Evaluation of food-drug interaction potential of *Aegle marmelos* (L.) Corrêa through metabolism mediated cytochrome P450 inhibition assay

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In the present study, possible cytochrome enzyme inhibition potential of *Aegle marmelos* (L.) Corrêa fruit and its bioactive compound was evaluated. Interaction potential of *Aegle marmelos* extract was evaluated through CYP450-carbonmonoxide complex (CYP450-CO) assay. Additionally, influence on individual major human cytochrome P450 drug metabolizing enzymes such as CYP3A4, CYP2D6, CYP2C9 and CYP1A2 were studied through fluorescence screening assay. Bioactive compound was quantified through RP-HPLC, in order to standardize the sample material. *Aegle marmelos* extract showed higher IC₅₀ value than the respective standard inhibitors and lower than the bioactive compound imperatorin. *Aegle marmelos* extract showed significantly lesser (P<0.001, P<0.01) interaction potential than the standard inhibitors. The research findings indicated that selected sample is unlikely to cause clinically relevant drug interactions involving inhibition of major CYP isozymes.

**Keywords**: Cytochrome P450, RP-HPLC, Imperatorin, *Aegle marmelos* (L.) Corrêa

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*Aegle marmelos* (L.) Corrêa (Family - Rutaceae) commonly known as ‘Bael’ in Hindi, ‘Bilwa’ in Sanskrit and Bael tree in English. It is used as very useful fruit in India particularly in summer season as an effective drink as fruit juice. Traditionally *A. marmelos* is used as dietary agent in the Indian subcontinent and also useful in treating pain, diabetes, fever, inflammation, respiratory disorders, cardiac disorders, dysentery and diarrhoea¹. Fruits contain functional and bioactive compounds such as phenolics, alkaloids, coumarins, flavonoids, terpenoids, carotenoids and vitamin C². Various research showed *A. marmelos* have different pharmacological activity such as anticancer, anti-diarrheal, anti-inflammatory, hepatoprotective, anti-pyretic, hypoglycemic, antioxidant and anti-fungal³ etc. Major bioactive chemical constituents isolated from the *A. marmelos* include aegelin, lupeol, skimmianine, fagarine, marmin, marmelide, marmesinin, marmelosin, psoralen, aurapten, cineol, citral, eugenol, cuminaldehyde, luvangetin and citronellal⁴,⁵.

Nutraceuticals and herbal products are often regarded as low risk because of their long history of human use. However, some herbal medicines have toxic effects in humans, which especially refer to extracts, concentrates or pure compounds obtained from plants⁶. Botanicals and nutraceuticals are mostly consumed without consultation of healthcare professionals. Several research reports highlights on potential herb–drug interactions due to the concomitant administration of food products and prescribed medicines. Cytochrome P450 enzymes are major drug metabolizing enzymes and are primary cause of herb-drug interactions⁷,⁸. Cytochrome P450 (CYP) inhibition or induction is the most common mechanism for the pharmacokinetic interactions of food and drugs⁹,¹⁰. Various herbs like *Ginkgo biloba* L., garlic, milk thistle and St. John’s wort have the potential to inhibit or induce human drug-metabolizing enzymes through cytochrome P450 (CYP) enzymes¹¹,¹². Interaction potential of *A. marmelos* with drug metabolizing enzymes like CYPs was investigated like CYP3A4, CYP2D6, CYP2C9 and CYP1A2 isozymes.

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Methodology

Chemicals

Vivid® CYP450 Screening Kit (CYP1A2, CYP2C9, CYP2D6 and CYP3A4) and specific Vivid® Substrates were purchased from Invitrogen Drug Discovery Solutions (USA) and used for this study. 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 used for the study. Ketoconazole and α-naphthoflavone were obtained from Merck (Mumbai, India). Quinidine and sulfaphenazole were procured from Sigma (Steinheim, Germany). Imperatorin was purchased from Sigma chemicals (Steinheim, Germany). All the other chemicals were of analytical grade.

Authentication and extraction of plant material

Fresh fruit of A. marmelos was collected from Kolkata, West Bengal, and authenticated by Dr. S Rajan, field Botanist, Ooty, Tamilnadu. Voucher specimen (SNPS-JU/1042) has been deposited at the School of Natural of Product Studies, Jadavpur University, Kolkata, for the future reference. The fruit pulp (500 gm) was air dried and defatted with hexane and extracted with the ethanol at ambient temperature (27 ± 1°C). The ethanol extract was prepared by refluxing the plant material with ethanol and this procedure was repeated thrice. The solvent was removed by using rotary vacuum evaporator (EYELA, Tokyo, Japan) in reduced pressure at 40°C, leaving semi-solid residue. The semisolid residue was dried under lyophilisation to get fine powder and final yield was 12.70 % w/w.

Standardization of extract

Extract of A. marmelos fruit, was standardized through reverse phase high-performance liquid chromatography (RP-HPLC). Lyophilized hydro-alcoholic extract of A. marmelos (AM) was accurately weighted and volume of the solution was adjusted in a volumetric flask to obtain a final concentration of 5 mg/mL. The optimized mobile phase considered of methanol, Milli-Q water and acetic acid in the ratio of 55:44:1 (v/v/v) with flow rate of 1 ml/min. The HPLC system comprised of rheodyne-7725 injection valve with a sample loop (20 mL), Vacuum degasser, quaternary pump and photo-diode array detector (PDA) was used, with data acquisition by Empower TM 2 software (Waters 600, Milford, MA, USA). Chromatography was performed on a Spherisorb C18 column (250mm × 4.6 mm, 5 mm; Waters, Ireland) fitted with a C18 guard column (10 × 3.0 mm). The sample elution was performed at 25 ºC and detected at the UV wavelength of 254 nm. Sample was analyzed by HPLC using 20 µL injection volume of a syringe (Hamilton Microliters; Switzerland). Amount of the phyto-constituent present in the sample was determined through the calibration curve. Stock solution of standard compound was prepared by dissolving the known amount of Imperatorin in methanol at a concentration of 10 mg/mL.

Cytochrome P450 inhibition study

Preparation of sample solutions

The lyophilized A. marmelos (AM) extract was solubilized in dimethyl sulfoxide (DMSO) and volume was adjusted to make a concentration of 10 mg/mL. The samples were examined to establish their influence on rat liver cytochrome P450 enzymes activity. Imperatorin (1 mg/mL) marker substrate was solubilized in ethanol and DMSO and also used for inhibition assay. Five µL ketoconazole (100 µM) was used as positive control to compare the inhibitory activity with standard and fruit pulp extract of A. marmelos. Microsomes without extract have been used as the negative control and appropriate solvent controls were used in the study.

Microsomes preparation

Rat Liver microsome (RLM) was isolated by the method described by Ponnusankar et al. (2011). In brief, the livers were perfused with 1.15% KCl solution and homogenized with four volumes (w/v) of ice-cold 1.15% KCl solution. The homogenate was centrifuged 600xg for 5 min and supernatant was again centrifuged at 9000xg for 20min to remove any broken mitochondrial fragments (Beckman Coulter 64R ALLEGRA). Supernatant was collected and further subjected to ultra centrifugation at 105,000xg for 1 h, at 4°C, (SORVALL RC100). The pellets were collected from the homogenates re-suspended in 1.15% KCl solution and stored at –80 °C for further use. Protein concentrations were determined by modified biuret method using bovine serum albumin as standard by AMS photoanalyzer PF-2 (CECIL, Japan).

Cytochrome P450-Carbon monoxide complex assay

CYP450-CO complex assay was performed with pooled RLM in 96 well microplate. Briefly, in this method, the RLM was diluted with phosphor-glycerol buffer (10 mM potassium phosphate, pH 7.4, 20% glycerol) and incubated with prepared extract...
The fluorogenic assay was performed based on earlier λ emission/excitation wavelength (BioTek FLx 800 T, U.S.A.) using an 96 microplates by microplate fluorescence reader Cytochrome P450 enzyme inhibition study (fluorogenic assays) analyzed using an excel spreadsheet. Sample solutions used for these assays and the data obtained were generated over the range was stopped by addition of 0.5 M tris base. reaction. After 30 min of incubation period reaction was stopped by addition of 0.5 M tris base. Generation of the products was linear over the range used for these assays and the data obtained were analyzed using an excel spreadsheet. Sample solutions and standard compounds were analyzed by their ability to inhibit the production of a fluorescent signal in the reactions using recombinant CYP isozymes and specific CYP Substrates. Product formation from the fluorogenic probes were determined from the fluorescence data at eight different concentrations of the inhibitors and the test and all measurements were performed in triplicate. Calculation of IC50 values was based on the curves of mean enzyme activity versus inhibitor concentration15. Percentage of inhibition and IC50 was calculated based on the following formula:

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

\[
\text{IC}_{50} = \frac{[(50-\text{LP}) \times (\text{HC}-\text{LC}) + \text{LC}]}{(\text{HP}-\text{LP})}
\]

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

Statistical analysis
All the tests were conducted in triplicate and results were presented as Mean ± SEM. The results were subjected to one-way analysis of variance (ANOVA) followed by the Bonferroni test for statistical analysis. The difference between the means was considered significant when p < 0.05. The statistical analysis was performed using the GraphPad InStat Version 5.

Results
Standardization through RP-HPLC
Extract of the sample material was standardized by RP-HPLC under isocratic conditions using the external standard calibration technique. The bioactive compound was identified by comparing with the respective retention time of the reference standard. Calibration curve was plotted by plotting peak areas against concentrations and five standard markers ranged from 100-500 µg/ml. Standard compound showed a good linearity between concentrations and the peak area, with the correlation coefficient (r^2) of 0.997. HPLC chromatogram of the extract and their respective markers was evaluated (Fig. 1). Optimum separation was achieved by using the same mobile system at the volume ratio of methanol: Milli-Q water and acetic acid in ratio of 55:44:1 (v/v/v) with a flow rate of 1mL/min. Retention time of imperatorin was found to be 9.032 min. Results were considered satisfactory and acceptable for subsequent quantitative analysis. The percentage of standard imperatorin present in the crude extract was found to be 1.94% (w/w) in the extract.
Cytochrome inhibition study

Cytochrome P450-CO complex assay

A CYP–CO complex assay was performed to assess the preliminary inhibitory potential of *A. marmelos* and its individual bioactive compound. The concentration of protein present in isolated rat liver microsomes was found to be 7.80 mg/ml which was estimated by modified Biuret method. Effects of individual component on the cytochrome P450 enzyme inhibition were studied by absorbance spectrum. The CYP450 concentration of RLM was found to be 0.618 nmol/mg protein. Study was performed by different concentrations of test solutions and a change in absorption spectra was measured. The percentage inhibition of CYP450 was calculated and the comparison of percentage of inhibition of the individual test substances with respect to positive control (Ketoconazole) is shown in Fig. 2.

To ensure the inhibition potential of the extract, suitable solvent control was used in this experiment and the percentage inhibition was calculated after nullifying the solvent effect. The observed result indicated the concentration-dependent inhibition of cytochrome enzymes in cytochrome P450 complex method\(^\text{16}\). The positive inhibitor ketoconazole showed higher inhibition potential than AM extract and imperatorin. It was observed that the plant extract and imperatorin showed significantly (P<0.001) less inhibition than positive inhibitor. Among all the test substances evaluated for their *in vitro* inhibitory activity, AM extract dissolved in DMSO showed the highest percentage of inhibition (35.107±2.829%). This change in inhibition was observed with all the DMSO solubilised extract, when compared with the ethanol solubilized extracts which may be due to the higher solubility of extract in DMSO than ethanol. The DMSO is considered as an industrial de facto solvent; it ensures complete solubility of the bioactive molecules present in the extract.

Lower inhibition (21.891±0.107 and 23.142±0.138%) was observed in imperatorin prepared in ethanol and DMSO respectively. The interaction with pooled CYP450 was more with the extract rather than its isolated molecule. This may be due to the synergistic effects of some other active molecules present in the extract\(^\text{17}\). Inhibition potential of AM extract showed higher percentage of inhibition than in ethanol but very less than standard inhibitor. This may be due to the DMSO, which is considered as an industrial de facto solvent; it ensures complete solubility of the bioactive molecules present in the extract.

Fluorogenic assays

For the characterization of the herb - drug interactions potential, CYP isozymes, specific high-throughput screening (HTS) assays is an important tool. AM extract and its major bioactive principle imperatorin were evaluated for their potential to affect the pharmacokinetics of conventional drugs when administered concomitantly. The assay was based on the ability of a drug to compete with different fluorogenic substrate for different CYP isozymes. Coumarin derivatives were used as the probe substrates, which generated fluorescent products after dealkylation by CYPs. Positive controls for each isozyme were used to confirm the assays precision.
Testing the pure compounds indicated the overall activity explainable by tested compounds or produced by other constituents in the extract.

All the test samples showed concentration-dependent inhibitory activity on CYP3A4, CYP2D6, CYP2C9 and CYP1A2 isozymes. All the samples were assayed in triplicate and IC$\textssub{50}$ values were calculated and represented in Table 1. Concentration dependant percentage inhibition of test solution on all the isozymes were found and shown in Fig. 3. The AM extract and its bioactive compound have less inhibition potential on the tested isozymes compared to their respective positive inhibitors. The study indicated that the extracts have higher inhibition potential comparing to their single bioactive compound. The higher enzyme inhibition potential by the extracts may be due to the synergistic effects of other constituents present in the extract. The test substances produced only minor inhibition of CYP3A4, CYP2D6, CYP2C9 and CYP1A2 isozymes. Among all the test substances AM extract showed the highest (IC$\textssub{50}$<0.01 mg/ml) interaction potential for all the isozymes; whereas, imperatorin (IC$\textssub{50}$>0.1 mg/ml) showed the least interaction potential. The study showed that AM extract have higher IC$\textssub{50}$ value than the standard inhibitors and lower than imperatorin for all the isozymes. The CYP450 inhibition of AM extract was more comparing to its single bioactive compound for all the isozymes. It was observed that the test substances have very less potential to interact with co-administered drug. In other word the A. marmelos fruit and their bioactive compound did not show much interaction potential.

![Fig. 3—Concentration dependant percentage inhibition of A. marmelos extract, imperatorin and positive inhibitors on drug modulating isozymes, CYP3A4 (A), CYP2D6 (B), CYP2C9 (C) and CYP1A2 (D); (Values are in Mean ± SEM).](image)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Solvent used</th>
<th>CYP3A4</th>
<th>CYP2D6</th>
<th>CYP2C9</th>
<th>CYP1A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. marmelos</td>
<td>DMSO</td>
<td>133.92 ± 1.56</td>
<td>141.28 ± 1.33</td>
<td>126.58 ± 1.09</td>
<td>121.72 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>149.62 ± 2.22</td>
<td>159.73 ± 1.43</td>
<td>137.45 ± 2.16</td>
<td>128.49 ± 1.27</td>
</tr>
<tr>
<td>Imperatorin</td>
<td>DMSO</td>
<td>189.83 ± 1.45</td>
<td>215.64 ± 2.04</td>
<td>223.39 ± 2.38</td>
<td>167.48 ± 2.59</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>182.23 ± 1.66</td>
<td>214.54 ± 2.32</td>
<td>213.96 ± 1.82</td>
<td>178.74 ± 2.93</td>
</tr>
<tr>
<td>Positive control</td>
<td>DMSO</td>
<td>5.85 ± 1.24</td>
<td>2.29 ± 0.94</td>
<td>25.81 ± 1.92</td>
<td>15.69 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>6.84 ± 1.02</td>
<td>3.06 ± 0.80</td>
<td>28.83 ± 1.59</td>
<td>19.07 ± 1.06</td>
</tr>
</tbody>
</table>

Table 1—IC$\textssub{50}$ (µg/ml) value of A. marmelos extract and imperatorin on the metabolism mediated by CYP3A4, CYP2D6, CYP2C9 and CYP1A2. (One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test)
not involve in interaction with other drug metabolism i.e. traditional use of these are safe.

**Discussion**

Consumption of food for therapeutic benefit is regulated by different laws in different countries, with limited concern on their safety, efficacy or standards of manufacture and quality control. Development of marker profiling for standardization is one of the most important parameter for maintaining the quality of herbals and to enrich the knowledge about the optimal concentrations of bio-active constituent’s present therein. Numerous case studies have described detrimental herb-drug interactions that can lead to morbidity or even mortality. Several such interactions have been reported earlier, for example CYP-modulating effects are described for St John’s wort, *Ginkgo biloba* and garlic. *A. marmelos* has a long history of use as a medicinal herb to treat several diseases. Through this study an approach has been made to evaluate the interaction potential of *A. marmelos* and its bioactive constituents with different CYP isozymes.

CYP450 has very significant role as metabolizing enzyme which is involved in the biotransformation of substance taken by oral route while the pre-systemic metabolism of these drugs takes place before entering into liver cell. The interaction between herb and drug with CYP450 enzyme may alter in plasma concentration and may lead to toxic effect. This is a useful screening method for the herbal medicine to get knowledge about herb-drug interaction. In the CYP450–CO method a concentration dependent inhibition of the enzyme was observed with the both *A. marmelos* extract and standard compound (imperatorine). Good linearity of concentration dependent inhibition was found in fluorescence screening assay at different concentrations of test and standard. Assay was performed in an accuracy mode in which the plate was incubated for the required reaction time. Organic solvent (DMSO and ethanol) strongly interferes at higher concentration (5%) with different isozymes (CYP3A4, CYP2D6, CYP2C9 and CYP1A2), whereas at low concentration (2%) act as alternative for solubilisation of tests samples. Ethanol and DMSO have been used as solvent and no interaction was observed with spectral absorption, proper solvent control was used and solvent effect was neutralized by deionised water. DMSO was used at low level to make sure the maximum solubility of the phyto-constituents. CYP-450 interaction potential was significantly higher in case of extract dissolved in DMSO compare to ethanol. The cytochrome P450 isozymes (CYP3A4, CYP2C9, CYP2D6 and CYP1A2), are clinically most important metabolizing enzymes. Investigation on AM extract and its biomarker against these isozymes showed very less inhibitory potential. AM extract and its individual component showed moderate inhibitory activity in a concentration dependent manner. Further *in vivo* studies are necessary to evaluate the clinical significance of the interactions.

**Conclusion**

The observed results by CYP-CO complex method and fluorogenic assays suggested that the AM extract and its bioactive compounds have less inhibitory effect on drug metabolising enzymes when consumed along with conventional therapeutics. The higher IC$_{50}$ values than positive control indicated that the test extracts and constituents have moderate interaction in drug metabolism. Test substances were likely to inhibit drug metabolizing enzymes, but less likely to produce significant drug interactions.

**Conflict of Interest:** The authors declare that there are no conflicts of interest.

**Acknowledgement**

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**References**


