HPLC Analysis and Standardization of Arjunarishta – An Ayurvedic Cardioprotective Formulation

Uma R. LAL, Shailendra M. TRIPATHI, Sanjay M. JACHAK, Kamlesh K. BHUTANI, Inder P. SINGH *

Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S. A. S. Nagar 160062, Punjab, India

* Corresponding author. E-mails: ipsingh@niper.ac.in or ipsingh67@yahoo.com (I. P. Singh)


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Abstract

Arjunarishta (Parthadyarishta) is an important Ayurvedic formulation used for cardiovascular disorders and is prepared by fermenting the decoction of specified plant materials using flowers of Woodfordia fruticosa. In present communication, an HPLC-PDA method was developed for the standardization of Arjunarishta by quantitative estimation of major antioxidant compounds, ellagic acid, gallic acid, ethyl gallate, quercetin and kaempferol as markers. The developed method was validated with respect to linearity, precision, accuracy, and robustness. The HPLC analysis showed an increase in amount of ellagic acid and gallic acid during preparation, i.e. decoction vs formulation. A similar increase in free radical scavenging activity of formulation vs decoction was also observed. Arjunolic acid and arjunic acid were not detected in the formulation.

Keywords

Parthadyarishta • Gallic acid • Ethyl gallate • Ellagitannins • Flavonoids

Introduction

Ayurveda ‘the science of life’ is a traditional system of medicine in India that dates back to ancient times. Ayurvedic preparations have been used to prevent diseases in both humans and animals for centuries in the Indian subcontinent; most of the formulations prepared by particular processing methods are compound preparations consisting of several herbs and
are available in different forms such as powder, decoction, fresh juices, tablets, oils, ghee (clarified butter preparations), ashavas and arishtas (alcoholic preparations). The official Ayurvedic Formulary of India (AFI) lists thirty-seven ashavas and arishtas (Anonymous, 2003) [1].

Arishtas are an important group of formulations used in Ayurveda. Arjunarishta (Parthadyarishta) is one of the ancient liquid oral formulations prescribed in Ayurveda for cardiovascular disorders. It nourishes and strengthens heart muscle and promotes cardiac functioning by regulating blood pressure and cholesterol. The plant ingredients in this formulation are Terminalia arjuna, Madhuca indica, Vitis vinifera, and Woodfordia fruticosa. The formulation is prepared by making a decoction of three plants in specified amounts as listed in AFI. Crushed jaggery and the flowers of Woodfordia fruticosa are then added and preparation is kept for a specified period of time during which it undergoes fermentation generating alcohol that helps extraction of active principles and also serves as preservative for these formulations. Ashavas and arishtas are very popular in India, probably due to their taste and alcoholic content in addition to their medicinal uses and physiological importance [2].

The major plant ingredient of this formulation, Terminalia arjuna (Combretaceae) is a native plant of India and south east Asia and has been traditionally used as a cardioprotective agent. It is commonly administered as arishta, ghrita (medicated ghee) or as a powder. The usefulness of this drug in cardiovascular disorders is well documented in literature. In vitro and in vivo studies on this plant have shown positive results as far as cardiovascular disorders are concerned [3]. The stem bark of TA has been used for alleviating angina and other cardiovascular conditions [4]. The extract also improved the symptoms of refractory chronic congestive heart failure [5]. Oral administration of TA bark also prevented ischemic-reperfusion injury induced oxidative stress and tissue injury of heart in rabbits indicating its beneficial therapeutilic effect in ischemic heart disease [6]. The plant is reported to contain triterpenoids, flavonoids, glycosides and tannins etc [3]. While the flavonoids have shown antioxidant and lipid lowering effects, the glycosides are claimed to be cardiotonic. Although there are numerous studies on cardioprotective activity of stem bark powder and extracts, the studies on isolated constituents are few. Arjunic acid was shown to have protective effect on arsenic-induced myocardial injury [7]. Similarly, the effect of arjunic acid, arjungenin, arjunetin and arjunglucoside II was studied on process of respiratory oxyburst [8]. Since Arjunarishta is prepared by decoction, it is likely that non-polar constituents are not present in the formulation and the claimed cardioprotective activities might be due to polar constituents of Terminalia arjuna. There are no scientific studies yet to establish the active constituents in this formulation.

Standardization is an important aspect for establishing the quality and/or efficacy of Ayurvedic formulations or any multiple ingredient herbal formulations. Generally, two approaches being used for standardization are fingerprint analysis by HPLC/HPTLC and quantitation of individual chemical markers. It ensures reproducible pharmaceutical quality of herbal products. Preliminary analysis of the three brands of marketed Arjunarishta showed varying patterns in HPLC chromatogram suggesting that there might be differences in the process of manufacturing and therefore a proper scientific validation (chromatographic fingerprinting, quantitation of major constituents) is needed for quality control purposes [9].
The aim of the present study was to develop a standardization method for Arjunarishta by determining the marker constituents and to study comparative composition of decoction and finished formulation w.r.t. these marker constituents and to correlate these with antioxidant activity. Keeping the above facts in mind the formulation Arjunarishta was prepared strictly as per Ayurvedic Formulary of India and the major phenolic constituents present in the formulation as well as decoction were determined using RP-HPLC-PDA.

There have been few reports on analysis of phenolics in different medicinal plants, for example, five phenolics were quantified in Chinese olive Canarium album by HPLC-DAD-ESIMS. The reported method did not show clear separation of ethyl gallate from hyperin [10]. Similarly, Singh et al [11] quantified phenolic components in extracts prepared from Acacia nilotica pods by HPLC-MS/MS and studied their antioxidant activity. However, to the best of our knowledge there have been no reports on quantification and antioxidant activities of these phenolics and flavonoids in Arjunarishta. This is the first report on standardization of Arjunarishta and investigation of chemical changes occurring during manufacturing of the formulation (decoction and formulation).

Results and Discussion

Reports on cardioprotective activity and phytochemical investigation of Terminalia arjuna, the major plant constituent of Arjunarishta are numerous [3]; however there are no reports on standardization of Arjunarishta. We have attempted to standardize the formulation with respect to its phenolic constituents that may be responsible for the therapeutic action.

First, an HPLC method was developed for the formulation after several trials for separation of phenolic acids and flavonoids. A reported method for analysis of polyphenolics of Terminalia chebula fruit, the major ingredient used in this formulation, achieved the separation of the hydrolysable tannins at pH 2.5–4.5 with acetonitrile as the suitable organic modifier [12]. However, the flavonoids showed very high retention time (>75 minutes) with the reported method. In the present study, a shorter run time (45 min) was achieved with gradual increase of organic phase (acetonitrile). Five phenolic compounds were identified in Arjunarishta; these were gallic acid, ethyl gallate, ellagic acid, quercetin and kaempferol (figure 1). A binary gradient system consisting of water–acetonitrile–acetic acid as mobile phase was able to separate these compounds as shown in figure 2B. The chromatogram also showed several other unidentified peaks.

**Calibration curves and method validation**

**Tab. 1.** Linearity, LOD, LOQ, regression curves of the HPLC method

<table>
<thead>
<tr>
<th>Parameter/ Marker</th>
<th>Linearity R range (µg/ml)</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
<th>Calibration curve</th>
<th>R²</th>
<th>t_R (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>0.8–100</td>
<td>0.13</td>
<td>0.4</td>
<td>y=63432x−238517</td>
<td>0.9990</td>
<td>3.93±0.040</td>
</tr>
<tr>
<td>Ethyl gallate</td>
<td>1–100</td>
<td>0.2</td>
<td>0.66</td>
<td>y=20665x+11405</td>
<td>0.9998</td>
<td>26.85±0.30</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>5–100</td>
<td>0.5</td>
<td>1.65</td>
<td>y=15057x−43378</td>
<td>0.9997</td>
<td>29.37±0.14</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.5–75</td>
<td>0.5</td>
<td>1.65</td>
<td>y=12415x+30310</td>
<td>0.9990</td>
<td>36.82±0.17</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.8–100</td>
<td>0.13</td>
<td>0.4</td>
<td>y=10778x−14454</td>
<td>0.9990</td>
<td>40.31±0.10</td>
</tr>
</tbody>
</table>
It was observed that the other constituents present in the formulation did not interfere with any of the five markers indicating specificity of the method. For qualitative purposes, the method was evaluated by determining the precision in retention time of all five markers in a mixture of five standard samples as well as in the formulation and a low RSD value (0.22 % to 1.33 %) indicated high precision of the method. The developed RP-HPLC method used for quantification of constituents in the formulation was validated by defining the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

The method showed a linear relationship between peak areas and concentrations over given range for all five compounds (Table 1). Standard solutions of five selected compounds were prepared and analyzed in a concentration range of 0.5 to 100 µg/ml. The regression equations of these curves are shown in Table 1 and their coefficients of regression ($R^2$) were >0.999 confirming the linearity of the method. A signal three times higher than noise was regarded as the detection limit. The LOD and LOQ values for these five phenolics ranged from 0.13 to 0.5 and 0.4 to 1.6 µg/ml, respectively.

The quantitative repeatability of the injection was determined by analyzing the quantity of markers in the formulation. A high repeatability was observed with RSD values lower than 1.9 % and 1.4 % for interday and intraday assay, respectively. Accuracy (expressed as recovery) of the method was determined by analyzing the percentage recovery of marker constituents. The high recovery values (93–107 %) obtained indicated satisfactory accuracy (Table 2). Finally, the robustness of the method was studied by changing the mobile phase; minor changes in mobile phase (solvent B from 80:20 to 90:10 and 70:30) showed no effect on peak resolution.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Intraday</th>
<th>Interday</th>
<th>Spike recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>451.4±1.6</td>
<td>438.0±8.7</td>
<td>20</td>
</tr>
<tr>
<td>Ethyl gallate</td>
<td>326.0±7.5</td>
<td>330.3±9.8</td>
<td>100</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>486.6±1.5</td>
<td>484.5±4.1</td>
<td>50</td>
</tr>
<tr>
<td>Quercetin</td>
<td>12.7±0.1</td>
<td>14.6±0.2</td>
<td>25</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>69.1±1.0</td>
<td>71.9±1.0</td>
<td>50</td>
</tr>
</tbody>
</table>

**RP-HPLC analysis and Quantitation of markers in formulation and decoction**

RP-HPLC analysis of the prepared formulation showed the presence of monomeric phenolic acids, ellagitannins and flavonoids as the major constituents. Five phenolics, gallic acid, ellagic acid, ethyl gallate, quercetin and kaempferol were identified in the formulation. The amounts of these marker constituents present per ml of the formulation are given in Table 3. The chromatogram (Fig 2B and 3A) of final processed formulation
showed many additional peaks due to ellagitannins in the region ($t_R$ 18.5 to 32.0), $\lambda_{\text{max}}$ at 254 nm and shoulder peak at 220–235 nm [13]. Chromatograms of decoction (Fig 2A) and formulation (Fig 2B) differ markedly, a comparison of the two chromatograms (formulation and decoction) indicated that the amounts of gallic acid and ellagic acid increased substantially during the fermentation. This increase in concentration of monomeric phenolics can be ascribed to possible hydrolysis of ellagitannins and gallotannins during fermentation. Also, additional peaks for ethyl gallate and two flavonoids, quercetin and kaempferol were identified in the formulation. Ethyl gallate is supposed to be formed from gallic acid while two flavonoids come from *Woodfordia fruticosa* flowers that are added to the decoction to start fermentation process. The absence of flavonoids in at least two of the marketed formulations suggested that flowers of *Woodfordia fruticosa* may not have been added in specified amounts or may not have been added at all to initiate the fermentation (Table 3).

### Tab. 3. Quantitation of marker constituents in the decoction and final processed formulation *Arjunarishta*

<table>
<thead>
<tr>
<th></th>
<th>Gallic acid</th>
<th>Ethyl gallate</th>
<th>Ellagic acid</th>
<th>Kaempferol</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction</td>
<td>116.4±11.7</td>
<td>–</td>
<td>174.9±9.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Formulation</td>
<td>438.4±10.4</td>
<td>328.3±10.4</td>
<td>537.9±13.5</td>
<td>68.6±1.5</td>
<td>15.0±1.8</td>
</tr>
<tr>
<td>$P_1$</td>
<td>39.2±1.2</td>
<td>–</td>
<td>–</td>
<td>3.14±0.1</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>$P_2$</td>
<td>38.3±3.1</td>
<td>–</td>
<td>154.6±12.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$P_3$</td>
<td>32.3±0.1</td>
<td>–</td>
<td>164±6</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*mean (µg/ml)±S.D of 9 runs. $^b$ $P_{1-3}$: Marketed formulations

The hexahydroxy diphenyl moiety of ellagitannins gives them water-soluble properties resulting in their easy extraction into the formulation [14]. These tannins are known to exhibit good antioxidant and vasorelaxation activity [15]. Other monomeric phenolics like gallic acid and ethyl gallate that are enhanced in final processed formulation also exhibit high antioxidant activity. The flavonoids (4-oxoflavonoids), quercetin and kaempferol which are introduced into the formulation during fermentation are known to show membrane stabilizing properties [16, 17], vasodilation capacity (by inhibiting protein kinase C) [18] and are found to prevent deposition of low density lipoproteins which are major cause of atherosclerosis [19]. Recently, kaempferol has been found to be responsible for majority of estrogenic activity in red wine which earlier was thought to be due to trans-resveratrol, suggesting the importance of polyphenolic composition of wine [20]. Free radical scavenging property of molecules has also been found to reduce the peripheral vascular resistance thus preventing the cardiovascular disorders [21].

The regular dose of this formulation as per Ayurvedic Formulary of India is 25 ml. However the triterpenoic acids (arjunolic acid and arjunic acid) could not be detected by HPLC method reported for these compounds [22] even in 25 ml of formulation. As *Arjunarishta* is prepared by decoction, it is likely that these non-polar components are not extracted into water. The results obtained indicate that the cardioprotective activity of *Arjunarishta* might be due to flavonoids and other phenolics that should therefore be regarded as index for quality assessment of *Arjunarishta*. 
Fig. 1. Chemical structures of major phenolic constituents present in formulation

Gallic acid, R=H  
Ethyl gallate, R=C₂H₅  
Ellagic acid  
Kaempferol, R=H  
Quercetin, R=OH

Fig. 2. Chromatogram of A) Decoction; B) Arjunarishta at 280 nm. 
**Antioxidant activity**

The prepared formulation showed good antioxidant activity (lipid peroxidation) and free radical scavenging activity (DPPH assay) (Table 4). There is almost 50% increase in free radical scavenging activity of the final prepared formulation compared to decoction. There is no substantial increment in the lipid peroxidation activity (TBA and FTC) in the final processed formulation as compared to the decoction. The results obtained from FTC and TBA assays indicate that monomeric phenolics, gallic acid and ethyl gallate showed higher lipid peroxidation activity than ellagic acid. Decreased lipid peroxidation activity of ellagic acid is due to presence of additional fused catechol structure with adjacent carbonyl group [23]. Quercetin showed slightly higher lipid peroxidation activity than kaempferol that could be ascribed to presence of additional hydroxyl group (Table 4). Further, increase in free radical scavenging activity of the formulation compared to decoction may be attributed to increase in monomeric phenolics during fermentation, and the constituents introduced through addition of *Woodfordia fruticosa* and Jaggery. The antioxidant activity of jaggery is documented [24]. Total percentage of triterpenoic acids determined by colorimetric method [25] was negligible again suggesting that the activity is due to phenolics and water soluble constituents.

**Tab. 4.** Antioxidant activity of major constituents of *Arjunarishta*

<table>
<thead>
<tr>
<th></th>
<th>DPPH µM, EC\textsubscript{50}</th>
<th>FTC % inhibition</th>
<th>TBA % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decoction\textsuperscript{a}</td>
<td>0.27</td>
<td>56.3</td>
<td>60.3</td>
</tr>
<tr>
<td>Formulation\textsuperscript{a}</td>
<td>0.16</td>
<td>59.3</td>
<td>65.3</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.23</td>
<td>69.5</td>
<td>71.5</td>
</tr>
<tr>
<td>Ethyl gallate</td>
<td>0.26</td>
<td>69.3</td>
<td>72.5</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>0.23</td>
<td>35.0</td>
<td>38.0</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>1.89</td>
<td>60.0</td>
<td>53.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.36</td>
<td>71.0</td>
<td>65.0</td>
</tr>
<tr>
<td>Trolox</td>
<td>0.19</td>
<td>76.7</td>
<td>72.0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.12</td>
<td>62.7</td>
<td>53.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} results are expressed as µl/µM for formulation and decoction

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Fig. 3. Chromatogram of *Arjunarishta* at 360 nm showing ellagitannins (EA) and their UV-spectrum.
Experimental

Reagents
DPPH (1,1-Diphenyl-2-picrylhydrazyl), linoleic acid, trolox, ascorbic acid were procured from Aldrich Chemicals; ferric chloride, trichloroacetic acid, thiobarbituric acid, ammonium thiocynate were obtained from CDH chemicals, India. Triterpenoic acids (arjunic acid and arjunic acid) were isolated from stem bark of Terminalia arjuna and their structures were confirmed by comparison with the data in literature [26, 27]. Gallic acid, ethyl gallate, ellagic acid, quercetin, kaempferol were isolated and characterized as per literature [28, 29]. Three popular brands of Arjunarishta were purchased from the local market.

Samples
The formulation ingredients were accurately weighed and powdered (Terminalia arjuna, bark powder; 2040 g) was passed through # 22 and Woodfrodia fruticosa (flowers, 408 g) were passed through # 60. Other ingredients (Vitis vinifera, fruits; 1020 g and Madhuca indica, flowers; 408 g) were weighed and the decoction was prepared by boiling listed plant ingredients in water till the volume was reduced to one fourth. Jaggery and Woodfordia fruticosa were added to the decoction taken in the wooden vat (teak wood) and the mixture was kept at room temperature 25 ± 2° C for one and half month for fermentation [1].

HPLC System and conditions
A Shimadzu HPLC system (Japan), consisting LC-10AT pump, automated gradient controller, Shimadzu SPD-M10 A (Class VP-series, version 6.10), Rheodyne 7725 I manual injector (CA, USA) and phenomenex C-18 column (250 x 4.6 µm ID, 5 µm with a compatible guard column was used. The mobile phase was filtered through a 0.45 µm-filter membrane, and was degassed prior to use.

Sample preparation for HPLC analysis
1 ml of sample (formulation or decoction) was dried on rotavapor for half an hr to remove the alcohol. 5 ml of methanol was added to it and sonicated for 10 minutes and centrifuged at 3000 rpm to settle down the precipitated sugars. 1 ml of supernatant was passed through 0.45 µm filter (Millipore) and 20 µl was injected for quantification.

Analysis of phenolics
A binary gradient with mobile phase containing: (solvent A) water-acetic acid (0.5 % v/v) and (solvent B) acetonitrile: water-acetic acid (95.5-0.5) = 80: 20 (v/v). The flow-rate was maintained constant at 1.5 ml/min and the solvent gradient elution program was as follows: 0–10 min, 90 % A, 10% B; 10–20 min, 90-80 % A, 10-20 % B; 20–30 min, 80-60 % A, 20-40 % B; 30–40 min, 60-40 % A, 40-60% B; 40–45 min, 40-30 % A, 60-70 % B; 45-50 min, 30-90 % A, 70-10% B.

Free radical scavenging experiment
DPPH assay
DPPH was dissolved in methanol (40 µg per ml). 50 µl of the (formulation/decoction) was diluted with methanol (4 ml) and centrifuged. An aliquot of 10, 20, 30, 40, 50 µl of the supernatant solution were added to DPPH solution. After mixing gently and leaving for 30
minutes at room temperature the absorbance was measured at 520 nm using UV-vis spectrophotometer. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 520 nm, which reflects the amount of DPPH radical remaining in the solution. The scavenging activity was expressed as the EC\textsubscript{50}, the concentration of samples required for scavenging 50% of DPPH radical in the solution. The free radical scavenging capacity of each sample was determined by comparing its absorbance with that of blank solution [30].

**Ferric Thiocyanate Method (FTC)**

Dilution was performed as above and 50 µl of the centrifuged solution was added to the mixture of 680 µl of 2.51 % of linoleic acid solution in ethanol and 2.25 mL of 40 mM phosphate buffer (pH 7.0). The mixture was incubated at 40° C in constant temperature oven. After every 24 h during incubation for 5 days a 20 µl aliquot was taken from the mixture and diluted with 2 ml of ethanol followed by addition of 20 µl of 30 % ammonium thiocyanate and 20 µl of 20 mM FeCl\textsubscript{2} in 3.5 % HCl, and exactly after 5 min the absorbance was measured at 500 nm. The level of lipid per oxidation inhibition by each sample was calculated from absorbance ratio to that of control without any sample [31].

**Thiobarbituric acid test (TBA)**

The samples prepared for FTC method were used for this assay. After incubation for 5 days, 250 µl of sample solution was taken from the mixture and 500 µl of 10 % aqueous trichloroacetic acid and 500 µl of 0.8 % aqueous thiobarbituric acid solution were added. The mixture was placed in boiling water bath for 10 min. After cooling it was centrifuged for 3000 rpm for 30 min and the absorbance of supernatant was measured at 532 nm [32].

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**Authors’ Statement**

**Competing Interests**

The authors declare no conflict of interest.

**References**


