Phytochemical characterization of *ayurvedic* formulations of *Terminalia arjuna*: A potential tool for quality assurance

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Ayurveda has gained worldwide attention due to its efficacy. With the growing need for safer drugs, attention has been drawn to their quality and standards of the Ayurvedic formulations. Ayurveda describes the formulation of *Terminalia arjuna* (*T. arjuna*) as a potent drug for dyslipidemia, cardiac disorders, and diabetes. It is administered as arishta, ghritam (medicated ghee) or as a powder. Thus, the main objective of the present work was to characterize Arjunarishta (AA) and Arjuna ghritah (AG) - ayurvedic formulations using arjunetin and arjungenin by High-Performance Liquid Chromatography-Photodiode Array Detector (HPLC-PDA) method. The presence of marker contents in the AA, AG, and *T. arjuna* hydroalcoholic extract (TAHA) was identified using retention time (Rt) and UV spectra matching with corresponding reference standards. The content of arjunetin and arjungenin was 0.47 and 8.22 mg/g in TAHA; 206.38 and 0.00 µg/mL in AA; 190.58 and 285.48 µg/mL in AG. These quantitative estimations were consistent with earlier reports on TAHA. The results of the present study indicate the characterization of Arjunetin and Arjungenin phytochemical markers in AG, and AA formulations, which have not been, reported so far. These finding will certainly help in the quality assurance during manufacturing of AG and AA formulations.

Keywords: Arishta, Arjunetin, Arjungenin, Ayurveda, Ghritah, HPLC, Standardization.

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*Ayurveda* is a traditional system of medicine in India. Ayurvedic preparations were used to prevent diseases for centuries¹². Formulations were prepared using well-documented methods consisting of several herbs in different forms like decoction, asavas, fresh juices, tablets, oils, ghee, and arishtas¹³. Ayurveda has gained worldwide attention due to its efficacy. With rising requirement for better drugs, attention has been focussed on the quality and standards of the Ayurvedic formulations⁵. However, different constituents with diverse physical and chemical properties made the quality control of formulations difficult⁶. Ayurvedic pharmacy advocates the use of quality control tests to make sure that the prepared medicines adhere to the standards mentioned in Ayurveda. Those were based on observation parameters. Hence, characterization of Ayurvedic formulations using modern techniques of analysis is extremely important⁵.

The bark of *Terminalia arjuna* (*T. arjuna*) is used in traditional Indian medicines either alone or as a constituent in lots of herbal medicines for treating various ailments. Ayurveda describes the formulation of *T. arjuna* as a potent drug for dyslipidemia, cardiac disorders, and diabetes. It is administered as arishta, ghritam (medicated ghee) or as a powder³. The usefulness of this drug in cardiovascular disorders is well documented in the literature⁷-¹⁰. The plant was reported to contain triterpenoids, flavonoids, glycosides, and tannins, etc. *T. arjuna* contains polyphenols such gallic acid, ellagic acid and triterpenoids like arjunolic acid, arjunic acid, arjunetin, arjungenin, arjunglucoside I and II. However characterized and validated methods for quantification were not available for Ayurvedic formulations such as Arjunarishta (AA) and Arjun...
ghritam (AG)\textsuperscript{11-13}. Pharmacopoeial standards for Ayurvedic formulations published by the Central Council for Research in Ayurveda and Siddha provide physicochemical parameters (pH, total solids, sugar content), thin layer chromatography profiling and gallic acid content of the AA formulation. Therefore, it is essential that there are definite and accurate analytical tools to ascertain consistency and quality of AA and AG\textsuperscript{14-16}. Thus, the objective of this work was to characterize AA and AG using specific phytochemical analytical markers.

**Methodology**

**Instrumentation**

A Dionex P680 HPLC system including autosampler, thermostatted column compartment and a Dionex UVD 170U/340U photodiode array detector (Dionex Corp., Germering, Germany) was used to acquire chromatograms. The column used was RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) from Thermo Electron Corp. (Bellefonte, PA). Chromeleon 6.70 software from Dionex was used to acquire data for fingerprint analysis.

**Chemicals**

HPLC grade methanol (Merck, Mumbai, India) was used to prepare samples; water was purified by using the Milli-Q (Millipore, USA) system; and HPLC grade acetonitrile (Merck) was used to prepare the mobile phase. Arjuneatin and Arjungenin were purchased as HPLC markers from the Natural Remedies Pvt. Ltd. Bangalore, India.

**Plant materials and extraction**

The *T. arjuna* bark sample was collected from a local market in Pune, India and authenticated by Agharkar Research Institute, Pune, India. The sample was deposited as voucher specimens no. S/B-109. The dried bark was extracted with ethanol: water (70:30 v/v) using Soxhlet extraction for 8 hours for consecutive three days at 65°C. The extract was dried under vacuum using rotary evaporator at 45°C and stored at 2-8°C until use.

**Formulations**

Marketed formulations, i.e., Arjunarishta (Batch No. GA-06, Sandu Pharmaceuticals Ltd. Goa, India) and Arjun Ghritam (Batch No. 116, Nagarjun Pharmaceuticals P. Ltd. Ahmedabad, India) manufactured by Indian-based Ayurvedic drug manufacturing companies were used for the analysis. These formulations were procured from the market.

**Preparation of standard solution**

Stock solutions of both markers prepared separately in methanol. Final concentration (31.25, 62.5, 125, 250, 500, 1000 µg/mL) was made up by further diluting with methanol.

**Preparation of sample**

AA 5 mL sample was added to 50 mL centrifuge tube, and then 10 mL of methanol added and vigorously shook for 5 min then stand for 10 min to settle down the precipitated sugars. After 10 min, methanol extract was pipetted out. Then, 1 mL of supernatant was passed through 0.45 µm filter (millipore) and 20 µL was injected for quantification. AG 10 mL sample was soaked on 10 g silica gel powder in 50 mL centrifuge tube, and 20 mL n-hexane was added and vigorously shaken for 5 min then allowed to stand for 10 min to settle down the precipitated ghritah component and silica gel powder. After that, n-hexane was pipetted out and discarded without disturbing silica gel powder. This procedure (wash with n-hexane) was repeated thrice. Then added 20 mL acetonitrile solvent and vigorously shaken for 5 min then allowed to stand for 30 min to settle down the precipitated silica gel powder. After that, acetonitrile was pipetted out. 1 mL of supernatant was passed through 0.45 µm filter (millipore) and 20 µL was injected for quantification.

**HPLC system parameters**

The analysis was performed with an RP C18 BDS Hypersil column (250 × 4.6 mm, 5 µm particle size) at a column temperature 26°C. Separation was achieved with two pumps. The gradient program for pump A (acetonitrile: water, 30:70) and pump B (acetonitrile: water, 70:30) as follows: initially 30% B, flow rate 0.8 mL/min; increased gradually to 50-70% B up to 30 min, flow rate 1.2 mL/min. Then washed the column for 20 min, 30% B, flow rate 0.8 mL/min. The detection wavelength was 220 nm, the absorption close to both the compounds. Injection volume for standard and sample was 20 µL\textsuperscript{17}.

**Data analysis of chromatogram**

Data analysis was performed by variations and similarity observed in retention time values, peak areas and spectral patterns of the peaks obtained in the
chromatograms of ayurvedic formulations and T. arjuna hydroalcoholic bark extract (TAHA).

Method validation
A complete method validation was carried out according to the industrial guideline for analytical method validation.

Specificity and selectivity
The specificity of the method was evaluated by analyzing methanol samples from at least six different lots to investigate the potential interferences at the LC peak region for analytes. The acceptance criterion for the experiment was that at least four out of six lots should have response less than five times of the LLOQ level response in the same solvent.

Linearity
The linearity of the method was generated by analysis of five calibration curves containing six non-zero concentrations. The six-point calibration curve for arjunetin and arjungenin (31.25, 62.5, 125, 250, 500, 1000 µg/mL) was constructed by plotting the peak area each analyte against the nominal concentration of calibration standards in methanol. This operation was repeated on six consecutive days with freshly prepared calibration standards to select the most appropriate regression model. Each calibration curves were analyzed individually by fitting the area response for analyte as a function of standard concentration, using no weighted or least square weighted (1/x or 1/x²) linear regression and excluding the point of origin. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

Precision and accuracy of the analytical method
Arjunetin and Arjungenin samples (at a concentration of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) were prepared. Intra- and inter-day assays were repetitively carried out on the samples at three different times of the same day and six different days, respectively. The standard spiked solutions were examined six times within one day to determine the intra-day precision. The inter-day precision was established by analyzing each sample on six consecutive days; each sample was injected three times on each day. Analytical precision was evaluated by calculating the % CV of variances Intra- and inter-day while accuracy was calculated from the nominal concentration (Cnom) and the mean value of observed concentration (Cobs) as follows: accuracy (bias, %) = [(Cnom - Cobs) / Cnom] x 100%

Statistical analysis
The experimental data were expressed as Mean ± SD. Precision was expressed as % coefficient of variation using Graph Pad Prism 5.00 Software (San Diego, USA).

Results and discussion
Standardization is an important aspect for establishing the quality of the any traditional formulations. Reports on cardioprotective activity and phytochemical investigation of TAHA and AA are numerous however there are no reports on standardization of AG. We have attempted to standardize the formulation with respect to its specific phytochemical constituents like arjungenin and arjunetin that may be responsible for the therapeutic action. The chromatograms of standards, TAHA, AA, and AG, were compared. Arjunetin and Arjungenin were separated under the optimum conditions. No interference peaks from the endogenous constituents of the TAHA, AA, and AG were found in the region of the investigated compounds.

Validation parameters
The retention time for arjunetin and arjungenin were found to be 4.95 and 7.68 min respectively. The spectral overlays showed the presence of UV spectra at 220 nm and were matched with the UV spectra of the reference standard. Quantitative estimation of these marker compounds was carried out using external standard calibration method. The calibration plots of concentration versus peak area were constructed in the range of 31.25-1000 µg/mL.

Specificity
Specificity of the developed method was checked by changing the solvent B (from methanol to acetonitrile). Retention time, lower limits of quantification were determined and are shown in Table 1, together with regression equations and their coefficients of regression (R²). The value of R² confirmed the linearity of the method. The robustness of the method was studied by changing the mobile phase (solvent B from 80:20 to 90:10); minor changes in mobile phase showed no effect on peak resolution. The linearity range of arjunetin and arjungenin solutions were obtained as 31.25-1000.0 µg/mL as shown in Table 1. The calibration curve was
constructed using a linear regression of the theoretical concentration of an analyte versus the corrected peak area. The mean regression equations and their correlation coefficients were calculated to be 
\[ Y = 0.069 X + 0.660 \] 
and 
\[ Y = 0.046 X + 0.628 \]
for arjunetin, and 
\[ Y = 0.069 X + 0.660 \] 
and 
\[ Y = 0.046 X + 0.628 \]
for arjungenin.

**Precision and accuracy**

The precision was considered at two levels, the repeatability, and the intermediate precision. The repeatability of the sample application was determined as an intra-day variation, whereas the intermediate precision was determined by carrying out the inter-day variation for the determination of arjunetin and arjungenin at different concentration levels of 31.25, 62.5, 125, 250, 500 and 1000 µg/mL. The standard solutions, at four different concentration levels, were analyzed at least six times within the same day, and % CV values obtained were in the range between 5.19 - 16.86% and 5.57 - 10.90% for arjunetin and arjungenin. Similarly, to measure the inter-day variability, the same concentration of the two standards was run over at least six consecutive days, and the values were in the range between 6.86 and 9.29% and 5.74 and 10.47% for arjunetin and arjungenin. The accuracy (% bias) values ranged 8.98 to 6.94 and -9.79 to 8.08 for arjunetin and arjungenin respectively. The results of the repeatability and intermediate precision were expressed in CV (%) and shown in Table 2; the low values of the CV (%) indicated the repeatability of the proposed method.

**Phytochemical characterization of AA and AG**

Phytochemical characterization of AA, AG, and TAHA was carried out using selected marker-based approach. Arjunetin and arjungenin were used as phytochemical markers for characterization purpose. The marker contents were estimated using earlier reported HPLC-PDA method suitably modified on column and mobile phase gradient. The optimized chromatographic conditions showed good resolution of the all the peaks. The presence of marker contents in the AA, AG, and TAHA was identified using retention time and UV spectra matching with corresponding reference standards (Fig. 1). The content of arjunetin and arjungenin was 0.47 and 8.22mg/g in TAHA; 206.38 and 0.00 µg/mL in AA; 190.58 and 285.48 µg/mL in AG (Table 3). These quantitative estimations were consistent with earlier reports on TAHA³. Previously, standardisation of AA reported using HPLC method with gallic acid, ellagic acid, and quercetin markers. Lal et al mentioned that AA formulation did not contain any specific T. arjuna phytochemical markers ³. This may be due to less amount of arjunetin present in the formulation or non-specific sample processing and analysis method.

<table>
<thead>
<tr>
<th>Phytochemical Marker</th>
<th>Retention Time (min)</th>
<th>R²</th>
<th>Concentration Range (µg/mL)</th>
<th>LLOQ µg/mL</th>
<th>Linear Regression Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunetin</td>
<td>4.945 ± 0.002</td>
<td>0.991</td>
<td>31.25-1000</td>
<td>31.25</td>
<td>Y=0.069X + 0.660</td>
</tr>
<tr>
<td>Arjungenin</td>
<td>7.685 ± 0.003</td>
<td>0.992</td>
<td>31.25-1000</td>
<td>31.25</td>
<td>Y=0.046X + 0.628</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>C_{nom} (µg/mL)</th>
<th>Precision Intra-day (n=6)</th>
<th>Accuracy</th>
<th>Precision Inter-day (n=6)</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C_{obs} (µg/mL)</td>
<td>Precision (% CV)</td>
<td>% bias</td>
<td>C_{obs} (µg/mL)</td>
</tr>
<tr>
<td>Arjunetin</td>
<td>1000</td>
<td>1047 ± 54.28</td>
<td>5.19</td>
<td>-4.7</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>245.5 ± 21.73</td>
<td>8.85</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>68.11 ± 4.9</td>
<td>7.19</td>
<td>-8.98</td>
</tr>
<tr>
<td></td>
<td>31.25</td>
<td>29.08 ± 4.91</td>
<td>16.86</td>
<td>6.94</td>
</tr>
<tr>
<td>Arjungenin</td>
<td>1000</td>
<td>1084 ± 60.39</td>
<td>5.57</td>
<td>-8.4</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>229.8 ± 19.82</td>
<td>8.62</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>68.62 ± 6.25</td>
<td>9.11</td>
<td>-9.79</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.
Fig. 1 — Phytochemical profiling HPLC chromatograms of arjunetin and arjungenin HPLC chromatograms at 220 nm. (I) extract of *T. arjuna* (TAHA); (II) Arjunarishta formulation (AA-F); (III) Arjuna ghritha formulation (AG-F); (IV) chromatogram of Spiked Arjunetin (A) and Arjungenin markers (B).

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Rt (min) AG</th>
<th>Rt (min) AA</th>
<th>Rt (min) TAHA</th>
<th>(µg/mL of AG)</th>
<th>(µg/mL of AA)</th>
<th>(mg/g of TAHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arjunetin</td>
<td>4.95 ± 0.0</td>
<td>5.06 ± 0.01</td>
<td>5.10 ± 0.02</td>
<td>5.09 ± 0.02</td>
<td>166.6 ± 2.69</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>Arjungenin</td>
<td>7.69 ± 0.0</td>
<td>7.87 ± 0.02</td>
<td>ND</td>
<td>7.88 ± 0.04</td>
<td>228.7 ± 9.15</td>
<td>0.85 ± 0.01</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.
Conclusion
The results of the present study indicate the characterization of Arjunetin and Arjungenin phytochemical markers in AG, and AA formulations, which have not been, reported so far. This work can be utilized as baseline data of characterization and quality assurance of T. arjuna formulations with specific phytochemical markers. These finding will certainly help in the quality control process of manufacturing of AG and AA formulations.

Conflict of interest
The authors declare that they have no conflict of interest.

References