Metabolism-mediated interaction potential of standardized extract of *Tinospora cordifolia* through rat and human liver microsomes

Shiv Bahadur, Pulok K. Mukherjee, S. K. Milan Ahmmed, Amit Kar, Ranjit K. Harwansh, Subrata Pandit

Abstract:

**Objective:** *Tinospora cordifolia* is used for treatment of several diseases in Indian system of medicine. In the present study, the inhibition potential of *T. cordifolia* extracts and its constituent tinosporaside to cause herb-drug interactions through rat and human liver cytochrome enzymes was evaluated.

**Materials and Methods:** Bioactive compound was quantified through reverse phase high-performance liquid chromatography, to standardize the plant extracts and interaction potential of standardized extract. Interaction potential of the test sample was evaluated through cytochrome P450–carbon monoxide complex (CYP450-CO) assay with pooled rat liver microsome. Influence on individual recombinant human liver microsomes such as CYP3A4, CYP2D6, CYP2C9, and CYP1A2 isozymes was analyzed through fluorescence microplate assay, and respective IC\(_{50}\) values were determined.

**Results:** The content of tinosporaside was found to be 1.64% (w/w) in *T. cordifolia* extract. Concentration-dependent inhibition was observed through *T. cordifolia* extract. Observed IC\(_{50}\) (µg/ml) value was 136.45 (CYP3A4), 144.37 (CYP2D6), 127.55 (CYP2C9), and 141.82 (CYP1A2). Tinosporaside and extract showed higher IC\(_{50}\) (µg/ml) value than the known inhibitors. *T. cordifolia* extract showed significantly less interaction potential and indicates that the selected plant has not significant herb-drug interactions relating to the inhibition of major CYP450 isozymes.

**Conclusions:** Plant extract showed significantly higher IC\(_{50}\) value than respective positive inhibitors against CYP3A4, 2D6, 2C9, and 1A2 isozymes. Consumption of *T. cordifolia* may not cause any adverse effects when consumed along with other xenobiotics.

**Key words:** Cytochrome P450, human liver microsome, reverse phase high-performance liquid chromatography, *Tinospora cordifolia*, tinosporaside

* *Tinospora cordifolia* (Family: Menispermaceae), also known as guduchi, is found in the tropics of Asia, Africa, and Australia. It is distributed throughout the tropical Indian subcontinent and most commonly used in Indian ayurvedic medicine as a tonic, vitalizer, and remedy for metabolic disorders.[1] This plant has been traditionally used in Ayurveda for the treatment of several diseases such as anti-diabetic, dyspepsia, bile secretion stimulation, burning sensation, vomiting, vaginal and urethral discharges, and urinary diseases.[2] The major phytoconstituents are tinosporone, tinosporaside, tinosporic acid, cordifolisides A to E, syringen, giloin, giloininand, picrotene, bergenin, and arabinogalactan. *T. cordifolia* has been reported to exhibit diverse pharmacological activities such as immunomodulatory, anticancer, antiulcer, memory enhancer, antidepressant, anti-ischemic, antifertility, chemopreventive, hypolipidemic, neuroprotective, blood purifier, antipyretic, antihepatitis, cardiotonic, antimicrobial, antileishmanial, antiinflammatory, antispasmodic, antiarthritic, analgesic, diuretic, etc.[3]

The rate of metabolic biotransformation is one of the most important pharmacokinetic parameters that control the pharmacological action of the drug. Concomitantly administered dietary supplements or food substances may either induce or inhibit drug metabolizing enzymes.

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**Address for correspondence:**
Dr. Pulok K. Mukherjee,
E-mail: naturalproductm@gmail.com

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resulting in changes in the rate of drug metabolism.\[^{14}\] For instance, one drug can inhibit the metabolism of another target drug, resulting in increased blood concentration of the target drug, which may lead to excessive accumulation and toxicity. Conversely, one drug can stimulate the metabolism of another victim drug, resulting in subtherapeutic plasma levels of that drug.\[^{15}\] Cytochrome P450 (CYP450) enzymes are superfamily of monooxygenases located primarily in hepatocytes are important drug metabolizing systems in mammals.\[^{16}\] Due to the central role of CYP450 catalyzed metabolism in drug clearance, the identification of potential of drug candidates as CYP substrate modulator (e.g., inhibitor and inducer) has been integrated, and the results will be helpful into the process of drug discovery and development. Therefore, the CYP450 enzymes inhibition might result alteration in drug metabolism and leading to various adverse effects.\[^{17}\] The present study was performed to investigate the inhibition potential of *T. cordifolia* and its active constituent tinosporaside on the activities of CYP3A4, CYP2D6, CYP2C9, and CYP1A2 and in human liver microsomes and the mechanisms *in vitro*, which could be used to predict the probability of herb-drug interaction.

**Materials and Methods**

### Chemicals and Reagents

High-performance liquid chromatography (HPLC) grade methanol, acetic acid, triethylamine, and other analytical grade solvents were procured from Merck (Mumbai, India). 0.45 µm syringe filter and membrane filter were obtained from Phenomenex (Torrance, CA) and Millipore (Billerica, MA), respectively. Tinosporaside was purchased from Sigma chemicals (Steinheim, Germany). Vivid\(^{\text{®}}\) CYP450 Screening kit and Vivid\(^{\text{®}}\) substrates were purchased from Invitrogen Drug Discovery Solutions, USA. 96-well black microplate was obtained from NUNC (Roskilde, Denmark). Quinidine and sulfaphenozol were procured from Sigma (Steinheim, Germany). Ketoconazole and α-naphthoflavone were obtained from Merck (Mumbai, India).

### Collection, Identification, and Processing of Plant Material

The aerial part of *T. cordifolia* was collected from Kolkata, India, and identified and authenticated by Dr. S. Rajan, field botanist, Ooty, Tamilnadu, India. A voucher specimen (JU-SNPS-1047) was deposited at the School of Natural product studies, Jadavpur University, Kolkata. The collected plant material was washed with distilled water, dried in room temperature, and then milled to a fine powder for preparation of extract.

### Extraction of Plant Material

Hydroalcoholic extract of *T. cordifolia* was prepared by immersing 200 g coarsely powdered plant material in conical flasks containing 500 mL 70% aqueous ethanol, with occasional shaking at room temperature. The mixture was kept for 72 h and filtered, and the process was repeated until remaining residues colorless. The filtrate was concentrated under reduced pressure using a rotary evaporator (Yiela, Tokyo, Japan). The obtained extract was lyophilized to get a dry powder, and the final yield was found to be 11.78% (w/w). The dried extract was stored at 4°C in air-tight container until further use.

### Standardization of *Tinospora cordifolia* Extract

*T. cordifolia* extract was standardized through reverse phase HPLC (RP-HPLC). Lyophilized hydroalcoholic extract of *T. cordifolia* was accurately weighted, and volume of the solution was adjusted in a volumetric flask to obtain a concentration of 10 mg/mL with methanol as a stock solution. Then, it was subsequently diluted to get 1 mg/mL concentration HPLC analysis. The optimized mobile phase was composed of acetonitrile, and Milli-Q water in the ratio of 25:75 (v/v) with flow rate of 1 ml/min.

The HPLC system was comprised rhodeyne-7725 injection valve with a sample loop (20 µL), vacuum degasser, quaternary pump, and photodiode array detector was used, with data acquisition by Empower\(^\text{TM}\) 2 software (Waters 600, Milford, MA, USA). Chromatography was performed on a Spherisorb C18 column (250 mm × 4.6 mm, 5 mm; Waters, Ireland) fitted with a C18 guard column (10 mm × 3.0 mm). The sample elution was performed at 25°C and detected at the ultraviolet wavelength of 254 nm. The sample was analyzed by HPLC using 20 µL injection volume of a syringe (Hamilton Microliters; Switzerland). The pH meter (Orion 3–Star, Thermo-Scientific) was used to adjust the pH of the mobile phase. The amount of the phytoconstituent present in the sample was determined through the calibration curve. Calibration curve constructed by corresponding peak area of the standard was plotted against the concentration of standards by means of linear regression. Stock solution of standard compound was prepared by dissolving the known amount of tinosporaside in methanol at a concentration of 1 mg/mL.

### Cytochrome P450 Inhibition Assay

#### Preparation of Sample Solutions

The lyophilized extract of *T. cordifolia* was solubilized in ethanol and dimethyl sulphoxide (DMSO), and volume was adjusted to make a concentration of 10 mg/mL. The samples were examined to establish their influence on rat liver CYP450 enzymes activity. Tinosporaside (1 mg/mL) was also solubilized in ethanol and DMSO and used for inhibition assay. 5 µL of ketoconazole (100 µM) was used as positive control to compare the inhibitory activity with standard and extract of *T. cordifolia*. The experiment was performed as approved by the Institutional Animal Ethical Committee with the ethical guidelines as provided by the Committee (approval number: AEC/PHARM/1501/05/2015) for the Purpose of Control and Supervision of Experiment on Animals, India. Rat liver microsome (RLM) was isolated by the method described by Ponnusankar et al.\[^{18,19}\]

#### Cytochrome P450-Carbon Monoxide Complex Assay

CYP450-carbon monoxide complex (CYP450-CO) assay was performed with pooled RLM in 96-well microplate.\[^{20}\] The RLM was diluted with a required phosphoglycerol buffer (10 mM potassium phosphate, pH 7.4, 20% glycerol) and incubated with prepared extract (dissolved in ethanol and DMSO). On addition of NADPH-generating system (4.20 mg/ml of NADP\(^{\text{+}}\) in solution of 100 mM glucose-6-phosphate, 100 mM MgCl\(_2\), and 100 U/ml glucose-6-phosphate dehydrogenase) reaction between extract and CYP450 was initiated. Two
aliquots were placed in microplate wells, and one plate was designed as reduced P450 (P), and another one was reduced P450-CO complex (PC). The P-well was sealed with tape and kept out side of the chamber, whereas PC well was kept in CO chamber and incubated for 15 min (because both well should be separate during incubation period). After that the samples were reduced by addition of 0.5 M sodium hydrosulphite fresh solution. The visible appearance of PC sample was yellow while P sample remained colorless. The difference in the absorbance of samples at 450 and 490 nm was monitored by using BIORAD microplate reader (Model 680XR). Ketoconazole was used as positive control, and proper solvent controls were used for the study.\[10,11\]

**Cytochrome P450 Enzymes Inhibition Assay**

This study was performed in black 96 microplates through fluorogenic assays. The assay method was based on the previous reports by Pandit et al.\[12\] Briefly, 145 µl NADPH-CO factor mixture (1.3 mM NADP⁺, 66 mM MgCl₂, and 66 mM glucose 6-phosphate) was added to each well of the first row of black 96-microwell plates. 7-benzoxymethoxy-3-cyanocoumarin (BOMCC) was used as substrate for CYP3A4 and CYP2C9. In case of CYP2D6 and CYP1A2, 7-ethoxymethoxy-3-cyanocoumarin (EOMCC) was used as substrate. 7-hydroxycoumarin and 7-hydroxy-3-cyanocoumarin by product were formed against BOMCC and EOMCC after the enzymatic reaction. Test samples were prepared in 0.1% DMSO and ethanol solvent. The result revealed that the interaction potential of T. cordifolia extract and biomarker tinosporaside. Inhibition potential as compared to ethanol [Figure 2]. Tinosporaside showed lower inhibition potential than the standardized extracts of T. cordifolia and its bioactive compound for CYP450 inhibiting activity. In the present study, we assayed the quantity of major bioactive constituent tinosporaside of T. cordifolia extract and their inhibition potential against different CYP isozymes.

**Statistical Analysis**

Statistical analysis was performed using the statistical functions of the GraphPad Prism® 5.0 software (CA, USA). The results were expressed in terms of mean ± standard error of mean. One-way analysis of variance test followed by Dunnett multiple comparisons test was followed. The values (P < 0.05) were considered statistically significant.

**Results**

Consumption of herbal products for therapeutic benefit has been regulated by several laws in different countries and mostly lack of proper documentation for their safety, efficacy or standards, and quality control. The objective of the present work was to establish a rational procedure to screen the standardized extracts of T. cordifolia and its bioactive compound for CYP450 inhibiting activity. In the present study, we assayed the quantity of major bioactive constituent tinosporaside of T. cordifolia extract and their inhibition potential against different CYP isozymes.

**Standardization of Tinospora cordifolia Extract**

*T. cordifolia* extract was standardized by RP-HPLC under the isocratic conditions using the external standard calibration technique. Bioactive compound was identified by comparing with the respective retention time (Rt) of the tinosporaside as standard. Calibration curve was plotted by plotting peak areas against concentrations, and five standard marker ranges from 100 to 500 µg/ml. Standard compound shows a good linearity between concentrations and the peak area, with the correlation coefficient (r²) of 0.998. Chromatograms from HPLC analyses of the extract and their respective markers are shown in Figure 1. The optimum separation was achieved using the mobile system at the volume ratio of acetonitrile and Milli-Q water in the ratio of 75:25 (v/v) with a flow rate of 1 mL/min. Rt of tinosporaside was found to be 9.13 min. The percentage amounts of standard constituent (tinosporaside) present in the crude extract were found to be 1.64% (w/w).

**Interaction Study through CYP Enzymes**

**Cytochrome P450-carbon monoxide complex assay**

The protein concentration in isolated RLMs was found to be 5.47 mg/ml that was estimated by modified biuret method. The CYP450 concentration of RLM was found to be 0.428 nmol/mg. The results on cytochrome inhibition assay through CYP450-CO complex method showed a concentration-dependent inhibition of cytochrome enzymes. Ketoconazole was used as positive inhibitor showed much more inhibition potential than *T. cordifolia* extract and biomarker tinosporaside. Inhibition potential of *T. cordifolia* extract that being dissolve in DMSO showed higher percentage of inhibition than in ethanol [Figure 2]. Tinosporaside showed lower inhibition potential as compared to *T. cordifolia* extract in both DMSO and ethanol solvent. The result revealed that the interaction potential of *T. cordifolia* extract was more rather than its individually bioactive molecule tinosporaside with CYP450 that may be due to the effects of some other bioactive molecules present in the hydroalcoholic extract of *T. cordifolia*.
Cytochrome P450 enzymes inhibition assay

Interactions potential of CYP microsomes has been performed through specific high-throughput screening assays. Concentrations range of the test substance and tinosporaside for the assay was from 12.5 to 400 µg/ml. *T. cordifolia* extract and its bioactive compound tinosporaside were evaluated for their capability to affect the pharmacokinetics properties of other conventional drugs when administered concomitantly. The interaction potential of the *T. cordifolia* extract has been deliberated through different CYP isozymes such as CYP3A4, CYP2D6, CYP2C9, and CYP1A2 to assay their inhibition potential. The principle of the assay was the ability of substrates to compete with different fluorogenic substrate for different CYP450 isozymes. Coumarin derivative generates fluorescent products after dealkylation by CYP isozymes, therefore, used as probe substrates. Positive controls for individual microsomes were used to confirm the precision of the assay. Testing the pure compounds indicated the overall activity explainable by compounds or caused by other constituents in the extract. Samples were assayed in triplicate, and IC$_{50}$ (µg/ml) values were calculated. The results of the present study revealed that the *T. cordifolia* extract and its bioactive compound have less inhibition potential on these isozymes compared to their respective positive inhibitors. Enzyme inhibition studies showed that inhibition of CYP3A4, CYP2D9, CYP29, and CYP1A2 by *T. cordifolia* was concentration dependent with IC$_{50}$ of 136.45, 144.37, 127.55, and 141.82 µg/ml, respectively.

Discussion

Herbal medicines have been exploited as an important source of clinically relevant drugs for the treatment of several diseases. *T. cordifolia* is a popular medicinal plant and is frequently used as folk medicine in Indian System of Medicine. However, its safety profile on herb-drug interaction aspects remained poorly understood. The results of the current study highlight the potential of *T. cordifolia* and its bioactive compound to interact with CYP450 isozymes, and this knowledge could be valuable for the development of targeted drug therapy.
understood. Till date, there is no safety evaluation or studies carried out to assess the effects of *T. cordifolia* on human drug metabolizing enzymes.

Considering the worldwide popularity of herbal medicines with the development of several herbal formulations with improved bioavailability, incidence of herb-drug interactions is predicted to be increased. Numerous case studies have been described detrimental herb-drug interactions which can lead to morbidity or even mortality.[6,13] Various herbs such as *Ginkgo biloba*, garlic, milk thistle, and St. John’s wort have the potential to inhibit or induce human drug metabolizing enzymes through CYP450.[6] In the previous research reports from our laboratory, several medicinal plants have been studied for the CYP450 interaction potential. Ahmed et al. (2016) has reported the CYP450 mediated inhibition potential of *Swertia chirata* (SC) and its active principle (ursolic acid). This study revealed that SC has less inhibition potential with two major drug metabolizing isozymes (CYP3A4 and CYP2D6). SC and ursolic acid showed significantly less inhibitory potential on RLM in a concentration-dependent manner.[13] In another study, Harwansh et al. (2014) reported the CYP450 interaction potential of trikatu, which is a very well-known polyherbal formulation in Ayurveda. Several concentrations of the trikatu formulation and its phytoconstituents showed less inhibitory activity on CYP3A4 and CYP2D6 isoenzymes as compared to the positive inhibitors.[14]

CYP450 isozymes (CYP3A4, CYP2C9, CYP2D6, and CYP1A2) are clinically most important metabolizing enzymes.[6] In the present study, an approach was made to evaluate the interaction potential of *T. cordifolia* through rat and human liver microsomes.[13] Standardization and chemoprofile of medicinal plants have more significance for validating its quality, safety, and to understand about the optimal concentrations of bioactive constituents present therein. The safety profile of *T. cordifolia* can be useful for both health-care professionals and consumers. The present study revealed that

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**Table 1: IC_{50} (µg/ml) value of *Tinospora cordifolia* extract, tinosporaside, and positive inhibitors on the major drug metabolizing enzymes such as CYP3A4, CYP2D6, CYP2C9, and CYP1A2**

| Samples            | Solvent used | CYP3A4       | CYP2D6       | CYP2C9       | CYP1A2       |
|--------------------|--------------|--------------|--------------|--------------|--------------|
| *Tinospora cordifolia* | DMSO         | 126.96±1.79  | 138.02±1.58  | 110.18±1.04  | 126.97±1.04  |
|                    | Ethanol      | 136.45±1.37  | 144.37±1.06  | 127.55±1.26  | 141.82±1.37  |
| *Tinospora*        | DMSO         | 155.67±1.08  | 167.37±1.73  | 231.19±1.58  | 171.38±1.47  |
|                    | Ethanol      | 164.39±1.41  | 178.88±1.97  | 240.17±1.69  | 187.14±1.38  |
| Positive control   |              | 3.89±0.88    | 2.29±0.94    | 6.87±0.92    | 7.69±0.38    |
|                    | DMSO         | 3.71±0.32    | 2.25±0.80    | 6.83±0.59    | 7.57±0.16    |
|                    | Ethanol      |              |              |              |              |

One-way ANOVA followed by Dunnett’s multiple comparison test. ANOVA: Analysis of variance, DMSO: Dimethyl sulfoxide
the concentration-dependent inhibition of the enzymes was observed with the both *T. cordifolia* extract and its bioactive compound tinosporaside. In fluorescence screening assay good linearity of concentration-dependent inhibition was found for *T. cordifolia* extract. Organic solvent (DMSO and ethanol) strongly interferes at higher concentration (5%) with isozymes (CYP3A4, CYP2D6, CYP2C9, and CYP1A2) therefore, a low concentration (0.01%) was used in this assay.\textsuperscript{[14,19]} Ethanol and DMSO have been used as solvent, and no significant interaction was observed with spectral absorption, proper solvent control was used, and further solvent effect was neutralized by deionized water.\textsuperscript{[13,18]} DMSO was used at lowest level to make sure the maximum solubility of the phytoconstituents. CYP450 interaction potential was slightly higher for the extract dissolved in DMSO compared to ethanol. *T. cordifolia* and its individual component showed less inhibitory activity in a concentration-dependent manner. The sites of interaction of active constituents from *T. cordifolia* extract with these isozymes are not known.

The present study demonstrated that *T. cordifolia* has less inhibition potential on the CYP3A4, CYP2C9, CYP2D6, and CYP1A2 isozymes in comparison with their respective inhibitors. Among all the test substances, *T. cordifolia* extract showed highest (IC\textsubscript{50} < 0.01 mg/ml) interaction potential for all the isozymes; whereas tinosporaside (IC\textsubscript{50} > 0.1 mg/ml) showed least interaction potential. IC\textsubscript{50} value indicated that there was no significant herb-drug interaction of *T. cordifolia* and tinosporaside. It can be concluded that *T. cordifolia* may have no significant interaction potential with the coadministered drugs. Thus, *T. cordifolia* can be used with the other medicines which are metabolized by these cytochrome enzymes. Therefore, the drugs clearance by these enzymes may not be influenced by the *T. cordifolia* extract. Further in vivo studies are necessary to evaluate the clinical significance of the interactions.

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**Conflicts of Interest**

There are no conflicts of interest.

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