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Possible herb-drug interaction of *Morus alba* L.- *a* potential anti-diabetic plant from Indian Traditional medicine

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In the Indian System of Medicine, *M. alba* L. leaves are widely used for the management diabetes and as a general tonic to enhance health. The present study was designed to standardize the extract of *Morus alba* by reverse phase high performance liquid chromatography (RP-HPLC) and exploration of the herb drug interaction potential through inhibition of Cytochrome P450 (CYP450) enzymes. Quantification of bioactive constituent in the plant extract was determined by RP-HPLC method. The plant extract and single bioactive molecule were studied for their inhibition potential on pooled CYP450, as well as recombinant human CYP450 is forms such as CYP3A4, CYP2D6, CYP2C9 and CYP1A2 isozymes. The inhibition potential of the plant extract is higher than the single bioactive molecule. Plant extract and its phyto-constituent showed significantly higher IC_{50} value than respective positive control against CYP3A4, 2D6, 2C9 and 1A2. Interaction potential of phyto-constituent and the plant extracts with pooled CYP450 were significantly lesser ($p<0.05$; $p<0.01$; $P<0.001$) than known CYP450 inhibitors. The present findings suggested that plant extract and its bioactive compound contributed negligible herb drug interactions and safe to use in diabetes management.

Keywords: Anti-diabetic, Medicinal plant, Rp-Hplc, Cytochrome P450, Herb drug interaction.

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Cytochrome P450 (CYPs) is a diverse group of enzyme involved in the oxidative metabolism of drugs and other xenobiotics. Most often unexpected adverse drug interactions occur due to CYP inhibition or induction and sometimes it may dramatically alter the metabolism of co-administered drugs[1]. Multi-drug combination therapy is pervasive for a number of diseases and the drug–drug, herb-drug and food-drug interactions are getting more focused in clinical practices[2,3]. On the regulation of *in vitro* human CYP enzyme activities several scientific evidences concerned about the action of phytoconstituents or whole extracts from herbals[4]. Plant extracts consist of many phyto-constituents which can cause pharmacokinetic herb-drug interaction with modern medicines when they were co-administrated with prescribed modern medicine[5]. Using *in vitro* assays of CYP inhibition a large number of isolated compounds as well as herbal extract can be conveniently evaluated for the screening of new drug from natural resources[6,7]. To screen herbal remedies, standardization and marker profiling of extract is essential for their quality evaluation. Also, strategies to interpret the data must be critically addressed, since active components cannot be reliably quantified in complex mixtures[8]. So, the development of marker profile as a standardization process for herbals is of great importance for quality control, proper scientific validation and batch to batch reproducibility of their therapeutic potency by the herbal drug manufacturers[9,10]. From the ancient time, *M. alba* is widely used for the management diabetes and as a general tonic to enhance health[11]. The popular medicinal plants, viz. *Morus alba*, along with medicinally active phyto-constituent (chlorogenic acid) was selected to explore the possibility of their drug interaction potential. *M. Alba* L. (Family-Moraceae) is an important herb, traditionally used to treat and prevent several diseases[12]. It is commonly used as a dietary supplement for its antioxidant properties and recently used as a pharmaceutical food[1]. All parts of this plant are used in the treatment of liver, spleen disorders and several cardiovascular complications[13]. The fruit juice acts as a cleansing agent with tonic properties. The stem and bark of this plant possesses hypoglycemic, antirheumatic,

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antispasmodic, diuretic, hypotensive activities[14]. There are several scientific documentation mentioning that this plant possesses anti-diabetic[15], hypolipidemic, antihypertensive[13], antimicrobial, neuroprotective[16], anticancer[17] (Choi and Hwang, 2005) and antiulcer[14] activities. *M. alba* and its bioactive molecule chlorogenic acid, rutin possesses potent anti-diabetic activity against type II diabetes[15] (Hunyadi *et al.* 2012). In the present study, the extract of *M. alba* was standardized by RP-HPLC its bio-active molecule. The standardized extracts of *M. alba* was screened for their inhibitory effects on CYP3A4, 2D6, 2C9, and 1A2 through fluorometric probe substrates with respect to their bioactive compounds.

Materials and methods

Chemicals

For this study, Vivid® CYP450 Screening Kit (CYP1A2, CYP2C9, CYP2D6 and CYP3A4) and specific Vivid® Substrates were purchased from Invitrogen Drug Discovery Solutions (USA). Vivid blue screening kit included baculosome (respective isozymes and NADPH-P450 reductase); regeneration system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase) and $NADP^+$ were also used for the study. Ketoconazole and α-naphthoflavone were obtained from Merck (Mumbai, India). Quinidine and sulfaphenazole were procured from Sigma (Steinheim, Germany). HPLC grade methanol, orthophosphoric acid and other analytical grade solvents procured from Merck were used for the experiments. Chlorogenic acid was procured from Sigma (Steinheim, Germany).

Preparation of plant extracts

The leaves of *M. alba* L. was purchased from local vendor and authenticated by Dr S Rajan, Field Botanist, Ooty, Tamilnadu, India. The voucher specimens (SNPS-1087) have been deposited at the School of Natural Product Studies; Jadavpur University for further references. Plant materials were washed with distilled water and shade dried at room temperature. Hydro alcoholic extraction of plant material was made by cold maceration method. The solvent was completely removed using rotary vacuum evaporator (EYELA, Tokyo, Japan) under reduced pressure, leaving semi-solid residue. These residues were lyophilized to fine powder and stored in desiccators for further use. Yield of the extract was 12.63 % (w/w).

Standardization of the *M. alba* extracts using RP-HPLC

The extract of *M. alba* was standardized and the quantity of the major bioactive compound present in the plant extract was determined through RP-HPLC. The HPLC system (Waters, Milford, MA, USA) consisting of 600 controller pump; a multiple-wavelength ultraviolet–visible (UV–vis) detector; an in-line degasser AF 2489; a rheodyne 7725i injector equipped with a 20µL loop and Empower™2 software. Separation was achieved using Waters spherisorb 5µm ODS2, 250×4.6 mm column (Ireland). The mobile phase was optimized with methanol: 0.1% orthophosphoric acid in miliQ water 75:25 (v/v), *p*H 3.6 and the elute was detected at 280nm. Marker compound and extract solution were dissolved in methanol and filtered through Whatman NYL 0.45 µM syringe filter, prior to injection. Amount of the phytoconstituents present in different plant extracts were determined by constructing calibration curve of respective marker compounds.

CYP450 enzyme inhibition assay

Effect of extract and marker compounds on pooled CYP450

The effect of the extract and bioactive compound on CYP450 was determined by CYP-CO complex assay[18]. In this method spectral property was employed for the specific estimation of CYP450 content and the possible interaction was studied with rat liver micro some. Reaction was performed in 96 well micro plates (NUNC, Roskilde, Denmark). Micro somes was diluted with a phospho glycerol buffer (10 mM potassium phosphate, *p*H 7.4, 20% glycerol) and incubated with the extract and marker compounds. Ketoconazole was used as positive control. One plate (P) was sealed with tape and kept in room temperature, while another plate (PC) was incubated in the CO chamber for 15 min. 0.5 M sodium hydrosulfite was used to reduce the sample. The absorbance was measured with a Spectra-max M5 (USA) at 450 nm and 490 nm and the absorbance difference were calculated. Concentration of CYPs was calculated using the formula; [CYP450] (mM) = (ΔAPC-ΔAP)/91 Where ΔAPC = absorbance difference of the PC sample, and ΔAP = absorbance difference of the P sample. Percentage inhibition was calculated using the following formula: Percentage inhibition = (Blank – Test) × 100/blank).

Fluorogenic assays

Fluorogenic assay was performed in black 96-well microplates (NUNC, Roskilde, Denmark); based on the earlier reported methods[19]. The assay mixture consisted of 50µl of prepared enzyme mixture and 40 µl test solution; incubate for 20 min at 37ºC. Inhibition potential was tested against CYP3A4 (5nMs), CYP2D6 (10 nM), CYP2C9 (10 nM) and CYP1A2 (5nM) isozymes. For determination of IC_{50} value, two fold serial dilutions of the tests were prepared. The enzymatic reaction was initiated by the addition of a mixture of $NADP^+$ and the appropriate substrate (10µl) and incubated for 10min at 37ºC. 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) and 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) fluorogenic substrate were used for CYP3A4, CYP2C9 and CYP2D6, CYP1A2, respectively. 10 µM substrate concentrations were used for CYP3A4, 2D6 and 2C9 to start the reaction. In case of CYP1A2 substrate concentration was 3 µM. Ketoconazole, quinidine, sulfaphenazole and α-naphthoflavone were used as positive inhibitors for CYP3A4, 2D6, 2C9 and 1A2 correspondingly. Product formation from the fluorogenic probes were measured on Spectra-max M5 (USA) using an excitation wavelength of 405 nm and emission wavelength of 460 nm. Percentage inhibition and IC_{50} value were determined using the following formula:

$$\text{Percentage inhibition} = \frac{100 - [(\text{Signal of well} - \text{Blank}) \times 100]}{(\text{Solvent control Blank})}$$

$$IC_{50} = \frac{(50 - \text{Low \% inhibition}) \text{ X } (\text{High concentration} - \text{Low concentration}) + \text{Low concentration}}{(\text{High \% inhibition} - \text{Low \% inhibition})}$$

Statistical analysis

All the tests were conducted in triplicate. The experimental data were expressed as mean ± S.E.M. The significance of difference between the groups was assessed by one-way analysis of variance (ANOVA). The statistical significance was calculated using GraphPad InStat Version 5.0. Dunnett's multiple comparison test were performed by fixing the significance level at $P<0.05$ and above.

Results and discussion

Several crude drugs and finished herbal products are being marketed as herbal medicines or dietary supplements for their therapeutic benefits. Unfortunately, herbal medicines or food materials are not free from the risk of interactions between them as well as with the modern medicines, which is an increasing concern and may have significant health hazards. Inhibitory effects of anti-diabetic medicinal plants (*M. alba*) along with its bioactive compound on CYP450 enzymes and its isozymes were investigated in this study to evaluate the possible herb drug interaction which affects the pharmacokinetics of other drugs when administered concomitantly. There are several scientific reports on standardization of medicinal plant with its corresponding bioactive compounds and their inhibitory effect on CYP450[4,2]. This study is in harmony with those reports to evaluate the herb-drug interaction of some herbs used as food as well as medicine in Indian System of Medicine.

HPLC analysis of *M. alba* extract and its active constituents

The quantitative analysis was performed using RP-HPLC under the isocratic conditions using the external standard calibration technique. Presence of bioactive compound in the *M. alba* extract was confirmed by comparing with the respective retention time of the reference standard. The standard chlorogenic acid exhibited good linearity within the range from 20 - 100 µg/ml of the calibration curve. Good correlations were found between concentrations and the peak area, with the coefficient of determinants (r^2) > 0.99. Figs. 1A & B showed the chromatograms obtained from HPLC analyses of marker and the *M. alba* extract. The contents chlorogenic acid present in the *M. alba* extracts was found 1.12 % (w/w).

CYP450 enzyme inhibition assay

CYP-CO complex assay

Cytochrome P450–CO complex method was used to assess the preliminary inhibitory potential of the plant extract and its bioactive compound. Reduced P450-CO complex showed an absorption spectrum, which was different than that of the reduced P450. Concentration of CYP450 was determined using a formula that incorporates the change in absorbance at 450 nm relative to 490 nm[20]. The concentration of protein present in isolated microsomes was found to be 8.3 mg/ml. The CYP450 concentration in the microsome was estimated to be 0.502 nmol/mg of protein. In order to study the interaction potential of the *M. alba* extract and chlorogenic acid, the concentration of pooled CYPs was determined by the incubation of microsomes with the test samples. Percentage inhibition of the test substances compared

to the positive control has been depicted in Fig. 2. *M. alba* showed the highest and chlorogenic acid showed the least interaction potential. Results indicated that the *M. alba* extract showed higher inhibition potential than its bioactive compound. This effect may be due to synergistic activity of some other active molecules present in the extract[21].

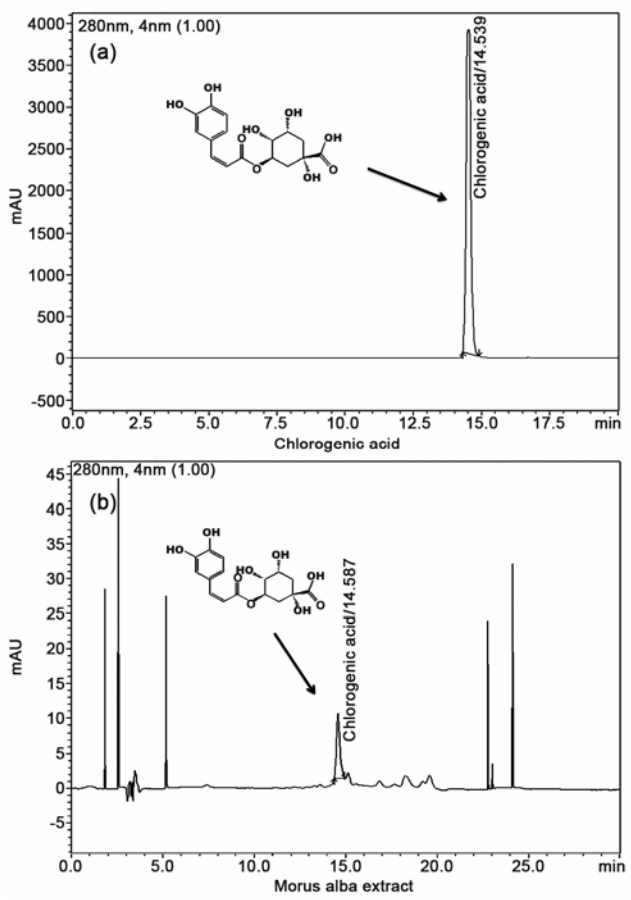

Fig. 1—HPLC analysis of the plant extract and its bioactive molecule. [1A: HPLC analysis of chlorogenic acid; 1B: HPLC analysis of *M. alba* extract]

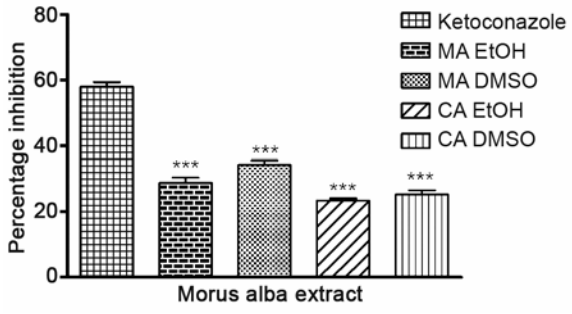

Fig. 2—Percentage inhibition of CYP 450 by the plant extracts and markers. [Values are mean ± S.E.M.; n=3. *** P<0.001, ** P<0.01 * P<0.05 versus positive control ketoconazole. MA – *Morus alba*]

Inhibitory profile of plant extracts and bioactive constituents on CYP isoforms

This assay was based on the ability of a drug to compete with the different fluorogenic substrate for different CYP is forms. The probe substrates used in these assays are derivatives of coumarin or resorufin, which after dealkylation by CYPs generates fluorescent products. Respective positive controls for each isozyme were used to confirm the assay precision. Pure compound indicates whether the overall activity was explainable by tested compound or caused by other constituents in the extract. *M. alba* extract and chlorogenic acid showed concentration-dependent inhibitory activity on CYP3A4, CYP2D6, CYP2C9 and CYP1A2. All the samples were assayed in triplicate and IC50 values were determined and represented in Table 1. *M. alba* extract showed lowest IC50 value against CYP3A4 (Table 1). Result indicated that chlorgenic acid showed least interaction potential against CYP3A4. Fig. 3A represents the concentration dependent inhibition of the extract and the bioactive compound against CYP3A4. CYP2D6 is the rate limiting enzyme for metabolism of many pharmaceuticals. Inhibition of CYP2D6 by the plant extracts has been shown in Fig. 3B. Chlorogenic acid showed the least interaction potential, with the higher IC_{50} value, where as the extract of *M. alba* showed lowest IC_{50} value (Table 1). CYP2C9 is a less common rate limiting enzyme. The result indicated that chlorogenic acid have highest interaction potential with lowest IC_{50} value against CYP2C9 and *M. alba* extract showed least interaction potential (Table 1). Concentration dependant percentage inhibitions of CYP2C9 by the botanical and bioactive compound have been shown in Fig. 3C. CYP1A2 is involved in the metabolic activation of aromatic hydrocarbons. Chlorogenic acid showed lowest inhibition against CYP1A2 among other isozymes. Concentration dependant percentage inhibition by the bioactive compound and plant extract against CYP1A2 has been shown in Fig. 3D. CYP inhibitory potential of plant materials and the bioactive compound as described in this work and performed by others[18,19,21] are potentially useful tools for accessing the herbal with respect to their herb-drug interaction. Present study revealed that the selected medicinal plant extract and the pure compound exhibit very less inhibition potential on CYP3A4, CYP2D6, CYP2C9 and CYP1A2; comparing to their respective positive inhibitors. CYP-CO complex assay also showed quite

Table 1—IC_{50} values of plant extract and its marker

Sample	Solvents Used	CYP3A4	CYP2D6	CYP2C9	CYP1A2
Morus alba	DMSO	144.37 ± 1.54	146.57 ± 2.23	174.68 ± 3.79	135.59 ± 1.46
	EtOH	132.96 ± 1.39	138.22 ± 3.18	157.23 ± 1.42	122.65 ± 2.14
Chlorogenic acid	DMSO	178.88 ± 2.53	178.87 ± 1.59	146.14 ± 1.39	107.17 ± 1.69
	EtOH	155.67 ± 2.46	166.36 ± 3.15	119.43 ± 1.15	92.42 ± 2.11
Positive control		Ketoconazole	Quinidine	Sulfaphenazole	α-naphthoflavone
	DMSO	7.12 ± 0.62	4.08 ± 0.42	11.41±1.01	15.64±2.11

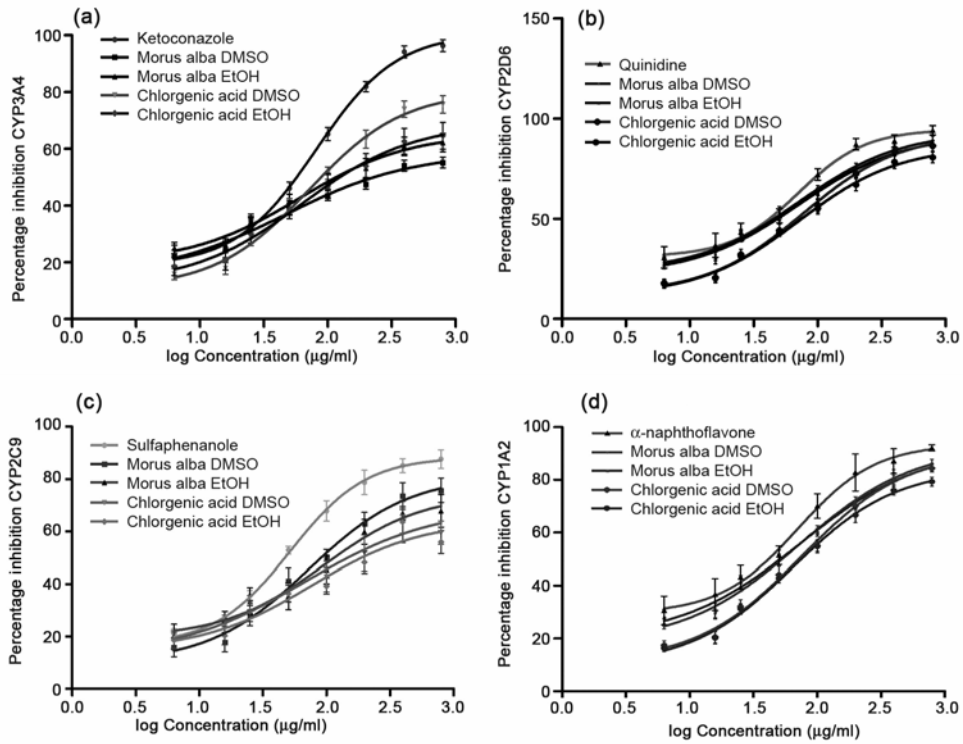

Fig. 3—Concentration dependent inhibition of CYP3A4 and CYP2D6 of the plant extracts and their bio-active compounds. [3A.Effect of the *Morus alba* (MA) extracts and chlorogenic acid (CA) on CYP3A4; 3B. Effect of the *Morus alba* extracts and chlorogenic acid on CYP2D6; 3C. Effect of the *Morus alba* (MA) extracts and chlorogenic acid (CA) on CYP2C9; 3D. Effect of the *Morus Alba* (MA) extracts and chlorogenic acid (CA) on CYP1A2]

similarity with fluorogenic assay. Higher CYP inhibition potential by the plant extract may be related to the synergistic effects of other constituents in the extract. From the above findings, it can be stated that the medicinal plant extract and phyto-constituent have very less potential to interact with co-administered drug metabolism.

Conclusion

Quality and safety assessment of medicinal plant is quite essential for increasing popularization of food as medicine throughout the world. Present study explored the possible herb-drug interaction potential of anti-diabetic medicinal plant with their bioactive compound used as food in Indian System of Medicine. Result indicated that test samples having higher IC_{50} values than respective positive inhibitors against different CYP isozymes. However, it can be concluded that possibilities of herb-drug interaction of the *M. alba* extract and chlorogenic acid are very less if these compounds are administered concomitantly with other herbal products or modern medicine and the botanical may not produce any toxic effect on their different therapeutic approaches. Some other major factors of metabolism like the competition between co-administered drugs, nonspecific interactions with proteins and enzyme induction due to chronic intake etc. need to be addressed further.

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