Indian Journal of Traditional Knowledge
Vol. 14(4), October 2015, pp. 614-619

Inhibition potential of *Moringa oleifera* Lam. on drug metabolizing enzymes

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Received 21 May 2015, revised 7 September 2015

The leaves of *Moringa oleifera* Lam. (Family: Moringaceae) has been traditionally used in Indian system of medicine (ISM) for its wide spectrum of pharmacological effects. The aim of this study was to evaluate the inhibitory effect of standardized *M. oleifera* extract and its biomolecule chlorogenic acid on drug metabolizing enzymes (CYP450) which may cause interaction. The amount of chlorogenic acid was determined by using high performance thin layer chromatography (HPTLC). CYP-CO complex assay and fluorescence assay were followed for this study by using rat liver micro some (RLM) and two key isozymes CYP3A4 and CYP2D6. The hydro-alcoholic extract of *M. oleifera* contains 2.99 % (w/w) of chlorogenic acid by HPTLC analysis. The CYP-CO complex assay showed 23.45 ± 0.78 % inhibition on RLM. The fluorescence study revealed that both the extract and chlorogenic acid (IC_{50}: 127.36 ± 2.98, 146.50 ± 3.46 µg/mL and 133.73 ± 1.79, 159.22 ± 3.53 µg/mL) had some inhibition with both isozymes CYP3A4 and CYP2D6 but very less in comparison to their respective positive inhibitors ketoconazole and quinidine. Thus it may be concluded that *M. oleifera* extract has very less potential to inhibit the CYP isozymes, so traditional use of this plant may be safe.

Keywords: Indian System of Medicine, *Moringa oleifera* Lam., Chlorogenic Acid, HPTLC, RLM, Cyp Isozymes

IPC Int. Cl.[8]: A61K 36/00, C01, C07, C12N 9/00, C12N 11/00

Moringa oleifera (Family: Moringaceae) commonly called as 'drumstick/horse-raddish tree' is the widely cultivated plant in Indian subcontinents. Traditionally, the leaves of *M. oleifera* are used to cure many diseases such as paralysis, hyperglycemia, hypertension, hyperlipidemia, fever, inflammation, wound, cough, asthma, nervous debility, diarrhea, cholera, spasm, enlarged liver and spleen, infection and ulcer[1]. Besides pharmacological activity, it has a wide nutritional property[2]. It has also been reported that it possesses nootropic activity and hence can improve memory[3] probably by altering brain monoamine levels and electrical activity[4]. It has also beneficial effect in CNS neurons (hippocampal origin) for formation of long-term memory. The leaves of *M. oleifera* are rich in various phytochemicals including ascorbic acid, chlorogenic acid, flavonoids, phenolics, alkaloids, terpenoids, phenylpropanes, amines, glucosinolates, glycosides, saponins, non-protein amino acids, carotenoids (including β-carotene) and β-sitosterol[5]. Cytochrome P450 (CYP450) is a group of principal enzymes for the metabolism of both endogenous and exogenous compounds such as drugs and other xenobiotics. Among several isoforms, CYP3A4 and CYP2D6 are most relevant for the metabolism of clinically significant drugs[6]. Inhibition of one or both isozymes may cause unexpected adverse drug interactions by altering the metabolic clearance of co-administered drugs. The literature study reveal that some drugs were withdrawn from market due to their life threatening interactions with other drugs. However, this interaction is not limited to prescribed drugs only. Herbal medicine such as *Silybum marianum* [*Silybum marianum* (L.) Gaertn.], *Ginkgo biloba* L., Grape fruit juice and St. John's wort has the potential to inhibit or induce hepatic CYP450 enzymes and show severe interaction[7].

Some reports revealed that the herb-drug, food-drug or spice-drug interaction occurred due to inhibition of CYP450 enzyme by the isolated molecule or plant extract[8,9]. Few study related to inhibition potential of Indian medicinal plants has been reported[10,11]. The biomarkers present in extract, can show either beneficial or harmful effect.

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Therefore it is essential to develop a marker profile and standardization of the medicinal plant to maintain quality control as well as to get the optimal concentrations of known active constituents presents therein[12]. Based on the above fact, attempt was made to standardize the *M. oleifera* extract by HPTLC analysis. The inhibition potential of *M. oleifera* leaves on CYP450 enzymes was evaluated through enzyme inhibition assay.

Methodology

Chemicals

All the solvents and reagents for standardization and inhibition assay were of analytical grade and purchased from Merck India Ltd, Mumbai. The kit for florescence assay was procured from BD Gentest™. Kit components contain cDNA expressed recombinant human CYP3A4, CYP2D6 using baculo virus (*Autographa californica*) infected insect cells (BTI-TN-5B1-4) along with potassium phosphate (*p*H 7.4) buffer; Tris base, $NADP^+$, $MgCl_2$, glucose 6-phosphate, glucose 6- phosphate dehydrogenase, AMMC (3-[2-(N,N-diethyl-N-methylamino) ethyl]-7-methoxy-4-methylcoumarin), quinidine and AHMC (3-[2-(N,N-diethylamino) ethyl]-7- hydroxy-4-methylcoumarine hydrochloride); BFC (7-benzyloxy-trifluromethylcoumarin); HFC (7-hydroxy-trifluromethylcoumarin), ketoconazole (≥ 98%) and quinidine (≥ 98%). 96-well black microplates (NUNC, Denmark) were procured from local supplier. Chlorogenic acid was procured from Sigma Chemical Co, St Louis, MO, USA

Plant material and extraction

Leaves of *M. olifera* were collected from Bankura and authenticated by Dr S Rajan, Botanist, department of AYUSH, Tamil Nadu, India and the voucher specimens (SNPS-JU/2013/1087) has been deposited at School of Natural Product Studies, Jadavpur University, Kolkata, India. The leaves were dried under the shade and powdered by using grinder. Then the crude powder was extracted with 70% methanol followed by cold maceration. The extract was concentrated under reduced pressure in rotary vacuum evaporator at 45°C. Then the extract was lyophilized and kept in desiccator for further use.

HPTLC analysis of *M. oleifera*

The hydro-alcoholic extract of *M. oleifera* was standardized by using Camag HPTLC and the biomarker chlorogenic acid was used as standard. Camag (Muttenz, Switzerland) HPTLC system, made up of a Linomat 5 sample applicator, twin-trough plate development chamber, TLC Scanner III and winCATS integration software was used for standardization. Aluminium backed HPTLC plates 10 X 10 cm with 0.2 mm layers of silica gel 60 F254 (E. Merck, Darmstadt, Germany), were used. Solution of *M. oleifera (*10 mg/mL*)* was prepared by using ethanol and the solution was filtered through nylon syringe filter (0.45 µm). Same procedure was followed to make the solution of chlorogenic acid (1mg/mL). Different volumes of the standard and test solution were applied by Linomat 5 sample applicator to the plate at 2 cm above the edge using a bandwidth of 6 mm and a distance between tracks of 10 mm. The development chamber was saturated by optimized solvent system (ethyl acetate: water: formic acid = 8:1:1.5) and the plate was kept inside the chamber for development (up to 8 cm). Then the developed plate was air dried and scanned in a TLC scanner III at 254nm. Quantitative assessment was performed by densitometric scanning to determine the quantity of chlorogenic acid present in *M. oleifera* extract.

Sample preparation for enzyme inhibition study

Two mg extract of *M. oleifera* was dissolved in 2 mL of both ethanol and dimethyl sulfoxide (DMSO) solvent to make a concentration of 1 mg/mL. Same method was followed to make the concentration of 1 mg/ mL of chlorogenic acid. Ketoconazole was used as positive control for CYP-CO complex assay. Both extract and biomarker were studied to establish their effect on RLM. Microsome without extract was used as the negative control.

Preparation of Rat Liver Microsomes (RLM)

Two male Swiss Wistar rats (~ 200 gm) were used to isolate RLM. The experiment was performed based on the guidelines of the Animal Ethical Committee (Animal ethical committee approval no AEC/PHARM/1418/2014). Animals were anesthetized and liver was quickly removed and perfused with 1.15% KCl solution and homogenized with four volumes (w/v) of ice cold 1.15% KCl solution. 20% homogenate (w/v) was centrifuged at 9000×g for 20 min (Beckman Coulter 64R ALLEGRA) and the supernatant was collected. Then it was subjected to ultra centrifugation at 105,000 x gm for 1 hr at 4 °C (SORVALL RC100) and the microsomal fractions (105,000 x gm pellet) were

collected from the homogenates. It was resuspended in 1.15% KCl solution and stored at -80 °C for further use[13]. Bovine serum albumin was used as standard to estimate the protein concentration by following Bradford method and using AMS photo analyzer (AMS-2, Roma, Italy).

Cytochrome enzyme inhibition study

CYP450-CO complex assay

P450-CO complex assay was perfomed in 96 well black microtitreplate[14]. Normally the reduced P450-CO complex shows a distinct spectrum which is different from the absorption spectrum of P450. This property was employed for estimation of CYP450 and evaluation of enzyme inhibition with RLM. Microsome was diluted by using phospho glycerol buffer (mixture 10 mM potassium phosphate at *p*H 7.4 with 20% glycerol). The extract and the biomarker solution was mixed with diluted microsome and incubated for 10 min at 37° C. Ketoconazole was used as positive control. NADPH generating system (4.20 mg/mL of NADP+ in solution of 100 mM glucose-6-phosphate, 100 mM $MgCl_2$ and 100 U/ mL glucose-6-phosphate dehydrogenase) was added to initiate the enzyme reaction. One plate (P) was sealed with tape and kept outside of the CO chamber in room temperature, while the other plate (PC) was incubated in the CO chamber for 15 min. Then the sample was reduced by adding 10µL of sodium hydrosulfite (0.5 M) as reducing agent. The absorbance was measured using Spectra Max® M5 (USA) at 450 and 490 nm. The absorbance differences were calculated by using the following 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) x 100/Blank).

Fluorimetric assay

Inhibition of CYP450 isozyme (CYP3A4 and CYP2D6) by extract or marker was assayed by using High Throughput screening kit procured from BD Gentest™ was used to evaluate of CYP450 enzyme inhibition activity[15]. 1.3 mM $NADP^+$, 66 mM $MgCl_2$ and 66 mM glucose 6-phosphate were used to prepare NADPH-Cofactor mixture. 144µL of this mixture was added to the first row of the microplate. Solvent and 100µL cofactor mixture were added to the remaining well except blank. 6µL of test sample was added to first row of test compound. Same was followed in case of biomarker and positive control. Two fold serial dilutions were done by transferring 50µL mixture from 1st to 8th column and the rest 50 µL at the 8^{th} column was discarded. The NADPH-Cofactor mixture was pre-incubated for 10 min at 37 °C. The substrates 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC), 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) used for CYP3A4 and CYP2D6 was added with enzymes to make enzyme - substrate mixture respectively. Ketoconazole and quinidine were used as positive inhibitors for this assay. 50µL of this mixture was added to every column and mixed thoroughly and then incubated for 20 min at 37 °C. After incubation, stop reagent (75 µL) was added to each row of IC_{50}. The plate reading was taken at a suitable excitation and emission wavelength (409, 530 nm for CYP3A4 and 390, 460 nm for CYP2D6) by using Spectra Max® M5 (USA). Percentage inhibition was calculated by using the following formula:

Percentage inhibition = 100- [(Signal of well-Blank)/ (Solvent control-Blank) × 100]

IC_{50} = [{(50-LP) × (HC-LC) + LC}/ {HP-LP}]

where, LP = Low percentage of inhibition; HP = High percentage of inhibition; LC = Low concentration; HC = High concentration.

Statistical analysis

The data was plotted and statistical analysis was performed using the Graph Pad Prism, version 6.0. All the studies were conducted in triplicate and results were expressed as Mean ± SEM. The results obtained from the study were analyzed through one-way analysis of variance (ANOVA) followed by the Bonferroni test for statistical analysis. The difference between the means were considered significance when $p < 0.05$.

Results and discussion

Quantitative analysis of *M. oleifera* through HPLTC

The chromatogram obtained from HPTLC analysis of standard chlorogenic acid and hydro-alcoholic extract of *M. oleifera* has been shown in Figs. 1A & B. Standard chlorogenic acid showed good linearity (2-10 µL) in the calibration curve. The correlation coefficient (r^2) value was 0.994 which showed a good

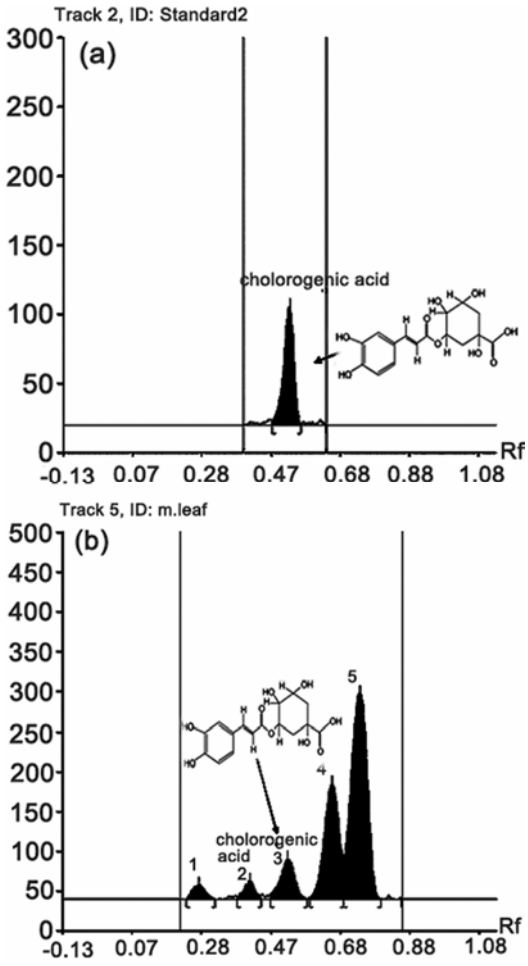

Fig. 1—HPTLC chromatograms of standard chlorogenic acid (A), extract of *M. oleifera* (B), [Eluent; Ethyl acetate: water: formic acid = 8:1:1.5 (v/v)].

correlation between drug content and peak area. The calibration plot was best described by the linear equation Y= 1131.61 + 763.697 X where Y is response and X the amount of chlorogenic acid. The peak of chlorogenic acid in the extract was identified by comparison with the R_f of reference standard at 0.49. The result was satisfactory and acceptable for subsequent quantitative analysis. The content of chlorogenic acid was found to be 2.99 % (w/w) in *M. oleifera* extract.

Cytochrome inhibition study

CYP450-CO complex assay

This assay revealed some information about the effect of *M. oleifera* extract and chlorogenic acid on RLM. The protein concentration of RLM was measured and it was found to be 8.12 mg/mL. The CYP450 concentration of microsome sample was

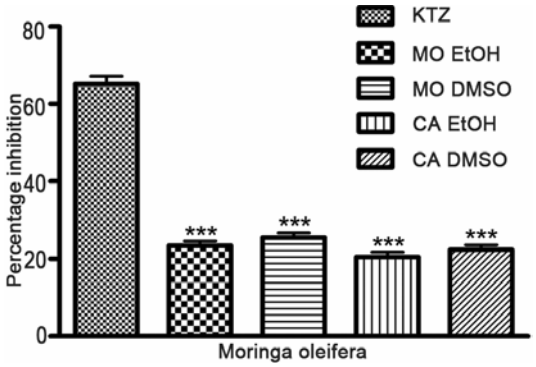

Fig. 2—CYP-CO Complex Assay. Percentage inhibitory effect of *M.oleifera* extract and chlorogenic acid on RLM. [Values are Mean ± SEM where n=3. $^{***}P$ <0.001 versus positive control ketoconazole. KTZ- Ketoconazole. MO DMSO- *M.oleifera* extract dissolved in DMSO, MO EtOH- *M.oleifera* extract dissolved in ethanol, CA DMSO- chlorogenic acid dissolved in DMSO and CA EtOH- Chlorogenic acid dissolved in ethanol].

0.416 nM/mg protein. Here, ketoconazole was used as positive inhibitor. The extract showed 23.45% inhibition on RLM which is very less compared to ketoconazole. It was also found that the chlorogenic acid has lesser inhibition than the extract. This screening gives an idea of the inhibition potential of *M. oleifera* extract and chlorogenic acid on pooled rat liver microsome. It was also observed that the inhibition potential of *M. oleifera* extract and chlorogenic acid dissolved in DMSO was comparatively higher than that of ethanol which is shown in Fig. 2. It might be due to the solubility of the phytoconstituents present in extract in DMSO is higher than ethanol.

Fluorescence assay

This High throughput screening (HTS) method is rapid, cost-effective and potentially useful tool for Fluorometric CYP inhibition assay[8]. Ketoconazole and quinidine were used as standard for this study. IC_{50} values of *M. oleifera* extract, chlorogenic acid, ketoconazole and quinidine dissolved in both solvent (DMSO and ethanol) has been represented on Table 1. *M. oleifera* extract and chlorogenic acid showed lower inhibition (IC_{50}: 127.36 ± 2.98, 146.50 ± 3.46 and 133.73 ± 1.79, 159.22 ± 3.53 µg/mL) against CYP3A4 and CYP2D6 isozyme than the positive inhibitors (7.46 ± 1.02, 4.38 ± 0.74). Higher IC_{50} means lower inhibition potential. The *M. oleifera* extract and chlorogenic acid showed some inhibition on CYP3A4 and CYP2D6 isozymes which is concentration dependant and has been shown in Figs. 3A & B. Inhibition potential of CYP isozyme

Table 1—IC_{50} (μg/mL) values of *Moringa olifera* and chlorogenic acid on the metabolism mediated by CYP3A4 and CYP2D6 (Values are Mean ± SEM; n=3).

Sample	Solvent used	CYP3A4	CYP2D6
Moringa olifera	Ethanol	127.36 ± 2.98	133.73 ± 1.79
	DMSO	117.24 ± 1.68	127.05 ± 1.65
Chlorogenic acid	Ethanol	146.50 ± 3.46	159.22 ± 3.53
	DMSO	139.63 ± 2.23	147.19 ± 3.42
Positive control	--	Ketoconazole	Quinidine
	Ethanol	7.46 ± 1.02	4.38 ± 0.74
	DMSO	6.28 ± 1.14	3.42 ± 0.86

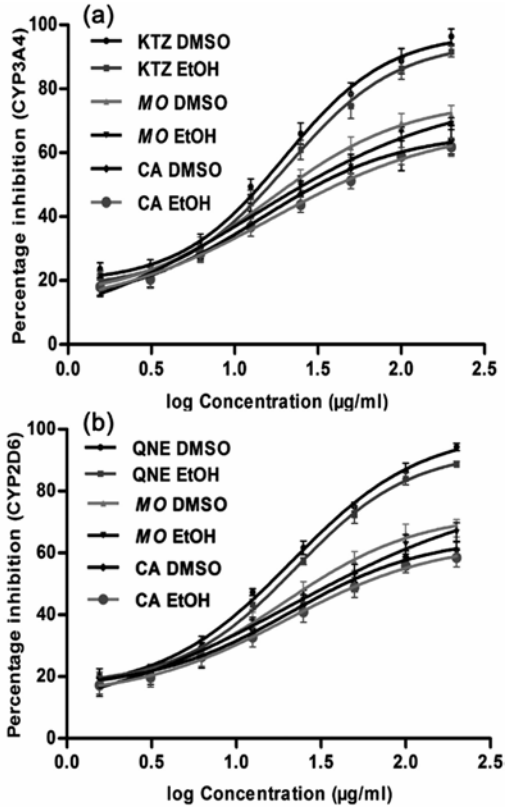

Fig. 3—Fluorescence Assay. Percentage inhibitory effects of *M.oleifera* extract, chlorogenic acid and positive inhibitors on drug modulating isozymes (A) CYP3A4 and (B) CYP2D6. [Values are Mean ± SEM; n=3). KTZ DMSO- Ketoconazole dissolved in DMSO. KTZ EtOH- Ketoconazole dissolved in ethanol. MO DMSO- *M.oleifera* dissolved in DMSO. MO EtOH- *M.oleifera* dissolved in ethanol. CA DMSO- chlorogenic acid dissolved in DMSO. CA EtOH- Chlorogenic acid dissolved in ethanol. QNE DMSO- Quinidine dissolved in DMSO. QNE EtOH-Quinidine dissolved in ethanol].

was observed in the order of chlorogenic acid <*M. oleifera* < positive inhibitors. It was also observed that the extract dissolved in DMSO showed comparatively higher inhibition than in ethanol. It might be due to synergistic effect of the phytomolecules present in extract which became more soluble in DMSO as compared to ethanol. Here it is obligatory to take care about the solvent because DMSO (concentration >5%) itself inhibit the drug metabolizing enzymes. It was shown that <2% DMSO has no effect on the drug metabolizing enzymes. Therefore, we used 1.5% DMSO to dissolve the extract, biomarker and positive inhibitors. Solvent effect was neutralized to make sure that there was no interference of solvent for this study. The study revealed that *M. oleifera* and chlorogenic acid had very less inhibition on both CYP3A4 and CYP2D6 isozymes, which suggested that the extract may have a low potential for herb-drug interaction mediated by CYP isozymes.

Conclusion

M. oleifera and its metabolite chlorogenic acid showed significantly less inhibitory effect on CYP3A4 and CYP2D6 as compared to their positive inhibitors (ketoconazole and quinidine). The inhibitory activity against CYP450 isozymes were in dose dependent manner. So, it may be safe to use *M. oleifera* traditionally. Some factors (co-administration of herb and drug, mechanism based enzyme inhibition, undesirable interactions with proteins, enzyme, etc.) need to be addressed in future for better understanding of herb-drug interaction.

Acknowledgement

The author would like to thank University grant Commission (UGC) for providing Maulana Azad National Fellowship for Minority Students (F1-17.1/2012-13/MANF-2012-13-MUS-WES-11557/(SAIII/Website)) to the 1st author working as Junior Research Fellow at School of Natural Product Studies, Dept of Pharmaceutical Technology, Jadavpur University.

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