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**Dr. Mrunmayee Toraskar**  
Department of Pharmaceutical  
Chemistry, Bharati Vidyapeeth's  
College of Pharmacy, Sector 8,  
C.B.D. Belapur, Navi Mumbai,  
Maharashtra, India

**Vineeta V Khanvilkar**  
Department of Pharmaceutical  
Chemistry, Bharati Vidyapeeth's  
College of Pharmacy, Sector 8,  
C.B.D. Belapur, Navi Mumbai,  
Maharashtra, India

## Evaluation of interaction potential of *Terminalia chebula* (Combretaceae) fruit extracts on rat hepatic enzymes

**Dr. Mrunmayee Toraskar and Vineeta V Khanvilkar**

### Abstract

The present study was aimed to evaluate *in vitro* interaction potential of *Terminalia chebula* fruit powder extracts on rat hepatic CYP enzymes. Alcoholic and hydroalcoholic extracts of *Terminalia chebula* fruit powder were prepared and standardized for their gallic acid content using RP- HPLC. *In vitro* metabolism studies were conducted to explore interaction potential of these extracts and marker gallic acid on rat CYP 2D6 enzymes. These extracts and gallic acid were explored for their CYP2D6 enzyme interaction potential by conducting *in vitro* metabolism study. The study was based on the probe reaction of conversion of dextromethorphan to dextrorphan in presence and absence of extracts. Quinidine sulphate, a known inhibitor, was used as positive control. RP-HPLC with UV detector was used to quantify the metabolite generated in the study. Alcoholic and hydroalcoholic extract fractions and gallic acid in methanol showed inhibitory effect on rat CYP2D6 enzyme activity. Gallic acid proved to be a potent inhibitor of rat CYP 2D6 enzyme.

**Keywords:** *Terminalia chebula*, gallic acid, alcoholic extract, hydroalcoholic extract, rat hepatic CYP 2D6

### Introduction

Herbal medicines are in great demand in both developed and developing countries for primary health care due to their efficacy, safety and lesser side effects. Also now a days there is growing awareness that herbal remedies and other phytoconstituents can affect the disposition of conventional pharmaceuticals [1]. Medical and scientific reports suggesting effect of concomitant administration of natural products and synthetic drugs on human drug metabolism originating risk of serious adverse reactions do exist in literature. This has led to several studies on evaluation of their effects on drug metabolizing enzymes, especially cytochrome oxygenase 450 (CYP 450) enzymes. CYP 450 is a superfamily of enzymes amongst which isoforms 2D6, 2C9 and 3A4 are found to play a significant role in metabolism of clinically significant drugs [2].

*Terminalia chebula* (*T. chebula*) Retz; (Family: Combretaceae) commonly known as 'Myrobalan' is well known in the Indian system of medicine. It is used as purgative and to cure bleeding and piles. It also shows cytoprotective, cardiogenic, antimutagenic and antifungal activity. Moreover, it is an important ingredient of 'Triphala', a popular Ayurvedic formulation used for the treatment of various ailments [3]. Literature cites data exploring the influence of *T. chebula* on Cytochrome P 450 (CYP 450) enzymes and suggests further investigation to clarify their interaction with individual isoforms [4].

The present study was designed to explore the effect of *T. chebula* fruit powder's alcoholic extract (AE) and hydroalcoholic extract (HE) on rat liver microsomes (RLM). *In vitro* metabolism studies, based on probe reaction of *O*-demethylation of Dextromethorphan [5] were conducted in presence and absence of extracts and gallic acid, biomarker of *T. chebula*. Metabolite dextrorphan formed in the *in vitro* studies was separated from the substrate and quantified using a simple RP-HPLC method with a UV detection.

### Materials and Methods

#### Drugs and Chemicals

Dextromethorphan, dextrorphan, quinidine sulphate and Tris-HCl were procured from Sigma-Aldrich Ltd. Carbon monoxide gas (reagent purity) was obtained from Alchemie Gases and Chemicals Pvt. Ltd. *T. chebula* fruit powder was obtained from Rajesh Chemicals and gallic acid from Research Lab Fine Chem Industries. All other chemicals were obtained from S. D. Fine Chemicals, Mumbai, India.

#### Correspondence

**Dr. Mrunmayee Toraskar**  
Department of Pharmaceutical  
Chemistry, Bharati Vidyapeeth's  
College of Pharmacy, Sector 8,  
C.B.D. Belapur, Navi Mumbai,  
Maharashtra, India

### Instrumentation

Biofuge Stratos cold centrifuge was used for isolation of microsomes from rat livers. Shimadzu-160A UV Visible spectrophotometer was used to record the absorbance. HPLC system, UFLC Shimadzu with multiple wavelength ultraviolet detector equipped with an inline degasser, an auto sampler, a rheodyne injector with variable microliter sample loop and Lab Solution software was used in the study.

### Preparation of *T. chebula* extracts

*T. chebula* fruit powder was authenticated from Department of Botany, Guru Nanak Khalsa College, Mumbai. AE was prepared by soaking weighed quantity of fruit powder in five volumes of ethanol and macerating for 24 hours with intermittent shaking. The extract obtained was filtered and dried on a rotary evaporator. Similar procedure was followed for preparation of HE using mixture of water and ethanol (1:1v/v) as an extracting medium.

### Standardization of extracts by RP-HPLC

Extracts were standardized using RP-HPLC with gallic acid as reference. Stock solution of gallic acid was prepared and diluted appropriately to get working standard solutions in the range of 1-12 $\mu$ g/ml. Weighed quantities of AE and HE were individually sonicated in methanol, the solutions were filtered through 0.45 $\mu$  syringe filter and subjected to RP-HPLC analysis. 10 $\mu$ l of each of the extract was injected on HPLC column and was detected at 270nm. Isocratic mobile phase used for separation consisted of methanol and phosphate buffer (10mM, pH 3.1 adjusted using *o*-phosphoric acid) in the ratio of 2:3v/v. The analysis was repeated twice with the flow rate of 1 ml/min. Gallic acid present in the extracts was identified by comparing the retention time (Rt) of chromatographic peaks of standard with the extracts. Percentage of gallic acid in extracts was determined by constructing a calibration curve.

### Evaluation of enzyme interaction potential

#### Preparation and characterization of RLM

RLM were isolated using calcium aggregation method [6]. Livers were obtained from Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, India. The protocol was approved by animal ethics committee (No. IAEC/PR/2014-15/13). Livers were perfused with ice-cold 0.9% w/v NaCl solution. These were thawed, finely chopped and homogenized with four volumes of ice-cold 10mM Tris-HCl buffer containing 0.25M sucrose (pH 7.4) in a Potter glass homogenizer equipped with a Teflon pestle [7]. The homogenate was centrifuged at 13000 $\times$ g for 10 min at 4°C in a refrigerated centrifuge and the precipitate was discarded. To the supernatant, calcium chloride was added to yield a final concentration of 10mM. The solution was stirred for 15-20 min, and then centrifuged at 25000 $\times$ g for 10 min at 4°C. The firmly packed microsomes pellets were resuspended by homogenization in 100mM Tris-HCl buffer containing 20% w/v glycerol and 10mM EDTA (pH 7.4). These were stored at -70°C until use. The entire isolation protocol was carried out at 0-4°C.

Spectral CYP450 content was determined by diluting the microsomes in the ratio of 1:9 with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% Triton X-100 and 1mM EDTA [8]. The solution was stirred thoroughly and divided into two five ml glass cuvettes. The sample and reference cuvettes containing the microsomal preparations were saturated with 30 to 40 bubbles of carbon monoxide, at a

rate of 1 bubble/sec. Sodium dithionite was added only to the sample cuvette to obtain reduced vs oxidized carbon monoxide difference spectrum. An extinction coefficient of 106 l/mmol/cm was used for the determination of CYP450 content.

Protein estimation of isolated RLM was done using Folin Lowry method [9]. Standard protein plot was prepared using bovine serum albumin. Absorbance was recorded at 625 nm.

### CYP2D6 Enzyme inhibition assay

CYP2D6 enzyme activity in RLM was measured by probe reaction of *O*-demethylation of dextromethorphan mediated by the polymorphic CYP2D6. 500 $\mu$ L standard incubation mixture containing RLM (0.5mg/ml), dextromethorphan (40 $\mu$ M) in 0.1M sodium phosphate buffer (pH 7.4) at 37°C was incubated for 60 min (solvent control). For inhibition study, probe substrate was incubated with RLM in presence of different concentrations of AE, HE and gallic acid. Positive control was run with a known CYP2D6 enzyme inhibitor quinidine sulphate solution (40 $\mu$ M). The reactions were initiated using NADPH solution (10mM) and then terminated with 500 $\mu$ l cold methanol at the end of 60 min. The samples were centrifuged at 10000  $\times$  g for 10 min at 4°C and the supernatant was subjected to RP-HPLC analysis. A modified RP-HPLC method was used for simultaneous estimation of dextromethorphan and dextrophan at a wavelength of 282 nm [10]. Samples were run on a Hemochrome Intsil C18 column 5 $\mu$  (4.6mm  $\times$  250mm) using acetonitrile and water (pH 3.8) as mobile phase in the ratio of 38:62v/v. Percentage inhibition of rat CYP2D6 by AE, HE and gallic acid was calculated by the formulae given below [11].

% inhibition by the sample = 100 - % of metabolite formed in the sample group

% of metabolite formed =  $[A_{\text{sample}} / A_{\text{control}}] \times 100$

Where,  $A_{\text{sample}}$  and  $A_{\text{control}}$  are areas of metabolite generated during *in vitro* metabolism in presence of sample (AE/HE/gallic acid) and in solvent control respectively.

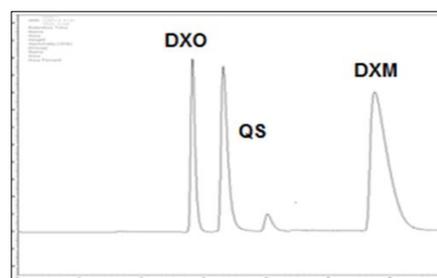
### Results

#### Standardization of *T. chebula* extracts by RP-HPLC

Rt of gallic acid was found to be 3 min. Calibration curve was linear with correlation co-efficient of 0.9994. Gallic acid content in AE and HE was found to be 2.47% w/w and 2.59% w/w respectively.

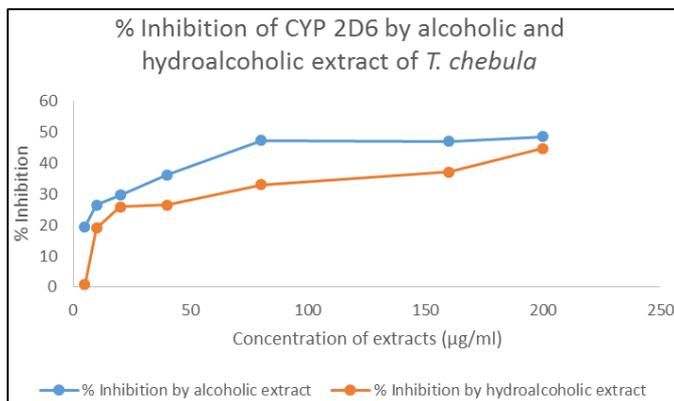
#### Evaluation of enzyme interaction potential

CYP content was found to be 3.676nmol/ml. Protein content of isolated RLM was found to be 15mg/ml. Retention time of dextrophan was found to be 2.9 $\pm$ 0.2 min, for quinidine sulphate 3.3 $\pm$ 0.2 min and for dextromethorphan 6.0 $\pm$ 0.2 min. HPLC chromatogram of dextrophan, quinidine sulphate and dextromethorphan is shown in figure 1.



**Fig 1:** HPLC chromatogram of dextrophan (DXO), quinidine sulphate (QS) and dextromethorphan (DXM) using optimized chromatographic conditions

AE, HE and gallic acid showed inhibitory potential on rat CYP2D6. Amount of metabolite generated in presence of AE, HE and gallic acid was found to be decreased as compared to the solvent control. Inhibitory effect of AE and HE on CYP2D6 enzyme is shown in figure 2. IC<sub>50</sub> values of AE, HE and gallic acid for rat CYP 2D6 enzymes were found to be 201, 225 and 43.41 µg/ml respectively.



**Fig 2:** Inhibitory effect of alcoholic and hydroalcoholic extract of *T. chebula* on CYP2D6 enzymes [The results are expressed as mean±SD (n=3)]

### Discussion

CYP 450 enzyme system plays a significant role in drug metabolism. These enzymes can be inhibited or induced by simultaneously administered herbs or drugs resulting into drug interactions. Concomitant administration of drugs and certain herbs have resulted into herb drug interactions (HDI) and few of such serious HDI are reported in literature. However the plants studied for their HDI potential are very less. Creation of a comprehensive and valid list of HDI is need of an hour. *T. chebula* has long been used as herbal medicine. Inhibitory effect of *T. chebula* hydroalcoholic extract fractions on CYP 450 enzymes is reported using Bio-Rad microplate fluorescence and absorbance reader. However no data is available on the extent of inhibition. Current research work was specifically aimed at determination of the inhibitory potential of alcoholic and hydroalcoholic extracts of *T. chebula* fruit powder towards rat CYP 450 2D6 isoform. Gallic acid is the major biomarker of *T. chebula* which is reported for several biological activities so both the extracts were standardized for the content of gallic acid. RP-HPLC method using methanol and phosphate buffer as a mobile phase proved to be effective in determining gallic acid content of AE and HE.

*In vitro* metabolism studies were carried out by incubating substrate dextromethorphan in necessary conditions in presence and absence of the extracts and gallic acid. Methanol content in all incubation samples was maintained below 1% and thus could not affect the *in vitro* metabolism study [12]. RLM are very commonly used in such type of studies. Rats are widely used animal models in HDI assessment because of ease of handling, relatively low cost, smaller amount of material required and a low inter animal variability. Both AE and HE exhibited inhibitory potential for rat CYP2D6 enzymes which was reflected through decreased amount of metabolite formed in their presence. Standard 2D6 inhibitor, Quinidine sulphate was used as positive control. Inhibitory effect observed was however insignificant in comparison with the Quinidine sulphate as higher concentrations of 200 µg/ml of each AE and HE could show percentage inhibition of only 48.6181% and 44.8248% respectively. Gallic acid is easily

soluble in water and alcohol. Therefore it was necessary to check whether Gallic acid possess any inhibitory potential. Our findings showed that gallic acid is the potent inhibitor of CYP 2D6 enzyme with dextromethorphan as the specific substrate. Concentration of 43.41 µg/ml of gallic acid was responsible for decline of CYP 2D6 enzyme activity to 50%. This signifies potential risk of interaction when substrates for this isoform are taken together with preparations containing high content of gallic acid. These results can merit further investigations to clarify their interactions with human liver microsomes and *in vivo* studies.

### Conclusion

*T. chebula* alcoholic and hydroalcoholic extracts and gallic acid exhibited inhibitory potential on rat CYP450 2D6 enzymes *in vitro*. A simple analytical technique was designed to identify this inhibitory potential. IC<sub>50</sub> value for gallic acid was calculated which showed that it is a potent inhibitor of rat CYP2D6 isoform. This implies potential risk of herb drug interaction when substrates for CYP2D6s are taken together with preparations containing higher content of gallic acid. Mode of inhibition and inhibition constant values of gallic acid still needs to be investigated further. Thus, this paved a way to carry out further research to investigate interactions with human liver microsomes, which could be translated to review studies for confirmation.

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### Conflict of interest

Authors have no conflict of interest

### List of Abbreviations

*T. chebula*, *Terminalia chebula*; AE, alcoholic extract; RLM, rat liver microsomes; HE, hydroalcoholic extract; NADPH, Nicotinamide adenine dinucleotide; Rt, Retention Time HDI, herb drug interaction

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