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(Special Issue- 6)**Innovation development and standardization of Novel Herbal Formulation****(September 24-25, 2018)*****In vitro* evaluation of inhibitory potential of *Terminalia chebula* (Combretaceae) fruit extracts on rat CYP enzymes****M Toraskar and Vineeta V Khanvilkar**DOI: <https://doi.org/10.22271/phyto.2018.v7.isp6.1.10>**Abstract**

The present study was aimed to evaluate *in vitro* inhibitory 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. These extracts and gallic acid were explored for their CYP3A4 enzyme inhibitory potential by conducting *in vitro* metabolism study. The study was based on the probe reaction of conversion of testosterone to 6 β -hydroxytestosterone in presence and absence of extracts. Ketoconazole, 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 CYP3A4 enzyme activity with IC₅₀ values of 160, 80 and 21.21 μ g/ml respectively. An analytical tool is designed to identify inhibitory potential of *Terminalia chebula* fruit powder extracts.

Keywords: *Terminalia chebula*, gallic acid, alcoholic extract, hydroalcoholic extract, rat hepatic CYP 3A4

Introduction

Many phytoconstituents isolated from herbs have been reported for their inhibitory activity on drug metabolizing enzymes, causing the possible herb drug interactions (HDI). Some common HDI are reviewed in literature [1]. Over 90% of drug metabolism in humans is mediated via Cytochrome P450 (CYP450) enzymes. Amidst lot of CYP450s identified, enzymes from CYP1, CYP2 and CYP3 families catalyze biotransformation of majority of the clinically used drugs [2]. Several *in vitro* tools to assess hepatic drug metabolism have been developed [3]. Literature displays methodologies and applications of such tools for predicting HDI [4].

Terminalia chebula (*T. chebula*) Retz. (Combretaceae), commonly known as chebulic myrobalan, is a popular medicinal plant in the Ayurvedic system of medicine. The plant is reported to exhibit anticancer, antidiabetic, antimutagenic, antibacterial, anti-inflammatory and cardio-protective activities [5]. Literature reports inhibitory effects of *T. chebula* hydroalcoholic extract in different organic solvent fractions and its formulation, Triphala churna on CYP450 enzymes. The research work is performed using BioRad microplate absorbance and fluorescence reader which is a highly sophisticated instrument but needs significant investment to carry out routine work [6, 7].

Thus, the present study aimed to explore the inhibitory potential of alcoholic extract (AE) and hydroalcoholic extract (HE) of *T. chebula* on rat hepatic CYP450 enzyme with an instrument that could be found routinely in any analytical laboratory. Gallic acid, one of the major phytoconstituent of *T. chebula*, is expected to be present in alcoholic extract (AE) and hydroalcoholic extract (HE). In this research work, *in vitro* metabolism studies were conducted with rat liver microsomes (RLM). Inhibitory potential of *T. chebula* extracts and gallic acid on CYP3A4 was investigated using reverse phase high performance liquid chromatography (RP-HPLC) method with UV detection.

Materials and methods

Drugs and Chemicals

Testosterone, 6 β -hydroxytestosterone, ketoconazole 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.

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 autosampler, 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 μ g/ml. Calibration curve was prepared for standard gallic acid by plotting its concentration on X axis against peak area on Y axis. Weighed quantities of AE and HE were individually sonicated in methanol, the solutions were filtered through 0.45 μ syringe filter and subjected to RP-HPLC analysis. 10 μ 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 in extracts was quantified by extrapolation method using calibration curve prepared as discussed above.

Evaluation of enzyme inhibition potential

Preparation and characterization of RLM

RLM were isolated using calcium aggregation method [8]. 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 [9]. The homogenate was centrifuged at 13000 \times g for 10 min at 4 $^{\circ}$ 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 $^{\circ}$ C. The firmly packed microsome 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 $^{\circ}$ C until use. The entire isolation protocol was carried out at 0-4 $^{\circ}$ 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 [10]. 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 [11]. Standard protein plot was prepared using bovine serum albumin. Absorbance was recorded at 625 nm.

CYP3A4 Enzyme inhibition assay

CYP3A4 enzyme activity in RLM was measured by 6 β -hydroxylation of testosterone by CYP3A4 [12]. 500 μ l of standard incubation mixture (solvent control) containing RLM (0.5mg/ml), testosterone (40 μ M) in 0.1M sodium phosphate buffer (pH 7.4) at 37 $^{\circ}$ C was incubated for 60 min. Positive control was run with known CYP3A4 enzyme inhibitor, ketoconazole (40 μ M) solution. The reactions were initiated using NADPH solution (10mM) and terminated with 500 μ l methanol containing caffeine as an internal standard (50 μ g/ml) at the end of 60 min. The samples were centrifuged at 10000 \times g for 10 min at 4 $^{\circ}$ C and the supernatant solutions were subjected to RP-HPLC analysis. A modified RP-HPLC method was used for simultaneous estimation of 6 β -hydroxytestosterone and testosterone at a wavelength of 245 nm [13, 14]. Samples were run on a C18 column using methanol and water (pH 3.1 adjusted with acetic acid) as mobile phase in the ratio of 3:1. The inhibitory effect was studied by conducting *in vitro* metabolism in presence of different concentrations of AE, HE and gallic acid. Metabolite formed was quantified by RP-HPLC method mentioned above. Percentage inhibition by AE, HE and gallic acid was calculated using following formula [15].

$$\% \text{ inhibition} = \frac{(\text{AR}_{\text{control}} - \text{AR}_{\text{sample}})}{\text{AR}_{\text{control}}} \times 100$$

Where, AR_{control} and AR_{sample} are area ratios of metabolite/internal standard in solvent control and in presence of AE/HE/gallic acid respectively. IC₅₀ values were calculated by constructing a nonlinear regression graph between percentage inhibitions versus concentration, using Graph Pad Prism Software.

Results

Standardization of *T. chebula* extracts

Retention time (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 inhibition potential

CYP content was found to be 3.676nmol/ml. Protein content of isolated RLM was found to be 15mg/ml. Rt of caffeine, 6 β -hydroxytestosterone and testosterone were found to be 3.1 \pm 0.2, 4.2 \pm 0.2 and 9.0 \pm 0.2 min respectively. HPLC

chromatogram of caffeine, 6 β -hydroxytestosterone and testosterone is shown in figure 1.

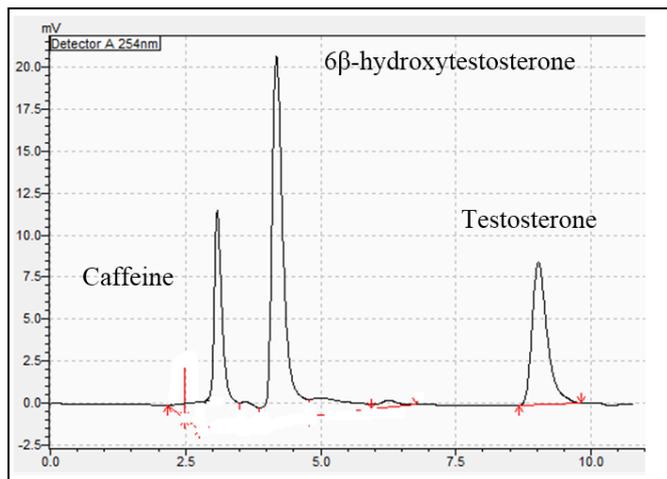


Fig 1: HPLC chromatogram of caffeine (internal standard), 6 β -hydroxytestosterone and testosterone using optimized chromatographic conditions

Various concentrations of AE, HE and gallic acid showed linear inhibition of CYP3A4 similar to ketoconazole. Inhibitory effect of AE and HE on CYP3A4 enzyme is shown in figure 2. Inhibitory effect of gallic acid on CYP3A4 enzyme is shown in figure 3. IC₅₀ values of AE, HE and gallic acid were found to be 160, 80 and 21.21 μ g/ml respectively.

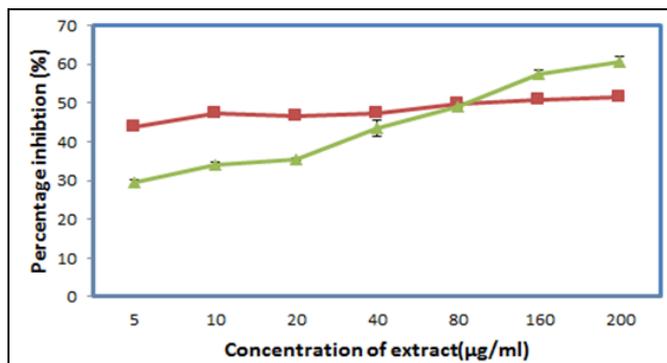


Fig 2: Inhibitory effect of alcoholic (■) and hydroalcoholic extract (▲) of *T. chebula* on CYP3A4 enzymes [The results are expressed as mean \pm SD (n=3)]

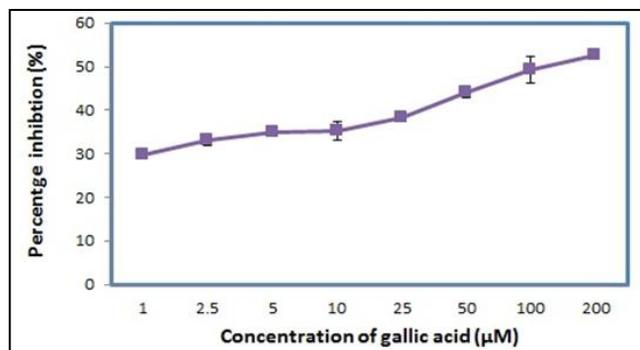


Fig 3: Inhibitory effect of gallic acid on CYP 3A4 enzyme [The results are expressed as mean \pm SD (n=3)]

Discussion

CYP 450 enzyme system plays a major role in the metabolism process. Amongst CYP 450 enzymes, 3A subfamily is the most prominent which involves metabolism of about 50% of clinically used drugs. Many herbal moieties are reported to

possess an ability to inhibit or induce CYP enzymes. Such moieties when administered concomitantly with the drugs, give rise to HDI. Various serious HDI are reported with St. John wart, ginkgo, grapes, saw palmetto, garlic, milk thistle etc [16]. However due to very less information established till date, it is need of an hour to investigate HDI potential of commonly co-administered herbs. *T. chebula* has long been used as herbal medicine. Inhibitory effect of *T. chebula* hydroalcoholic extract fractions on CYP 450 enzymes is reported using BioRad microplate fluorescence and absorbance reader.

Rat liver microsomes 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. Therefore goal of the study was to investigate effect of *T. chebula* alcoholic and hydroalcoholic extract on CYP3A enzyme activities in RLM with routinely available equipment such as HPLC with UV detector.

Gallic acid is one of the major phytoconstituent of *T. chebula* and is been reported as a reference for standardization of its extracts. *In vitro* metabolism study reports indicated that both the extracts and gallic acid had a significant inhibitory effect on CYP 3A4 mediated metabolism. Inhibition was reflected through decreased amount of metabolite formed in their presence. Ketoconazole was used as a positive control. Methanol concentration was maintained below 1% and thus could not affect the *in vitro* metabolism study [17]. IC₅₀ value of an inhibitor is its concentration causing 50% inhibition of enzyme activity. Gallic acid reported to be a highly potent inhibitor as compared to the two extracts with concentration of 21.21 μ g/ml responsible for reducing the enzyme activity by 50%. Difference in the activities of extracts and gallic acid indicates presence of other constituents in the alcoholic extracts with an ability to counteract the inhibitory potential of gallic acid. Amongst the two extracts, hydroalcoholic extract was found to have higher inhibitory potential indicating presence of some water soluble phytoconstituents like gallic acid responsible for the same. This is definitely an issue of potential safety due to wide use of *T. chebula*.

Conclusion

T. chebula alcoholic and hydroalcoholic extracts and gallic acid exhibited inhibitory potential on rat CYP450 3A4 enzymes *in vitro*. A simple analytical technique was designed to identify this inhibitory potential. IC₅₀ value for gallic acid was calculated which showed that it could be a major player in inhibiting the CYP3A4 isoform. This implies potential risk of herb drug interaction when substrates for CYP3A4 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*; HDI, herb drug interactions; RLM, rat liver microsomes; AE, alcoholic extract; HE, hydroalcoholic extract, NADPH, Nicotinamide adenine dinucleotide; Rt, Retention Time

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