Research Article

Effects of Ayurvedic Rasayana botanicals on CYP3A4 isoenzyme system

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ABSTRACT

OBJECTIVE: Consuming botanical dietary supplements or herbal drugs along with prescription drugs may lead to potential pharmacokinetic-pharmacodynamic (PK-PD) herb-drug interactions (HDI). The present study focuses on the importance of and novel approach for assessing HDI in integrative medicine with case examples of two frequently-used Ayurvedic Rasayana botanicals.

METHODS: The aqueous extracts of Asparagus racemosus (ARE) and Gymnema sylvester (GSE) were prepared as per Ayurvedic Pharmacopoeia of India. Chemoprofiling of these extracts was done using high-performance liquid chromatography (HPLC). Additionally, ARE was characterized for the presence of shatavarins IV and I using HPLC & mass spectroscopy respectively. Effects of ARE and GSE were investigated on rat liver microsome using testosterone probe drug assay. The changes in formation of metabolite (6-β hydroxy testosterone) were monitored on incubation of testosterone alone, testosterone with ketoconazole, ARE and GSE using HPLC. Half inhibitory concentration (IC₅₀) was used to predict plausible HDI.

RESULTS: ARE and GSE showed no inhibition with IC₅₀ values >1000 µg/mL while the standard inhibitor ketoconazole completely abolished CYP3A4-dependent activity at 0.531 µg/mL and IC₅₀ was found to be 0.036 µg/mL.

CONCLUSION: ARE and GSE prepared as per Ayurvedic Pharmacopoeia of India were found to be safe for CYP3A4-mediated inhibitory HDI in rats. Our in vitro study suggests the need of further in vivo investigation for HDI in order to provide clinical relevance.

Keywords: drugs, Chinese herbal; Ayurveda; Asparagus racemosus; Gymnema sylvester; plant extracts; cytochrome P-450 CYP3A; herb-drug interactions

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1 Introduction

Herbal medicines, whether used alone or in combination with conventional drugs, are increasingly thought of as attractive options for the treatment of chronic disease conditions¹ such as diabetes, cancer, and arthritis. Many patients take herbal medicines without a physician’s consent. They believe that herbal drugs are safer than conventional drugs and can be helpful in reducing side effects associated with conventional drugs. However, the use of herbal drugs in combination with conventional drugs may have a range of effects, varying from beneficial to sub-therapeutic, and

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even toxic effects\cite{5}. Like conventional drugs, these kinds of combination therapies should be tested for their herb-herb and herb-drug interactions in the early phases of herbal drug development. Current available data reported by the World Health Organization show that usage of herbal and/or complementary and alternative medicines vary between 30% to 90% throughout the world\cite{3}. A systematic approach is required in order to minimize negative interactions. In order to track such possible interactions many regulatory agencies such as the United States Food and Drug Administration (USFDA) and European Medicines Agency have outlined systematic procedures, which place the most emphasis on data gathered from in vitro drug interaction studies using an important cytochrome P450 (CYP) such as CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A\cite{4}. The significance of individual CYP enzymes in human drug metabolism varies, with CYP3A, CYP2D, and CYP2C being responsible for the metabolism of 50%, 25%, and 20%, respectively, for currently known drugs or xenobiotics\cite{5}. Several herbs such as Hypericum perforatum, Hydrastis canadensis, Gingko biloba, Echinacea purpure and phytoconstituents such as daidzein are now officially reported as CYP modulators by the USFDA\cite{4}. However, limited reports are available on the profile of Ayurvedic Rasayana botanical (ARB)-mediated CYP modulation. Hence we have selected two important ARBs: Asparagus racemosus (Shatavari) (AR), which is used for nearly all degenerative diseases, and Gymnema sylvestre (Gudmar) (GS), which is used in the treatment of diabetes\cite{6,7}. The present study was carried out to study CYP3A4-mediated herb-drug interactions (HDI) using the rat liver microsome (RLM) system, with testosterone probe drug assay.

2 Materials and methods

2.1 Chemicals, reagents and solvents

Testosterone, 6-β hydroxy testosterone, ketoconazole and standard shatavarin IV were received from the Synapse Laboratory Pvt. Ltd., as samples, while nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from the Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Methanol, acetonitrile and potassium dihydrogen phosphate were procured from Merck, Mumbai, India. All other chemicals used were of analytical grade.

2.2 Collection of plant materials

The roots of Asparagus racemosus and leaves of Gymnema sylvestre were obtained from Botanical Garden of Bharatiya Sanskriti Darshan Trust Ayurved Mahavidyalaya, Wagholi, Pune, Maharashtra, India. Roots were authenticated from Ayurvedic botanist Dr. S. Jagtap and samples were transferred to repository of Bioanalytical Department of Symbiosis School of Biomedical Sciences, and given a specimen voucher number SSBS/BD/2012/AR-001 or SSBS/BD/2012/GS-00, respectively.

2.3 Preparation of extracts

AR roots and leaves of GS were powdered and their extracts were prepared with distilled water using decoction method as described in the Ayurvedic Formulary of India\cite{8,9}. The menstrum obtained was concentrated under vacuum at 85 °C with 100 mmHg pressure. The resulting slurry was then spray dried (inlet temperature 180 °C, outlet temperature 117 °C and vacuum −10 mmHg). Aqueous extracts of AR (ARE) and GR (GSE) were dried and stored in aluminum-coated polyethylene bags. The percentage extract yield was calculated\cite{10}.

2.4 Chemical characterization of prepared extracts

Test samples of AR crude weighing 1.2 g and ARE weighing 130 mg were dissolved into 10 mL of high-performance liquid chromatography (HPLC)-grade methanol and vortexed for 5 min at room temperature. All the solutions were filtered through a 0.22-µm filter paper (Pall Life Science, India) before injecting in to the HPLC. Crude AR (root powder of AR) and ARE were chemically characterized for the presence of shatavarins IV and I using reported HPLC and HPLC-diode-array-detector (DAD)-mass spectrometry (MS) methods, respectively, from experience previously gathered by our laboratory research group\cite{11}. GSE was chemically characterized using previously reported HPLC-MS methods reported by our group\cite{12}.

2.5 Preparation of rat liver microsome homogenate

Animals were procured from the National Toxicity Centre, Pune, India. They were of Swiss Wistar strain. Liver microsome was isolated from Swiss Wistar strain rats (130–150 g) using previously reported method\cite{13} with slight modification. The study was approved by the Animal ethical committee affiliated to the Symbiosis School of Biomedical Sciences and Institutional Animal Ethical Committee (SSBS/IAEC/PH.BAL/PH.M/04/2011-12). Briefly, the rats were sacrificed by cervical dislocation, and the liver was quickly removed, perfused in ice-cold homogenization buffer, and homogenized with four volumes (w/v) of ice-cold 1.15% KCl solution. The 20% homogenate (w/v) was centrifuged at 9 000×g for 20 min and the supernatant was collected (Beckman Coulter, Allegra 64R), then subjected to ultra-centrifugation at 105 000 × g for 1 h. The complete process after liver removal was carried out below 4 °C. The microsomal fraction (105 000 × g pellet) was collected from the homogenates and it was resuspended in microsome buffer solution, stored in −80 °C, and used for protein concentration.

2.6 Estimation of protein content in RLM

Protein concentration was estimated in the RLM using bovine serum albumin as reference standard by using Lowery’s method\cite{14}. The method was optimized as per our
laboratory conditions and validated for the linearity and precision. The linear response of the detector versus concentration was considered on the basis of correlation of coefficient ($r^2$).

### 2.7 CYP3A4 inhibition activity assay

Testosterone was used as a probe substrate and formation of 6-β hydroxy testosterone was used as a measure of CYP3A4 activity\(^{[13]}\). Briefly, in 24-well plates (Nunc, USA) 250 µL reaction mixture containing 10 µL phosphate buffer (pH 7.4), 10 µL of test samples, 10 µL of testosterone (70 µmol/L) and 205 µL of RLM were added. The reaction was initiated with 25 µL of NADPH (2 mmol/L). The reaction mixture was incubated in a shaking water bath at 37 °C for 15 min. The reaction was stopped by the addition of ice-cold 100 µL of methanol and the assay was performed in triplicate.

### 2.8 Preparation of test materials

ARE and GSE (200 mg) were dissolved in 2 mL of deionized water. All solutions were pre-filtered through a 0.2-µm membrane filter. The organic contents were maintained at >1% during the assay.

### 2.9 Measurement of 6-β hydroxy testosterone using HPLC method

Testosterone and 6-β hydroxyl testosterone were monitored in the study samples, using previously reported HPLC-DAD method\(^{[16]}\). Briefly, chromatographic separations were carried out on RP C-18 Hypersil base-deactivated silanol column (250 mm × 4.6 mm, 5 µm particle size) using methanol and water (60:40, v/v) as mobile phase for 30 min with flow rate of 1.2 mL/min. Auto-sampler and column oven were maintained at temperatures of 8 and 30 °C respectively. Analysis was monitored at wavelength of 240 nm.

### 2.10 Determination of 50% inhibitory concentration value

Five different concentrations ranging from 200 to 1000 µg/mL of ARE and GSE were selected on the basis of one daily maximum human dose (ARE: 0.5–1.5 g/d; GSE: 3–6 g/d) of these herbs when diluted in 1 L of gastrointestinal fluid or 56 L of total body fluid to cover the in vivo concentrations. Ketoconazole was used from the concentration range 0.01–4.78 µg/mL respectively. Percentages (%) of control activity and inhibitory activity were calculated using formulae: Control activity (%) = (Peak area of 6-β hydroxy testosterone in the presence of extract or fractions/ peak area of 6-β hydroxy testosterone in control) × 100 and inhibition activity (%) = 100 – % control activity. Final 50% inhibitory concentration (IC\(_{50}\)) was calculated using GraphPad Co. Ltd., USA-5 by using nonlinear regression analysis.

### 2.11 Prediction of plausible clinical interactions

IC\(_{50}\) of testosterone oxidation activity was calculated graphically by nonlinear regression analysis of logarithmic inhibitor concentration (log conc.) versus % of inhibitory activity plot using GraphPad Prism 5. The data were expressed as mean ± standard deviation. Ratio of I/Ki was used to predict plausible clinical interactions and > 0.1 was considered for possible clinical interaction\(^{[17]}\). [I] is mean maximum surrogate plasma concentration (C\(_{max}\)) at steady state after administration of the highest clinical dose of inhibitor in human and used from previously published literature\(^{[18]}\). Inhibitor constant (Ki) was calculated using equation: $Ki = IC_{50}/1+[S]/[Km]$. [S] and [Km] are substrate concentration and Michaelis constant respectively, since the interaction between ketoconazole and testosterone is competitive. In our experiment, [S] and [Km] values were same as 70 µmol/L. [I] values for ketoconazole were used as 1 to 5 µg/mL (5 µg/mL was used in the experiment)\(^{[19]}\). It was not possible to calculate I/Ki ratio for ARE and GSE because of the absence of pharmacokinetics data, and specifically [I] values as these extracts are complex mixtures of phytoconstituents.

### 3 Results

#### 3.1 Extractive value

The extraction yield rates for ARE and GSE were found to be 11.2% (w/w) and 19.8% (w/w) respectively, which were satisfactory as per previous reports\(^{[20,21]}\).

#### 3.2 Chemical characterization of ARE and GSE

ARE was chemically characterized on the basis of its steroidal saponins and shatavarns IV and I by using HPLC and HPLC-DAD-MS in positive ionization mode method respectively. The representative HPLC-DAD chromatograms for crude AR, ARE and shatavarn IV are shown in Figure 1. HPLC-DAD fingerprints showed the presence of 9 total prominent peaks in crude AR and ARE. Therefore, the peaks could be used for monitoring quality of AR and its marketed samples. Previous reports suggested that the shatavarns IV and I are responsible for various pharmacological activities of AR and therefore considered as the active marker compounds\(^{[22]}\). In order to identify shatavarn IV in ARE and its crude AR sample, we have used an external standard method. Identification of shatavarn IV was done using retention time ($t_b$) and UV spectral data matching. The HPLC-DAD chromatogram of reference standard of shatavarn IV (as shown in Figure 1-C) has $t_b$ of 31.89 min and absorbs UV light data at 225 nm. Crude AR and ARE sample showed the presence of peak at $t_b$ of 31.89 min (peak No. 8) with UV spectral overlays (window W of Figure 1). Thus results confirmed the presence of shatavarn IV in both samples of crude AR and its extract. Both the chromatograms of A and B showed the presence of major peak (peak No.7) at $t_b$ of 29.36 min. The online UV spectra of this peak showed maximum wavelengths at 211 nm. This suggested that the peak could be of shatavarn I as previously reported\(^{[23]}\).
Further, to confirm this observation, we isolated this peak by collecting eluants after repetitive injections of ARE samples on the HPLC column. The collected eluants were concentrated under the vacuum. The concentrated eluant was injected into MS system for identification of mass. The MS showed the presence of \( m/z = 1071.45 \) in positive mode (Figure 2). The \( m/z \) value was found to be matched with the \( m/z \) value of shatavarin I in the literature\(^{[24]}\). Therefore, peak No. 7 was identified as shatavarin I and need further confirmation using other hyphenated analytical techniques.

HPLC-DAD chromatographic fingerprint was developed for chemical characterization of GSE either. The representative HPLC-DAD chromatogram was given in Figure 3 and showed the presence of 7 prominent peaks. Phytochemical investigations suggest the presence of gymnemic acids as major compounds in GSE\(^{[25]}\). Identification of these peaks could provide information on gymnemic acids and other important phytoconstituents which needs further investigation using tandem mass spectrometry analysis. These chemically standardised extracts of AR and GS were used for the evaluation of CYP3A4 inhibitory potential using RLM. The concentration of RLM in the assay was decided on the basis of protein content.

### 3.3 Estimation of protein content in RLM

The method was found to linear in the range of 50–3 200 \( \mu \)g/mL with \( r^2 = 0.99 \) suggesting excellent linearity (Figure 4) precision (intermediate precision and repeatability) of the method was calculated on the basis of relative standard deviation (\% RSD). The results showed that \% RSD was less than 5\% suggesting the method is precise. The total protein content in the RLM has been found to be 1.4 mg/mL.

### 3.4 Effects of ARE and GSE on CYP3A4-inhibitory activities

Effects of ARE and GSE on CYP3A4-inhibitory potential were studied on RLM using testosterone 6-β hydroxylase assay. RLM was incubated with test drugs and formation rate of metabolite 6-β hydroxy testosterone was monitored by using HPLC method (Figure 5). Ketoconazole, a known CYP3A4 inhibitor was used as the positive control. All samples were assayed in triplicate and the percentage inhibition versus logarithmic concentration graphs were plotted (Figure 6 and Table 1) for the calculation of IC\(_{50}\) values. Ketoconazole completely abolished CYP3A4-dependent activity at 0.531 \( \mu \)g/mL and IC\(_{50}\) was found to be 0.036 \( \mu \)g/mL. These findings were consistent with previous reports\(^{[26,27]}\). ARE and GSE did not show significant inhibitory activities even at high doses of 1 000 \( \mu \)g/mL. Several reports are available on prediction of clinically possible PK interactions based on inhibition constant (\( K_i \)) from \textit{in vitro} data and mean maximum surrogate plasma concentration at steady state (\( I \)) after administration of the highest clinical dose in humans\(^{[28]}\). \( K_i \) values were calculated from IC\(_{50}\). The \( I/K_i \) ratio > 0.1 suggested possible clinical interactions and requires

![Figure 1](https://www.jcimjournal.com/jim/)

**Figure 1** HPLC chromatograms for detecting chemical characterization of \textit{Asparagus racemosus} and its extracts

It shows that the presence of shatavarin IV in crude AR (A) and extract (B) with shatavarin IV (C) as marker. Spectral overlays of reference standard and extract are shown in window (W). HPLC: high-performance liquid chromatography.
in vivo evaluation for drug interactions. Ki value for ketoconazole was found to be 0.018 µg/mL with [I]/Ki ratio of 277.77, suggesting possibility of clinically significant pharmacokinetic interactions with CYP3A4 substrates. This finding is consistent with previously reported clinically significant PK interactions with CYP3A4 substrates such as ranolazine, ritonavir and imatinib, etc [29–31]. It was not possible to calculate I/Ki ratio for ARE and GSE because of the absence of pharmacokinetics data especially [I] values.

4 Discussion

Our study highlights the need for herbal pharmacokinetic profiling for prediction of clinically relevant phytoconstituents-mediated HDI, especially if these pharmacologically active constituents are administered individually. ARE and GSE prepared via traditional methods [9,11] showed absence of CYP3A4 inhibition, suggesting safe use during integration with chemotherapeutics – specifically with CYP3A4 substrate drugs. Since many researchers have shown that the method of aqueous extraction employed by Ayurvedic practitioners extracts very less proportion of phytoconstituents which might be responsible for insignificant inhibitory effects and/or HDI [10,32]. Recent reports by Patil et al [33] studied HDI using human recombinant CYP3A4 by taking aqueous extract of roots of Withania somnifera (WSE), stems
Table 1  Effect of different concentrations of ARE, GSE and ketoconazole on CYP3A4-mediated metabolism

<table>
<thead>
<tr>
<th>Log dose (μg/mL)</th>
<th>Inhibition</th>
<th>ARE</th>
<th>GSE</th>
<th>Log dose (μg/mL)</th>
<th>Inhibition of ketoconazole</th>
</tr>
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<tr>
<td>5.301 03</td>
<td>0.177 ±0.006 5</td>
<td>0.108 ±0.007 5</td>
<td>-1.973 52</td>
<td>1.917 ±0.024 8</td>
<td></td>
</tr>
<tr>
<td>5.602 06</td>
<td>0.268 ±0.007 6</td>
<td>0.221 ±0.009 6</td>
<td>-1.672 49</td>
<td>18.870 ±0.041 3</td>
<td></td>
</tr>
<tr>
<td>5.778 15</td>
<td>0.337 ±0.006 8</td>
<td>0.348 ±0.007 0</td>
<td>-1.233 15</td>
<td>59.640 ±3.761 0</td>
<td></td>
</tr>
<tr>
<td>5.903 09</td>
<td>0.569 ±0.008 3</td>
<td>0.551 ±0.010 0</td>
<td>-0.756 03</td>
<td>85.690 ±0.862 3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.606 ±0.005 7</td>
<td>0.598 ±0.008 0</td>
<td>-0.233 15</td>
<td>95.869 ±0.042 9</td>
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<tr>
<td></td>
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<td>0.202 58</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.679 70</td>
<td>99.235 ±0.190 9</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition data (%) are represented as mean ± standard deviation (n=3). ARE: extract of Asparagus racemosus; GSE: extract of Gymnema sylvester.
of *Tinospora cordifolia* (TCE) and ARE along with pharmacologically important phytoconstituents isolated from TCE. They found that there is significant inhibition by these individual phytoconstituents. TCE showed mild inhibition while no significant inhibition was observed in WSE and ARE. From the IC$_{50}$ value and recent published data, both these extracts have been shown to be safe and without inhibitory effects. Using these extracts at dosages typically prescribed by Ayurvedic physicians is within the safe range, and the possibility of inhibitory interactions would be very rare. Clinical studies would be needed in order to confirm this. The clinical studies can also focus on enzyme inhibition-induction or interplay, so that a complete HDI profile can be studied. Further research on novel approaches that can bypass the need of phytoconstituents and their molecule-dependent values (e.g., I, Ki, $K_{intac}$) would also be valuable.

### 5 Conclusion

The IC$_{50}$ values of aqueous extracts of both AR and GS have been found to be beyond 1 000 μg/mL. Since Ayurvedic physicians typically use much lower doses of these extracts than the doses used here, the chances of inhibitory interactions occurring — especially those that are mediated through CYP3A4 — are very rare. Therefore, it has been concluded that both the extracts are safe for CYP3A4-mediated inhibitory interactions. But in order to confirm this, clinical study is required which can also focus on enzyme induction and interplay, so that a complete HDI profile can be studied.

### 6 Acknowledgements

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### 7 Conflict of interests

The authors declare that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### REFERENCES

13. Ponnusankar S, Pandit S, Babu R, Bandyopadhyay A.


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