use of their primary health care [3]. A major problem resulting from a combination of Ayurvedic medicine in present medical practices is a lack of clinical and scientific data to provide evidence for their efficacy and safety. It is a requirement to carry out research on herbal mixtures, to develop simple bioassays for biological standardization and pharmacological evaluation.

However, polyherbal formulations (PHF) are well documented to have the potential of inducing or inhibiting enzyme expression and their activities [4-6]. Several native products have been studied for the activity on drug metabolizing enzymes (DMEs) by several researchers [7,8] and North American botanicals [9]. Furthermore, most of the studies on herb-drug interactions deal

with induction or inhibition of enzymes, and less attention has been paid toward the recovery of these modulated enzymes back to normal levels following the discontinuation of respective herbal drug [10]. The major DMEs, the cytochrome P450 (CYP), consist of hemeproteins superfamily that catalyzes the oxidative metabolism of a wide variety of exogenous and indigenous chemicals. Several isoforms such as CYP1A1, CYP1A2, CYP2C9, CYP2B1, CYP2C1, CYP2D6, CY2E1, and CYP3A4 appear to be most relevant isozymes participated in the metabolism of clinically significant drugs [11].

Five plants, all native in India were used in PHF. *Eugenia jambolana* Lam. is known for its anti-diabetic activity [12]. Fruit of *Momordica charantia* L. is a known for anti-diabetic activity [13]. *Psidium guajava* L. and *Allium sativum* L. are known for their anti-diabetic activity [14,15]. *Ocimum* sanctum Linn. (Holy basil) has been mentioned in Indian system of traditional medicine to be of value in the treatment of diabetes mellitus [16]. To achieve the aim, the effect of PHF administration on the transcriptional level as well as the functional activity of Phase-I and II DME's in dose and time dependent manner. The effect of PHF treatment discontinuation after multiple weeks on Phase-I and II DMEs was evaluated to assess the time required for the recovery of PHF modulated enzymes back to control levels. With this aim, the present work was designed to study the effect of PHF administration on Phase-I and II DMEs of liver and intestine to assess its drug interaction potential.

MATERIALS AND METHODS

Chemicals and Reagents

Acetaminophen, bufurolol, 1'-hydroxybufurolol, caffeine, dexamethasone, ethoxyresorufin, glutathione (reduced), mephenytoin, 4-hydroxymephenytoin, pentoxyresorufin, resorufin, phenacetin, warfarin, 7-hydroxywarfarin, phenylmethanesulfonyl fluoride, p-nitrophenol (PNP), UDP-glucuronic acid (ammonium salt) (UDPGA), 1-chloro-2, 4-dinitrobenzene (CDNB), 2-mercaptoethanol, 3'-Phosphoadenosine-5'-phosphosulfate (PAPS), flavin adenine dinucleotide, dicoumarol, 2,6 dichlorophenolindophenol, 2-naphthylsulfate, high performance liquid chromatography (HPLC) grade acetonitrile, and methanol were purchased from Sigma–Aldrich (St. Louis, MI, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) and dimethyl sulfoxide were purchased from SRL Pvt. Ltd (Mumbai, Maharashtra, India). Testosterone, 6β-hydroxytestosterone, chlorzoxazone, and 6-hydroxychlorzoxazone were purchased from Cayman chemical company (USA). Ultrapure water (18.2 M/Ω cm) was obtained from Milli-Q PLUS PF water. All other chemicals were commercially available or HPLC grade.

Preparation of PHF

Five herbs *E. jambolana* Lam. (Jamun) seeds, *M. charantia* L. (Bitter gourd) fruits, *O. sanctum* Linn. (Holy Basil) leaves, A. *sativum* L. (garlic), and *P. guajava* L. (guava) were obtained from the local vegetable market in the city of Lucknow, India. The selected herbal materials were shade dried and grinded by mixer grinder. The prepared hydroalcohol extracts were concentrated using rotary evaporator at 40°C temperature. The concentrated extracts were freeze-dried at −20°C for 12 h then lyophilized using lyophilizer. The lyophilized extracted powders were stored in an airtight glass box and kept in the desiccator until used. PHF was prepared by mixing 200 mg powder of each herb in a single formulation.

Animals

Male Sprague–Dawley rats of weight between 220 ± 20 g were provided by CSIR-IITR (India). Animals were maintained at 25°C temperature in steel cages with alternate 12 h of light and dark cycles and given a pallet diet and water. Before start of experiment, rats were acclimatized for 7 days then divided into two groups, PHF pre-treated ($n = 5$) and vehicle treated control ($n = 5$). Rats in pretreated group were gavaged (16-gauge gavage needle) with PHF (50, 100, and 200 mg/kg/day) for 7 days and multiples of weeks. The PHF suspension was made in 0.5% sodium carboxymethyl cellulose for oral administration. The control group was administered the same volume of vehicle for 7 days. Animals were allowed free access to food and water but before euthanasia, rats were overnight fasted to decrease the intestinal content. At the end of the experiment, rats were sacrificed by anesthetic ether inhalation. Experiments were carried out in accordance with current legislation on animal experiments as per Institutional Animal Ethical Committee at King George's Medical University Lucknow (IAEC approval no IAEC/2013/44).

Assessment of PHF Pretreatment on Hepatic and Intestinal Phase-I DMEs

The effect of PHF administration of Phase-I enzymes (CYPs) was evaluated at transcriptional and assessment of functional activity level was performed by various enzyme assays.

RNA Isolation, Primer Designing and Real-Time Polymerase Chain Reaction (RT-PCR) for CYPs

Rats were pretreated with PHF and vehicle for 7 days than rats were sacrificed with inhalation of anesthetic ether. A part of liver and intestine (10-50 mg) was homogenized with TRIzol (Invitrogen Life Technologies, USA) and RNA was isolated. Quantitation and purity check were accomplished using Nano drop (Thermo 6000, USA). Isolated RNA was instantly used for cyclic deoxyribose nucleic acid (cDNA) synthesis [17]. The cDNA sequences for various rat CYPs were obtained from Gene bank (gene bank as session number and sequences in [Table 1]. To make sure the specificity of primer sequences to target mRNA, each sequence was searched for homology with NCBI BLAST.

Effect of PHF pretreatment on the mRNA expression of CYPs was evaluated using RT-PCR. For quantitative RT-PCR, cDNA was amplified using Light Cycler 480 (Roche Molecular Biochemicals, USA) using SYBR green kit (Fermentas, USA).

Table 1: Oligonucleotide primer used for RT-PCR

CYP	Gene accession no	Forward primer sequence	Reverse primer sequence
CYP1A1	NM_012540.2	TTCAGTTCAGTCCTTCCTCACA	GAAGGCTGGGAATCCATACA
CYP1A2	NM_012541.3	CTACAACTCTGCCAGTCTCCAG	CCTCTCAACACCCAGAACACT
CYP2B1	NM_001134844.1	TTCAGTTCAGTCCTTCCTCACA	GAAGGCTGGGAATCCATACA
CYP2B2	NM_001198676.1	GGGGAACCTCCTGCAGTT	GTGAACACATCTCCATATTTTTCG
CYP2C11	U33173.1	GGAGGAACTGAGGAAGAGCA	AATGGAGCATATCACATTGCAG
CYP2D1	AB008422.1	TCAGGATGGTGAAACTAGTGGA	TGGGAACGTGTTAAGAACCTC
CYP2D2	NM_012730.1	GAAGGAGAGCTTTGGAGAGGA	AGAATTGGGATTGCGTTCAG
CYP2E1	AF061442.1	CTGACTGTCTCCTCATAGAGATGG	TCACAGAAACATTTTCCATTGTGT
CYP3A1	L24207.1	GCAGGAGGAGATCGACAGG	CCAGGTATTCCATTTCCATCA
CYP3A2	U09742.1	ACC CGT CTG GAT TCT AAG CA	TGGAATTATTATGAGCGTTCAGC
GAPDH	M17701.1	AGCTGGTCATCAATGGGAAA	ATTTGATGTTAGCGGGATCG

CYP: Cytochrome P450, RT-PCR: Real time polymerase chain reaction

The reaction was performed in 96-well PCR plate. The reaction mixture was consisted of 1X SYBR green, 200-300 nM primers, and 2 μl of cDNA in final reaction volume of 20 μl. The thermal cycle condition was 95°C for 15 min to activate Amplitaq Gold DNA polymerase, 95°C for 15 s and anneal/extension at 60°C for 1 min (40 cycles). Glyceraldihyde-3-phasphate dehydrogenase (GAPDH) was used as internal loading control. Fold changes in mRNA level were resultant after normalizing with GAPDH mRNA levels [17].

Evaluation of PHF Treatment on Function Activity of CYPs

To evaluate the effect of PHF pretreatment on CYPs functional activity various enzyme assays were performed with specific CYP probe substrates using microsomes structured from PHF treated and control animals.

Preparation of Microsome and Cytosolic Fractions

Liver and intestine were quickly excised, snap frozen, and stored at −80°C until used after perfusion with ice-cold normal saline. Microsome and cytosolic fractions were prepared [18]. Microsomal protein and cytosolic protein were determined using Lowry method of protein assessment using BSA as standard [19]. CYP concentrations in rat liver microsomes (RLM) and rat intestine microsomes were quantified [20].

Assessment of CYPs Mediated Phase-I Enzymatic Activity

Microsomes prepared from PHF pre-treated, recovery and control groups were used for assessing the effect of PHF pretreatment and discontinuation on the enzymatic activity of hepatic Phase-I DMEs. CYP-mediated Phase-I DMEs were analyzed with following enzymatic assays.

CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) and CYP2B-dependent pentoxyresorufin O-dealkylase (PROD) assay

EROD and PROD assays [18] were performed with slight modifications. In brief, the reaction mixture consisted of a 100 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mg/ml microsomal protein with a final reaction volume of 0.2 ml. Ethoxyresorufin (5 μM) and pentoxyresorufin (10 μM) were used as probe substrate for CYP1A1 and CYP2B, respectively. The reaction mixture was pre-incubated for 5 min at 37°C. After pre-incubation, the reaction was initiated with addition of NADPH (2 mM), followed by incubation at 37°C for 15 min. An equal amount of buffer was added to the blank reaction. The reaction was terminated by addition of 300 μl ice cold methanol. The reaction was mixed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as HPLC sample. The amount of formed metabolite (resorufin) was determined using HPLC with fluorescence detector. The activity was expressed as pMol of product formed/min/mg.

CYP1A2-dependent phenacetin O-deethylase (POD) assay

POD assay [21] was performed with slight modifications. Phenacetin (50 μM) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. Reaction was initiated with adding of NADPH (2 mM). After 20 min incubation at 37°C reaction was terminated by adding up equal volume of ice-cold acetonitrile (containing internal standard caffeine). The blank reaction consisted of equal amount of buffer. The mixture was vortexed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant obtained was used as HPLC sample. Quantification of product (acetaminophen) was done from standard curve using HPLC-UV. Activity was expressed as nMol of product formed/min/mg.

CYP2C19-dependent mephenytoin 4-hydroxylation assay

Mephenytoin 4-hydroxylation assay [22] was carried out with slight modifications. Mephenytoin (50 μM) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of 100 mM potassium phosphate buffer (pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and terminated by adding up the equal volume of ice-cold acetonitrile (containing internal standard beta-naphthol) after 40 min incubation at 37°C. The blank reaction consisted equal amount of buffer. The mixture was vortexed and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was used as HPLC sample. Quantification of product (4-hydroxymephenytoin) formed was

done using standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

CYP2D-dependent bufurolol 1-hydroxylation assay

Bufurolol (20 μM) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and reaction was terminated by adding equal volume of ice cold acetonitrile after 20 min incubation at 37°C. The blank reaction contained equal amount of buffer. The mixture was then sturdily mixed, chilled on ice for 15 min, and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (1-hydroxybufurolol) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

CYP2E1-dependent chlorzoxazone 6-hydroxylation assay

Chlorzoxazone 6-hydroxylation assay [23] was performed with slight modifications. Chlorzoxazone (50 μM) was incubated with RLM (1 mg/ml), 10 mM $MgCl_2$, in a total volume of 0.2 ml of potassium phosphate buffer (100 mM, pH 7.4). Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with the addition of NADPH (2 mM) and reaction was terminated by adding equal volume of ice cold acetonitrile (containing internal standard phenacetin) after 20 min incubation at 37°C. Blank reaction contained equal amount of buffer. The mixture was then vigorously mixed and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (6-hydroxychlorzoxazone) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

CYP3A-dependent testosterone 6β-hydroxylation assay

Testosterone 6β-hydroxylation assay was performed [24]. Testosterone (100 μM) was incubated with RLM (1 mg/ml), 5 mM $MgCl_2$, in a total volume of 0.2 ml of 100 mM potassium phosphate buffer, pH 7.4. Reaction mixture was pre-incubated for 5 min at 37°C. The reaction was started with addition of NADPH (2 mM) and reaction was terminated by adding of equal volume of ice-cold acetonitrile (containing internal standard dexamethasone) after 20 min of incubation at 37°C. Blank contains equal amount of buffer. The mixture was vigorously mixed and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant obtained was used as HPLC sample. Quantification of product (6β-hydroxytestosterone) formed was done from standard curve of metabolite using HPLC. Activity was expressed as nMol of product formed/min/mg.

Assessment of PHF Pretreatment on Hepatic and Intestinal Phase-II DMEs

To examine the effect of PHF pretreatment on hepatic and intestinal Phase-II DMEs, microsomal and cytosolic conjugation enzyme activities were evaluated. Animals experiment and microsomal and cytosolic fractions were prepared as described. Phase-II DME's were analyzed with following enzymatic assays.

UDP-glucuronyltransferase (UDPGT) assay

UDPGT activity was measured by determining the PNP conjugation with UDPGA [25]. Activity was performed by determining amount of PNP consumed. The reaction mixture was consisted of 0.5 mM PNP, 4 mM $MgCl_2$, 50 mM TrisHCl (pH 7.4) and 1 mg/ml microsomal protein. The samples were preincubated in shaking water bath for 5 min at 37°C and the reaction was initiated with addition of UDPGA (2 mM). Blank samples were performed in the absence of UDPGA, but the equal volume of buffer was added. After taking 15 min for incubation, reactions were broken by the addition of trichloroacetic acid (5% final concentration). Samples were then centrifuged at 12,000 rpm for 15 min; supernatant was collected and NaOH was added to the reaction mixture to achieve final concentration of 0.5 M. Consumed PNP was calculated by subtracting value from blank. PNP concentration was determined at 410 nm using standard curve of PNP. Activity was expressed as nMol of conjugate/min/mg.

Glutathione S-transferase (GST) assay

Cytosolic GST activity to conjugate CDNB with reduced glutathione (GSH) was estimated [26]. Reaction mixture contained potassium phosphate buffer (0.3 M, pH 6.5) GSH (30 mM) and CDNB (30 mM) in 96 well plate. Reaction was initiated with addition of cytosolic enzyme. Blank contained an equal volume of buffer. The reaction was continuously monitored at 37°C in plate reader at 340 nm immediately after addition of cytosolic protein. Activity was expressed as nMol conjugate/min/mg.

Sulfotransferase (SULT) assay

SULT activity in cytosolic fraction was measured using methylene blue extraction assay [27]. In brief, reaction mixture contains sodium acetate buffer pH 5.5, 7.5 μMol 2-mercaptoethanol, 65 μMol PAPS, 215 μMol of 2-naphthol. After pre-incubation for 5 min at 37°C, reaction was initiated with addition of cytosolic enzyme. Control reaction also contained equal volume of enzymes solution but the reaction was instantly stopped by addition of equal volume of methylene blue reagent. Chloroform (2.0 ml) was added, and the reaction mixture was vortex vigorously. Mixture was centrifuged at 1000 × *g* for 15 min. Sodium sulfate was added to collected organic phase. The absorbance of organic phase was measured at 651 nm and specific activity (nMol product/min/mg protein) was calculated (10 nMol of 2-naphthylsulfate yields 0.3 absorbance at 651 nm). Activity was expressed as nMol of product formed/min/mg.

Statistical Analysis

Statistical analyses were performed with help of Prism version 5.1. Student's *t*-test (non-parametric) and one-way analysis of

variance followed by the Student's Newman-Keuls multiple range tests were used as applicable. Data are obtained as mean with standard errors. The level of significance was set prior at $P < 0.05$.

RESULTS

Effect of PHF Administration on Hepatic Phase-I and Phase-II DMEs

To evaluate the effect of PHF administration on hepatic DMEs, dose and time dependent study was conducted. Similarly, the effect of PHF discontinuation after 1 week pretreatment was also evaluated to determine whether the modulated DMEs recovered back to normal level or not, when PHF administration was discontinued.

The treatment with PHF at lower dose (50 mg/kg/day) for 7 days only change mRNA expression of CYP1A1 (two-fold up-regulation) and CYP3A2 (1.9 fold down-regulation) as compared to vehicle treated control. However, the treatment with PHF (100 and 200 mg/kg/day dose) for 7 days significantly up-regulated the mRNA expression of CYP1A1 (5.1 and 5.9 fold), CYP1A2 (1.9 fold), CYP2D1 (2.2 and 2.7 fold), and CYP2D2 (3.5 and 3.9 fold), respectively compared to vehicle treated control. However, treatment with PHF (100 and 200 mg/kg/day dose), significantly down-regulation was found in the mRNA expression of CYP3A1 (1.9 fold) and CYP3A2 (2.6 and 2.4 fold), respectively compared to control. Interestingly, treatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days did not alter the mRNA expression level of CYP2B1, CYP2B2, CYP2C11 and CYP2E1 compared to vehicle-treated control [Figure 1].

Similarly, to validate the mRNA data, functional activity of CYPs were assessed after PHF treatment. The pretreatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days significantly increased the functional/catalytic activity of EROD (1.6, 3.7 and 5.4 fold), POD (1.5 fold), PROD (1.2, 1.4 and 1.5 fold) and bufurolol hydroxylase (2.9 fold), respectively, compared to control. However, pretreatment with PHF did not alter the enzymatic activity of, mephenytoin and chlorzoxazone hydroxylase enzymes. In addition, treatment with PHF (50, 100 and 200 mg/kg/day dose) for 7 days decreased the enzymatic activity of testosterone hydroxylase (2 fold), respectively, compared to control [Figure 2].

Likewise, when Phase-II conjugation activity was assessed in dose-response study, no alteration in UDPGT, GST and SULT was observed at lowest dose study (50 mg/kg/day for 1 week). However, increase in catalytic activity of UDPGT (1.6 and 2.3 fold) and GST (1.6 and 2.1 fold) was observed at 100 and 200 mg/kg/day, respectively, in comparison to control. Modulation in SULT functional activity (1.8 fold) was obtained at 200 mg/kg/day of PHF dose compared to control [Figure 3].

Time Dependent Response of PHF Administration on Hepatic Phase-I and II DMEs

To assess the time-dependent response of DMEs rats were administered with PHF (100 mg/kg/day) up to 4 weeks and

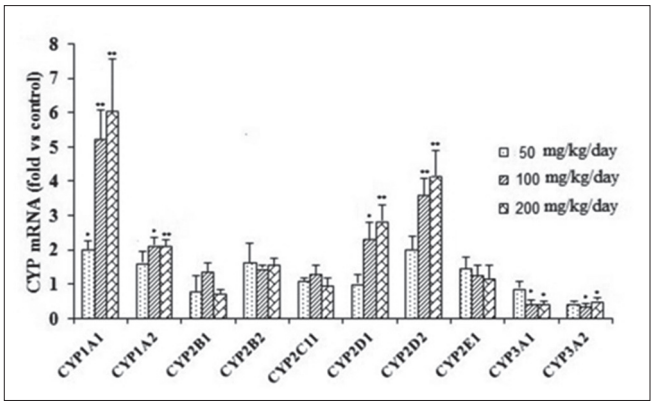

Figure 1: Dose response of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 mRNA level. Rats were given 50, 100 and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

activity was analyzed after every week. PHF pretreatment for 1, 2, 3 and 4 weeks increased the mRNA level of CYP1A1 (5.4, 5.5, 4.7 and 4.1 fold), CYP1A2 (1.7, 2, 1.9 and 2.9 fold), CYP2D1 (2, 2.1, 1.2 and 2.5 fold), and CYP2D1 (3.4, 2.9, 2.7 and 4.1 fold). Similarly, PHF treatment for 1, 2, 3 and 4 weeks decreased the mRNA levels of CYP3A1 (1.9, 2.8, 2.3 and 1.6 fold) and CYP3A2 (2, 2.5, 2 and 1.6 fold), respectively. No significant modulation in mRNA level of CYP2B1 and CYP2C11 was observed. However, CYP2E1 mRNA level was not altered by 2 weeks but after 3 and 4 weeks of PHF treatment mRNA level of CYP2E1 was increased by 1.9 fold in comparison to control [Figure 4].

In the same way, PHF treatment for 1, 2, 3 and 4 weeks increased the catalytic activities of CYP1A1 (3.5, 5, 7.2 and 8.6 fold), CYP1A2 (1.6, 1.5, 1.5 and 2.4 fold), and CYP2D (2.1, 2.1, 2.4 and 3 fold), respectively. Although increase in the mRNA level of CYP2B2 was observed, the CYP2B-dependent catalytic activity was increased significantly by 1.6 fold only after 4 weeks of PHF treatment. Similarly, CYP2E1-dependent catalytic activity was also increased by 1.4 fold after 3 weeks of PHF treatment. In addition, CYP3A-dependent catalytic activity significantly decreased by 1.8, 1.8, 2 and 2.1 fold after 1, 2, 3 and 4 weeks of PHF treatment [Figure 5]. However, no difference in CYP2C9 and CYP2C19-dependent catalytic activities was observed. When Phase-II conjugation enzymes activities were assessed, it was observed that UDPGT and GST activities were increased only by 1.5 fold after every 1 week of PHF treatment compared to control. No difference in catalytic activity of SULT was observed up to 2 weeks of PHF treatment. However, after 3 weeks of PHF treatment SULT activity was increased by 1.8 fold in comparison to control [Figure 6].

Effect of PHF Discontinuation on Recovery of Modulated Hepatic DMEs

To assess the recovery of modulated hepatic CYP enzymes with discontinuation of PHF, rats were first treated for 1 week with PHF (100 mg/kg/day) for induction/inhibition and then PHF treatment

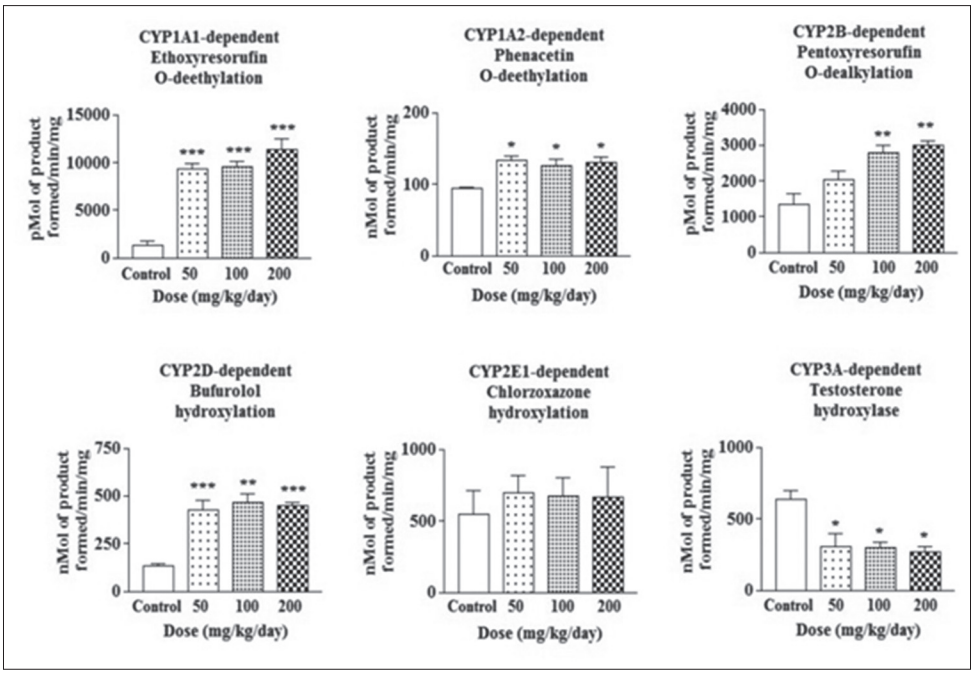

Figure 2: Dose response of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 functional activity. Rats were given 50, 100, and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same time period of time. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

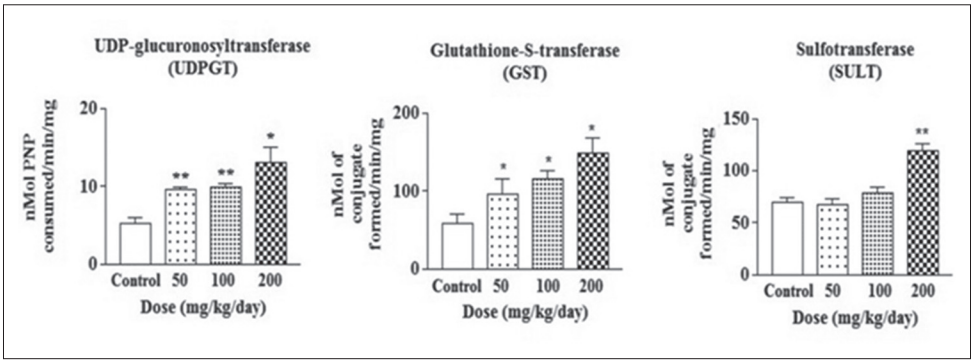

Figure 3: Dose response of polyherbal formulation (PHF) pretreatment on hepatic Phase II conjugation enzymes functional activity. Rats were given 50, 100 and 200 mg/kg/day of PHF for 1 week. Control rats received same volume of blank formulation for same period of time. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) from control

was discontinued. Hepatic CYP mRNA expression level was assessed after the induction period and each week of the recovery period. Pretreatment with PHF (100 mg/kg/day) for seven days significantly increased the mRNA expression levels of CYP1A1 (5.2 fold), CYP1A2 (1.7 fold), CYP2B2 (3.1 fold), CYP2D1 (2.2 fold), and CYP2D2 (3.4 fold) in comparison to control. However, a significant decrease of 2 fold in mRNA expression of CYP3A1 and CYP3A2 was observed compared to control. However, no significant difference in mRNA level (CYP1A1, CYP1A2, CYP2D1, CYP2D1, CYP2D2, CYP3A1, and CYP3A2) was observed after 1 week of last PHF dose in comparison to control. No alteration in mRNA levels of CYP2B1, CYP2C11, and CYP2E1 was observed with PHF treatment [Figure 7].

Similarly, all CYP enzyme activities and Phase-II conjugation enzyme activities that were modulated with PHF treatment reverted back to control level after 1 week of PHF discontinuation except CYP1A1 and CYP2D-dependent enzymatic activities. The CYP1A1-dependent and CYP2D-dependent catalytic activities recovered back to control level after 2 weeks of BM discontinuation [Table 2].

Dose-response of PHF Administration on Intestinal Phase-I and II DMEs

Pretreatment with PHF at 50, 100 and 200 mg/kg/day dose significantly induced the mRNA level of CYP1A1 by 3.2, 3 and 4.3 fold, respectively. Interestingly, PHF pretreatment for 1 week decreased the mRNA level of CYP3A2 (2, 1.9 and 2.2 fold) at 50, 100 and 200 mg/kg/day, respectively. However, the treatment with PHF (50, 100 and 200 mg/kg/day dose) for 1 week did not alter the mRNA expression level of CYP2C11, 2D1, 2D2 and 2E1 compared to vehicle-treated control [Figure 8].

To validate our mRNA data with active protein level, we measured the catalytic activity of respective CYPs with specific probe substrate and the result obtained supported the mRNA data. In dose response study a significant increase in CYP1A1-dependent catalytic activity by 1.4, 1.7 and 1.6 fold was observed at 50, 100 and 200 mg/kg/day, respectively. However, PHF pretreatment significantly decreased the CYP3A-dependent catalytic activity of testosterone 6β-hydroxylase by 1.5 fold at dose studied. No significant difference in the CYP2C9, CYP2C19, CYP2D, and CYP2E1-dependent catalytic activity was observed [Figure 9].

When Phase-II conjugation activity was assessed in dose-response study, no alteration in conjugation enzymes was observed at low PHF dose (50 mg/kg/day) when administered for 1 week. However, PHF pretreatment for one week significantly increased UDPGT (3.4 and 4.2 fold) and GST (1.4 and 1.9 fold) activity at 100 and 200 mg/kg/day, respectively. No alteration was observed in SULT activity with PHF treatment [Figure 10].

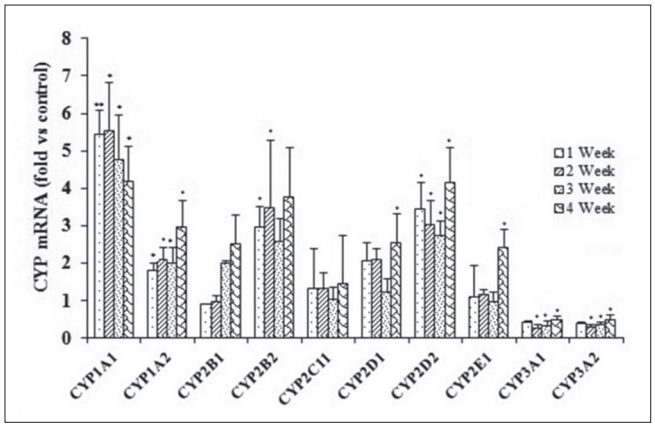

Figure 4: Time dependent effect of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 mRNA level. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$,**$P < 0.01$,***$P < 0.001$) from control

Time-dependent Response of PHF Administration on Intestinal Phase-I and II DMEs

Similarly to evaluate the effect of different pretreatment duration on intestinal DMEs, rats were pretreated with PHF (100 mg/kg/day) for different time duration and any alteration in DMEs mRNA level and functional activity was measured and compared with control. In study, it was observed that mRNA level of CYP1A1 was increased by 3.3, 3.6, 3.5 and 5.4 fold after 1, 2, 3 and 4 weeks of treatment with PHF, respectively, compared to control. However, no significant difference was observed among the treatment duration. Similarly, no treatment time dependency was observed for CYP3A2 (2.2, 2.3, 2.1 and 2.1 fold decrease). In addition, PHF failed to alter the mRNA level of CYP2C11, 2D1, 2D2 and 2E1 compared to vehicle-treated control [Figure 11].

To confirm the RT-PCR results with functional activity of respective CYP enzymes, it was observed that CYP1A-mediated catalytic activity (EROD) was increased by 1.2, 1.2, 1.3 and 1.5 fold after 1, 2, 3 and 4 weeks of PHF treatment, respectively. The CYP3A-mediated testosterone hydroxylase catalytic activity was decreased by 1.7, 1.9, 2.4 and 2.2 fold after 1, 2, 3 and 4 weeks of PHF treatment, respectively. Similar to CYP1A-mediated catalytic activity no treatment duration dependency was observed. No significant difference was observed in CYP2E1, however, catalytic activity was significantly increased by 2.1 after 3 and 4 weeks of PHF treatment. Furthermore, no

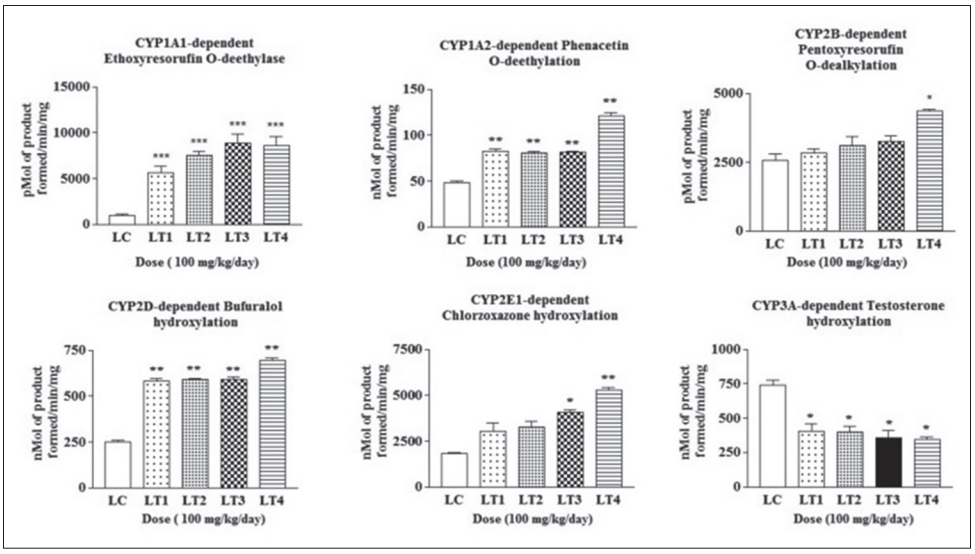

Figure 5: TTime dependent effect of polyherbal formulation (PHF) pretreatment on hepatic cytochrome P450 functional activity. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank for same time duration. LC, LT1, LT2, LT3 and LT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from control

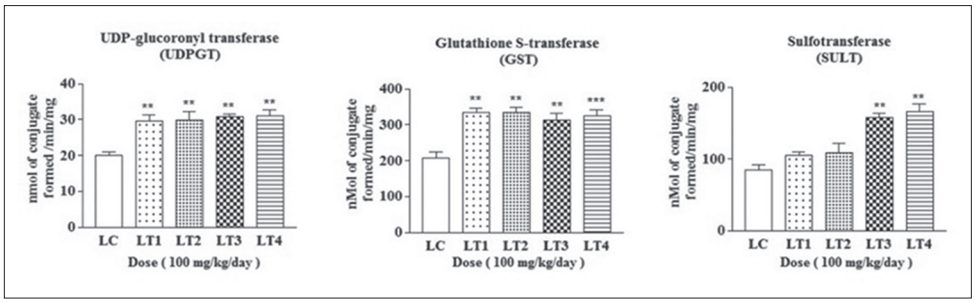

Figure 6: Time dependent effect of polyherbal formulation (PHF) pretreatment on hepatic Phase II conjugation enzyme functional activity. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacriced after every week. Control rats received the same volume of blank formulation for same time duration. LC, LT1, LT2, LT3 and LT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) from control

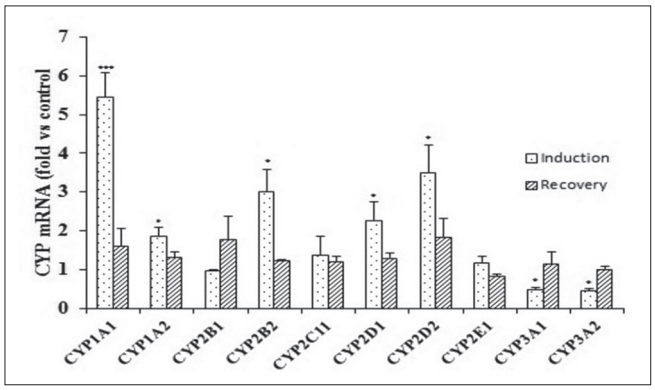

Figure 7: Effect of one week discontinuation of polyherbal formulation (PHF) treatment on hepatic CYPs mRNA level. Rats were treated with PHF (p.o 100 mg/kg/day) for one week and then PHF administration was discontinued for 1-week. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ****P* < 0.001) as compared to control

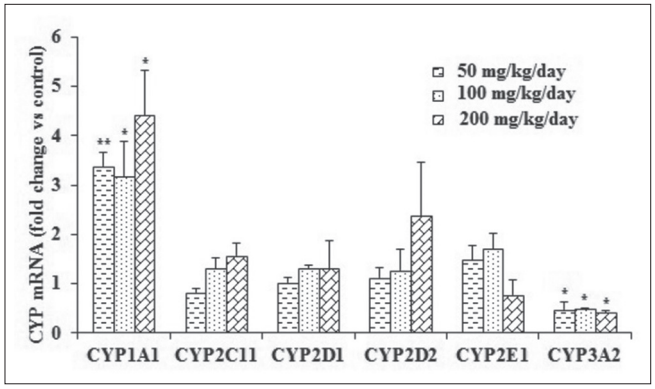

Figure 8: Dose response of polyherbal formulation (PHF) pretreatment on intestinal CYPs mRNA level. Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1-week and sacrificed on 8th day. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01) from control

significant difference in CYP2C9, CYP2C19, and CYP2D-dependent catalytic activity was observed even after 4 weeks of PHF treatment [Figure 12].

DISCUSSION

Recent studies have explained the importance and putative effect of anti-diabetic herbs in a single formulation on CYPs and conjugating enzymes. In this study, we used self-prepared polyherbal formulation to evaluate its effect on hepatic and intestinal CYP and conjugation enzymes. This herbal preparation was prepared by five herbs that have been traditionally used as an option for the treatment of diabetes. For effective and safe use of potential herbs, DMEs mediated herb-drug interactions must be studied. To the best of our knowledge, there is no such report available on hepatic and intestinal CYP and Phase-II (conjugation) enzymes for this type of polyherbal formulation. Therefore, the mechanism of herb-drug interactions that might occur with herbal consumption, alterations at transcriptional and/or translational level of DMEs must be studied. The true measure of enzyme response is their catalytic activity instead of total protein. Therefore, in the present study, we directly measured the enzymatic activities of individual CYP enzymes along with individual mRNA level instead of protein level to assess the fundamental mechanism of PHF mediated herb-drug interactions.

In vivo modulation of hepatic and intestinal DMEs was studied in a dose response manner using different doses, *viz*., 50, 100, and 200 mg/kg/day. Dose response experiment with PHF on CYP suggested that a lower dose (50 mg/kg/day) was adequate to produce effect on hepatic CYP enzymes. PHF pretreatment increased mRNA expression levels of hepatic CYPs to CYP1A1 > CYP2D2 > CYP2D1 > CYP2B2 > CYP1A2 that correspond well with catalytic activities of each CYP (CYP1A1-dependent EROD > CYP2D-dependent bufurolol hydroxylase > CYP1A2-depenedent phenacetin-O-dethylase). However, CYP1A1 is the only intestinal CYP that has been considerably upregulated with PHF treatment. Moreover, PHF pretreatment decreased the mRNA expression of hepatic and intestinal CYP3A1 and CYP3A2 genes and this decrease in mRNA level correspond well with CYP3A-dependent testosterone hydroxylase catalytic activity. Interestingly, no dose dependency was observed at mRNA level as well as the catalytic activity level of any CYPs. Alteration at mRNA level corresponds directly with the activity of respective

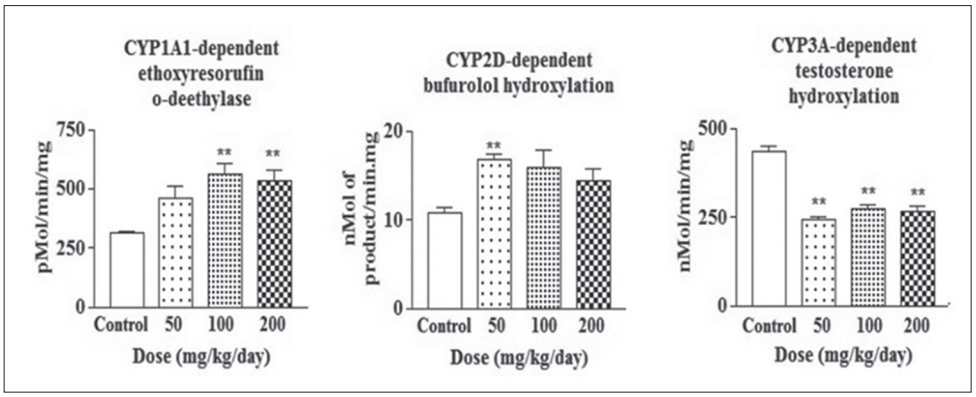

Figure 9: Dose dependent changes in intestinal cytochrome P450 activity in rats treated with standardized extract of polyherbal formulation (PHF). Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1-week and sacrificed on 8th day. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

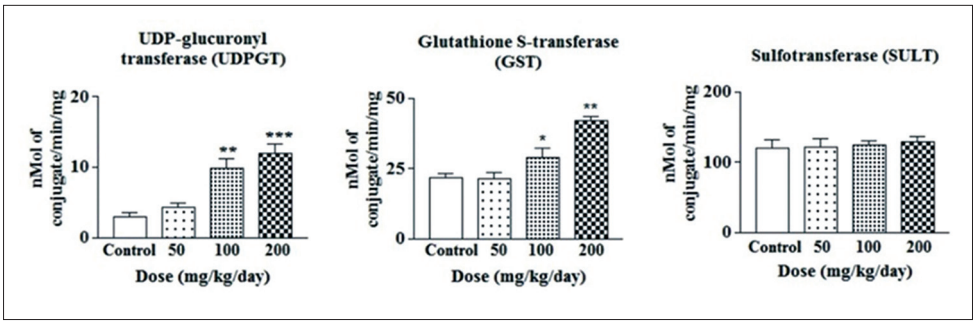

Figure 10: Dose dependent changes in intestinal conjugation Phase II enzyme activity in rats treated with extract of polyherbal formulation (PHF). Rats were given 50, 100 and 200 mg/kg/day of standardized PHF extract for 1 week and sacrificed on 8th day. Control rats received same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

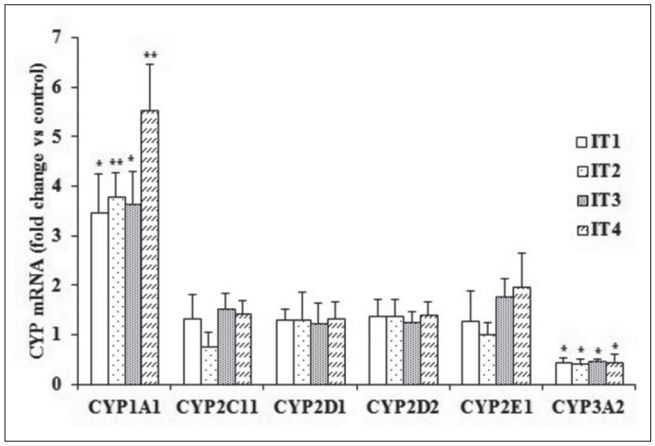

Figure 11: Time dependent effect of polyherbal formulation (PHF) pretreatment on intestinal cytochrome P450 mRNA level. Rats were given PHF (p.o 100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. IC, IT1, IT2, IT3 and IT4 represents control group, 1-week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with PHF, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

CYP that may be attributed to alteration at the translational level. However, PHF at dose studied (50-200 mg/kg/day) failed to modulate the expression of hepatic and intestinal CYP2C11 and CYP2E1 activities at transcriptional and activity level. Besides, CYP2C11 and CYP2E1, intestinal CYP2D isoform was also unaffected both at transcriptional and catalytic activity level. Similarly, time response study with PHF (100 mg/kg/day) exhibited that 1 week pretreatment was sufficient to produce the alteration in CYPs at transcriptional and catalytic activity level, and no further modulation was observed with increased treatment duration. In addition, no dose and time dependency was observed (except CYP2E1, upregulated after 3 weeks of treatment) that suggests low dose and 1 week treatment was sufficient for absorption of PHF to concentration level and that too up to saturation level to put forth its effect. However, upregulation of CYP2E1 could be attributed to time taken by responsible PHF components to accumulate up to the level at which they can exert their effect. Even at high concentration pretreatment for 1 week, level that could alter the CYP2E1 was not achieved to exert its effect on CYP2E1. These alterations in the catalytic activity of various CYP enzymes with PHF administration may affect their own metabolism or drugs that are metabolized through these CYP's [28].

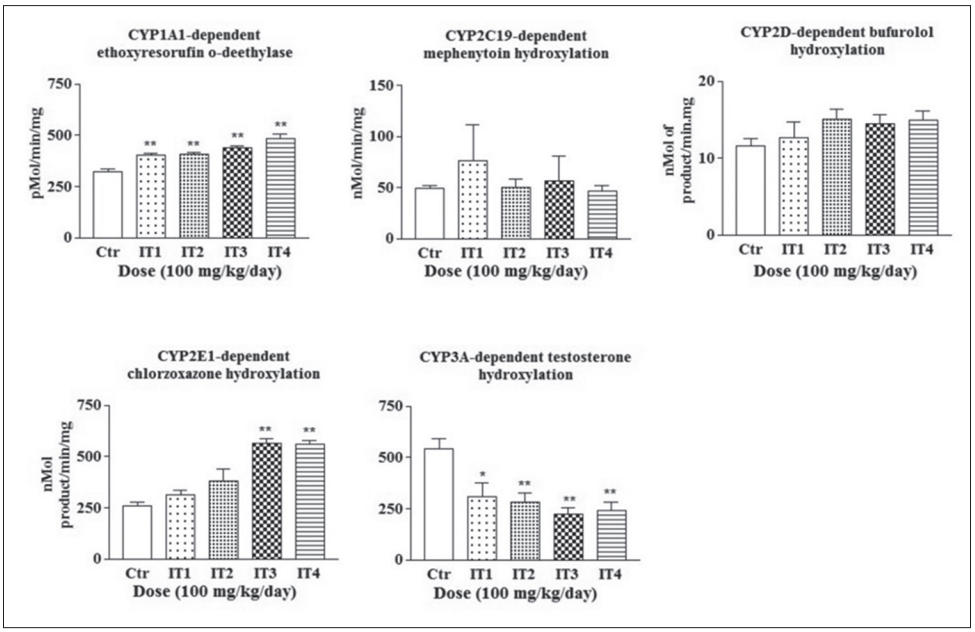

Figure 12: Time dependent effect of polyherbal formulation (PHF) pretreatment on intestinal cytochrome P450 functional activity. Rats were given PHF (100 mg/kg/day) for 4 weeks and rats were sacrificed after every week. Control rats received the same volume of blank formulation for same time duration. IC, IT1, IT2, IT3 and IT4 represents control group, 1 week pretreated group, 2 week pretreated group, 3 week pretreated group and 4 week pretreated group with BM, respectively. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) from control

Table 2: Induction and recovery of hepatic CYP activities and Phase-II conjugation reactions in rats treated with PHF extract

DMEs	Untreated control	Treatment 1 week	Recovery (after 1 week oral administration of PHF)		
			1 week	2 week	3 week
CYP					
CYP1A1 (pmol/mg/min)	926.56±412.76	6333.04±1230.25**	4680.0±916.74*	2590.09±323.29	1068.0±145.59
CYP1A2 (nmol/mg/min)	55.70±5.13	77.29±1.34	69.37±3.75	62.76±12.83	58.23±5.80
CYP2B (pmol/mg/min)	3002.29±117.16	1842.17±34.03	2791.59±345.22	2850.12±456.89	2919.29±567.65
CYP2D (nmol/mg/min)	249.66±12.66	582.66±21***	574.25±44.23*	483.41±57.63	280.21±23.0
CYP2E1 (nmol/mg/min)	1830.06±106.8	3029.47±795.96	2031.8±133.35	1927.7±186.24	1930.90±198.87
CYP3A (nmol/mg/min)	743.48±63.053	403.22±111.84*	450.14±49.06	510.91±30.45	650.45±19.98
Conjugation assay					
UDPGT (nmol of PNP consumed/mg/min)	20.03±2.56	29.52±3.78	24.25±2.76	25.23±4.23	21.94±5.23
GST (nmol of conjuagte/mg/min)	206.65±30.09	334.80±21.98*	221.76±19.76	208.87±31.26	210.65±40.09
SULT (nmol of conjuagte/mg/min)	85.04±1.14	88.91±3.56	96.23±2.67	86.56±5.23	

Rats were given 100 mg/kg of PHF for 7 days and then PHF was discontinued. Intestinal CYP and conjugation enzymatic activities were determined after 1, 2 and 3 weeks of PHF discontinuation. Each value is expressed as mean±standard error from three independent experiments. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) from control. CYP: Cytochrome P450, PHF: Polyherbal formulation, SULT: Sulfotransferase, GST: Glutathione S-transferase, PNP: p-nitrophenol, UDPGT: UDP-glucuronyltransferase

Similarly, a dose-dependent study with Phase-II conjugation activities revealed that PHF administration increased the hepatic and intestinal UDPGT and GST activity. No alteration was observed in intestinal SULT activity with various PHF doses and time-dependent durations. However, hepatic SULT activities were increased (1.8 fold) at higher doses (200 mg/kg/day). Conjugation enzymes (UDPGT and SULT) play a major role in drug metabolism. Therefore, an increase in activity of these enzymes might result in increased efficacy toward their role such as decreasing the risk of carcinogenicity and toxicity of many drugs, and their metabolites. Nevertheless, increased activity of the Phase-II DMEs (conjugation enzymes) might also affect the efficacy of many substrate drugs, e.g., Acetaminophen (UDPGT) 2-naphthol (SULT) [27].

We performed a recovery experiment to assess whether the altered level of DME's with PHF pretreatment recovered back to normal level or not after the discontinuation of PHF treatment. Although no considerable difference at mRNA and catalytic activity level of all CYP isoforms (hepatic) was observed in between control group and 1 week recovery group, the hepatic CYP1A1-dependent and hepatic CYP2D-dependent enzymatic activities were recovered back to control level after 2 weeks of PHF discontinuation. This late recovery could be attributed to increased amount of CYP1A1 and CYP2D protein that must be degraded to attain normal level. Similarly, to evaluate the effect of discontinuation of PHF after 1 week pre-treatment on Phase-II metabolizing enzymes, we observed a fast recovery of UDPGT, GST and SULT to control level with a recovery

period of 7 days. Such fast recovery from induced to control level ruled out the possibilities of Phase-II enzyme mediated herb-drug interaction following the PHF treatment. However, further investigation in human microsomes, human hepatocytes are required to validate their effect to clinical implementation.

CONCLUSIONS

Findings from the present study suggest that the PHF formulation has both inductive and inhibitory effect on DMEs when orally administered. PHF are likely to induce DMEs except CYP3A but less likely to produce significant drug interactions. Certain major factors of metabolism such as competition between co-administered drugs, unspecific interactions with proteins and enzyme induction due to chronic intake are not addressed *in vitro* assay. However, data presented in this study clearly demonstrate that PHF might have the potential to inhibit or induce the metabolism of certain co-administered antidiabetic drugs which are metabolized by CYPs and Phase-II conjugated enzymes.

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