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Development and Validation of Improved RP-HPLC method for Identification and Estimation of Ellagic and Gallic acid in Triphala churna

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Abstract: Triphala is an anti-oxidant-rich herbal formulation containing fruits of *Phyllanthus emblica*, *Terminalia belerica* and *Terminalia chebula* in equal proportions. The preparation is frequently used in Ayurvedic medicine to treat diseases such as constipation, jaundice, anemia, asthma, fever, chronic ulcers and many more. A simple reverse phase high-performance liquid chromatography (RP-HPLC) method for the separation and quantitative determination of the Gallic acid and Ellagic acid from Triphala has been developed and validated. The use of an RP18 column with a gradient acidic mobile phase enabled the efficient separation of gallic acid and ellagic acid within a 30 min analysis. Validation of the method was performed in order to demonstrate its selectivity, linearity, precision, accuracy and robustness. The proposed RP-HPLC method was found to be simple, precise and accurate and can be used for the quality control of the raw materials as well as formulations.
Key-words: Triphala, Ellagic acid, Gallic acid, RP-HPLC, Method Validation.

Introduction

Herbal medicines are in great demand in the developed as well as developing countries for primary healthcare because of their wide biological activities, higher safety margins and lesser costs. Herbal medicines has been enjoying renaissance among the customers throughout the world. In India, the herbal drug market is about $ one billion and the export of plant based crude drugs is around $ 80 million[1].

However, one of the impediments in the acceptance of the Ayurvedic or Herbal formulations is the lack of standard quality control profiles[2]. The quality control of herbal medicine that is, the profile of the constituents in the final product has implications in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques like HPLC and HPTLC are expected to help in circumventing this problem.

Triphala is an antioxidant-rich herbal formulation, frequently used in Ayurvedic medicine to treat diseases such as constipation, anemia, jaundice, asthma, fever, chronic ulcers and many more. The word Triphala means a mixture of three fruits and thus the preparation is a composite mixture of fruits of three medicinal herbs, Amla (*Phyllanthus emblica* Linn.), Baheda (*Terminalia belerica* Roxb., Fam. Combretaceae) and Harde (*Terminalia chebula* Retz., Fam. Combretaceae), in equal proportions. It is an important medicine of the 'Rasayana' group of Ayurveda and is believed to promote health, immunity and longevity[3]. Triphala churna is an herbal formulation used extensively in Ayurveda system of medicine as a rasayana. Since it contains enormous amount of tannins such as Ellagic acid and Gallic acid,

it is required to maintain their quality and purity for safety and efficacy[4].

Hence, in the present paper we report the development of an optimized, validated and simple HPLC method for the standardization of Triphala using polyphenols as markers and attempt was made to compare and evaluate the authentic laboratory triphala formulation (L) with the commercial marketed formulations of triphala (M1, M2 & M3) of different reputed companies also with the raw material evaluation by using modern analytical techniques such as HPLC.

Materials and Method

Materials:

Triphala and its individual components were procured from a local market in Ahmedabad city, Gujarat and authenticated by comparison with herbarium specimens[5-7]. Standard Gallic acid was procured from Tetrahedron Ltd., Chennai, India and standard Ellagic acid was from Yucca Enterprises Ltd., Mumbai. HPLC grade acetonitrile, O-Phosphoric acid and water were obtained from Merck (Darmstadt, Germany).

Instrumentation:

Analysis were performed on a HPLC of Shimadzu LC-20AD model equipped with an online degasser DGU-20 As, a Rheodyne 7725 injection valve furnished with 20 µl loop, a SPD-M20A photodiode array detector and a Class-VP software. Separation was carried out using a Phenomenax column (250 × 4.6 mm i.d., 5 µm pore size). The column was maintained at 27^{o} C throughout the analysis and detection was carried at 254 nm.

Calibration curve for Ellagic acid and Gallic acid:

The content of the markers were determined using a calibration curve established with six dilutions of each standard, at concentrations ranging from 10 to 100 µg/ml. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the markers injected. Peak identification was achieved by comparison of both the retention time (Rt) and UV absorption spectrum for standards.

Sample preparation:

A 100 mg amount of powder from Triphala churna (L, M1-M3) and 100 mg of its three ingredients, Amla, Baheda and Harde, were extracted three times with 100 ml methanol. The extract were combined and concentrated at reduce temperature (50^{o} C) on Rotary evaporator (Equitron rotevar, Medica instrument mfg. co.) upto 100 ml. Prior to use, all samples were filtered through a 0.45 µm nylon membrane filter.

Validation parameters:

The method was validated according to ICH guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification[8].

Selectivity was checked using an extract of Triphala churna and a mixture of standards in order to optimize separation and detection. Linearity of the method was performed by analyzing a standard solution of markers by the proposed method in the concentration range 10-100 µg/ml. The accuracy of the proposed method was determined by a recovery study, while was carried out by adding standard markers in the Triphala churna extract. The samples were spiked with three different amounts of standard compounds prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used as the actual values in order to calculate the spike recoveries. Precision was determined by repeatability and interday & intraday reproducibility experiments of the proposed method. A standard solution containing two markers was injected three times; Triphala churna was also extracted three times to evaluate the repeatability of the extraction process. The mean amounts and SD value of each constitute were calculated. The LOD and LOQ of marker compounds were calculated at signal-to-noise ratio of approximate 3:1 and 10:1 respectively.

Result and Discussions

Optimization of HPLC chromatographic conditions:

Optimum chromatographic conditions were obtained after running different mobile phase with a reverse phase C18 column. Acetonitrile was preferred over methanol as mobile phase because its use resulted in improved separation. Many different gradient systems of mobile phase were tried for the best separation of peaks. Selecting 254 nm as the detection wavelength resulted in an acceptable responses and enable the detection of compounds used in this study. The column was used at 27^{o}C. An HPLC fingerprint for the Triphala churna was developed. Elution was carried out at a flow rate of 0.8 ml/min with acetonitrile as solvent A and O-Phosphoric acid in Water (0.3%) as solvent B using gradients elution in 0-5 min with 90-88 % B, 5-6 min with 88-86 % B, 6-9.5 min with 86-80 % B, 9.5-10.5 min with 80-79 % B, 10.5-12 min with 79-78 % B, 12-22 min with 78-76 % B and 22-30 min with 76-90% B. Each run was followed by a 10 min wash with 10 % acetonitrile & 90 % O-Phosphoric acid in Water (0.3%) and an equilibration period of 15 min.

Quantification of markers present in Triphala churna:

The content of the active compounds were quantified using calibration curve (Figure 1, 2) of each markers individually (Figure 3, 4). The two markers used for quantification were found and well separated by proposed method in Triphala churna (Figure 5, 6). The

chromatogram of Triphala churna was quantified with respect to Ellagic acid and Gallic acid. The chromatograms of the individual components of Triphala churna were also quantified (Figure 7, 8, 9) with respect to the standards. The results obtained are shown in (Table 1).

Method validation for HPLC fingerprinting:

The HPLC method was validated by defining the selectivity, linearity, accuracy, precision, limits of detection and limit of quantification. For qualitative purposes, the method was evaluated by taking into account the precision in the retention time and selectivity of marker compounds eluted. A high repeatability in the retention time was obtained both for standards and extracts even at high concentration. For quantitative purpose linearity, accuracy, precision, LOD and LOQ were evaluated. LOD and LOQ values for Ellagic acid 0.42µg/ml and 1.9µg/ml and for Gallic acid 0.66µg/ml and 2.4µg/ml respectively. Linear correlation was obtained between peak area and concentration of two markers in the range of 10-100 µg/ml. Values of the regression coefficients (r^2) of the markers were higher than 0.99, thus confirming the linearity of the methods (Table 2). The calibration curve was drawn for concentration v/s AUC for Ellagic acid (Figure 1) and for Gallic acid (Figure 2). The high recovery values (94.60-101.4%) indicated a satisfactory accuracy (Table 3). Relative standard deviation of all the parameter was less than 3.5% for the degree of repeatability, indicating the high repeatability of the proposed method. The low coefficient of variation values of intraday and interday precision reveals that the proposed method is precise (Table 4). Therefore, this HPLC method can be regarded as selective, accurate and precise for the quantification of Ellagic acid and Gallic acid of triphala.

Table 1: Quantification of Ellagic acid and Gallic acid in Triphala churna and its ingredients by HPLC

Sample	Amount	
	Ellagic acid[a] (% w/w)	Gallic acid[a] (% w/w)
Laboratory sample	2.17 ± 0.02	1.06 ± 0.04
Market sample - 1	1.97 ± 0.05	0.91 ± 0.03
Market sample - 2	2.04 ± 0.02	1.03 ± 0.02
Market sample - 3	2.00 ± 0.04	1.00 ± 0.01
Amla	1.77 ± 0.02	0.87 ± 0.04
Baheda	2.09 ± 0.06	0.93 ± 0.05
Harde	2.24 ± 0.02	1.05 ± 0.02

[a] Mean ± SD (n=3)

Table 2: Regression parameter, Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) of the proposed HPLC method

Compound	Conc. range (µg/ml)	R_t (min) [a]	Regression equation	R^2	LOD	LOQ
Ellagic acid	10-100	20.44 ± 0.03	y = 18632x + 60268	0.996	0.42	1.9
Gallic acid	10-100	5.29 ± 0.04	y = 27132x + 14337	0.997	0.66	2.4

[a] Mean ± SD (n=3)

Table 3: Repeatability and Recovery tests for the markers (Ellagic acid and Gallic acid) in Triphala churna

Compound	Contents[a] (mg/g)	Added amt. (mg)	Recorded amt[a] (mg)	Recovery rate[a] (%)	RSD (%)
Ellagic acid	21.7 ± 0.02	10	31.42 ± 0.52	97.20 ± 1.08	1.65
		20	41.96 ± 0.47	101.3 ± 2.30	1.12
		30	51.68 ± 1.10	99.93 ± 0.71	2.12
Gallic acid	10.4 ± 0.04	5	15.13 ± 0.36	94.60 ± 2.04	2.37
		10	20.35 ± 0.51	99.50 ± 0.71	2.50
		15	25.61 ± 0.59	101.4 ± 1.55	2.30

[a] Mean ± SD (n=3)

Table 4: Precision of the Intra-day and Inter-day HPLC measurement for marker compounds in Triphala churna

Compound	Intra-day[b]		Inter-day[c]	
	Contents[a] (% w/w)	RSD (%)	Contents[a] (% w/w)	RSD (%)
Ellagic acid	2.17 ± 0.02	0.92	2.16 ± 0.02	0.93
Gallic acid	1.04 ± 0.02	1.92	1.06 ± 0.02	1.88

[a] Mean ± SD (n=3)
[b] Sample were analyzed three times a day
[c] Sample were analyzed once a day over three consecutive days

Figure 1: Calibration curve for Ellagic acid by HPLC

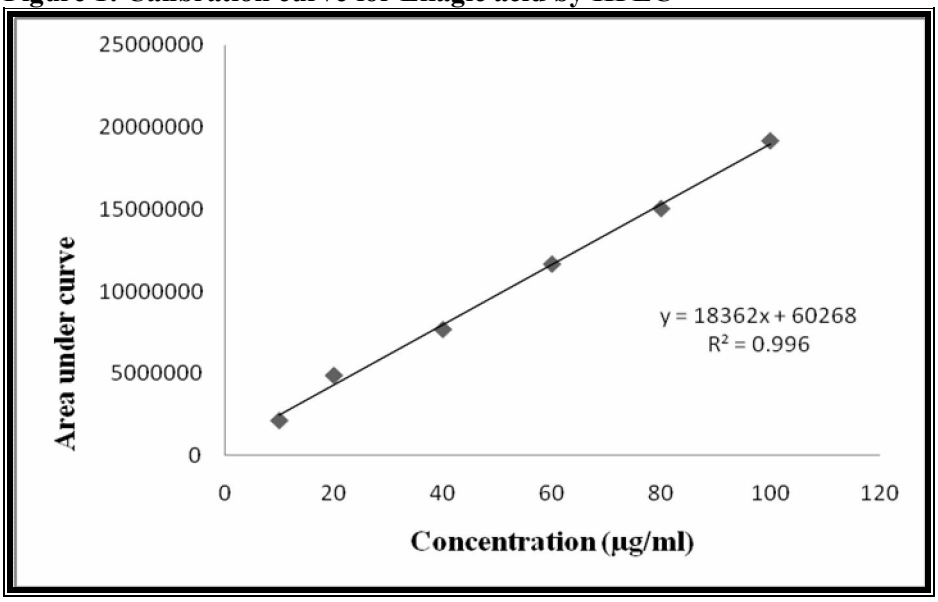


Figure 2: Calibration curve for Gallic acid by HPLC

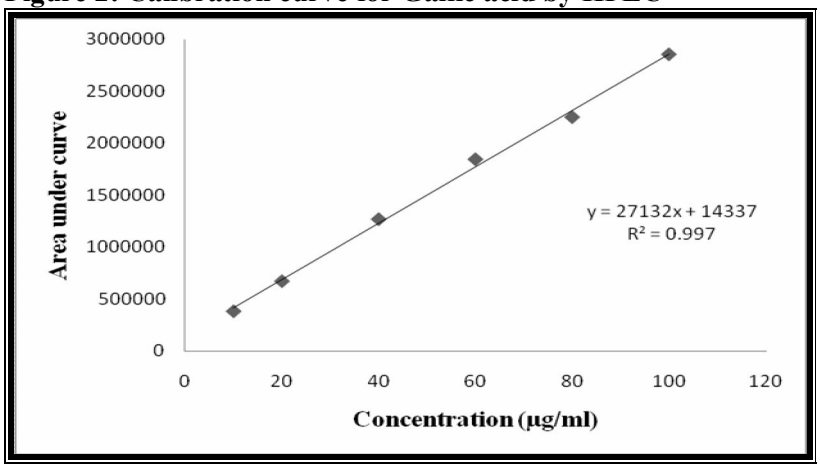


Figure 3: HPLC chromatogram for standard Ellagic acid

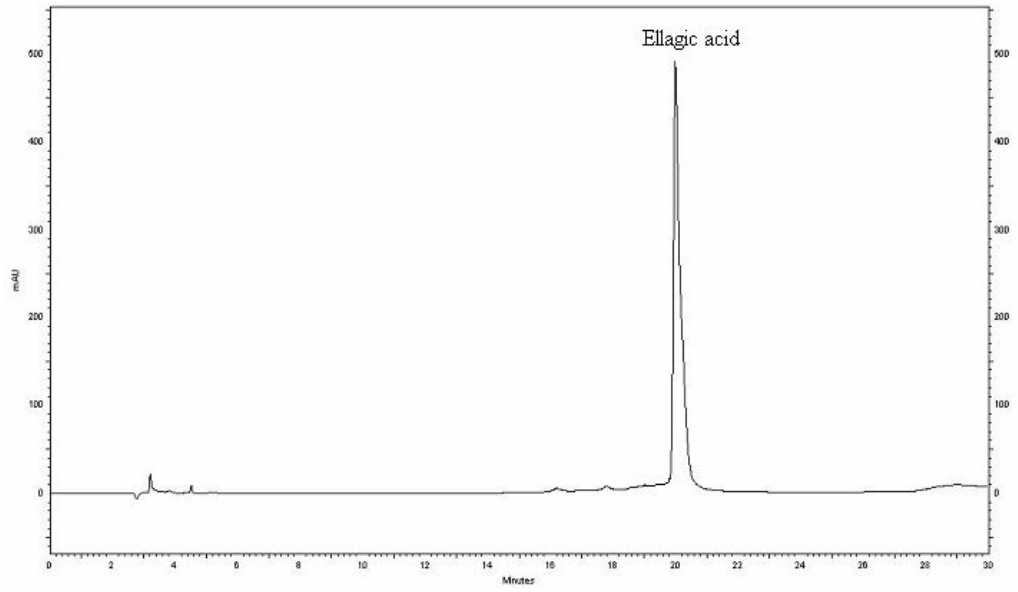


Figure 4: HPLC chromatogram for standard Gallic acid

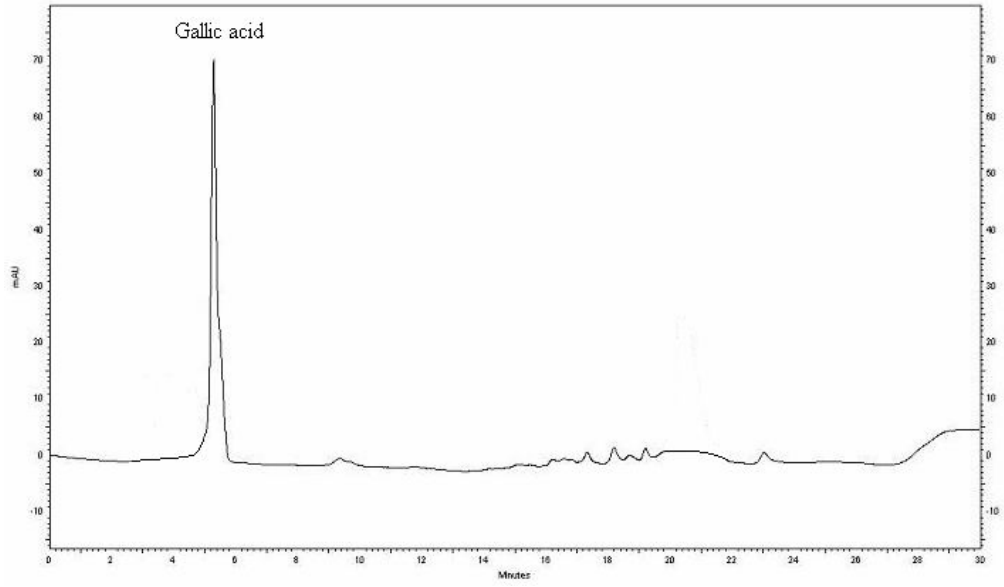


Figure 5: HPLC chromatogram of laboratory Triphala sample

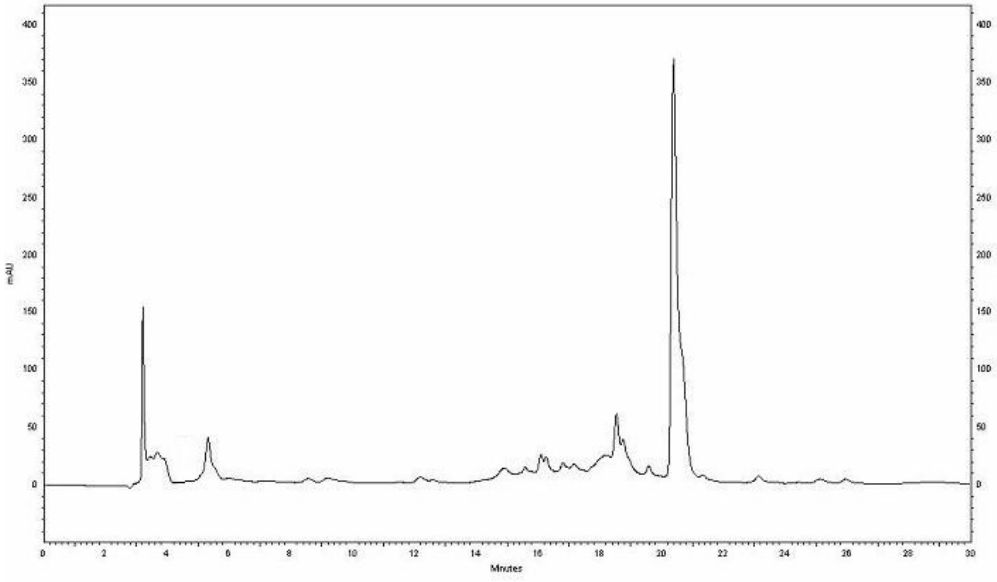


Figure 6: HPLC chromatogram of market Triphala sample.

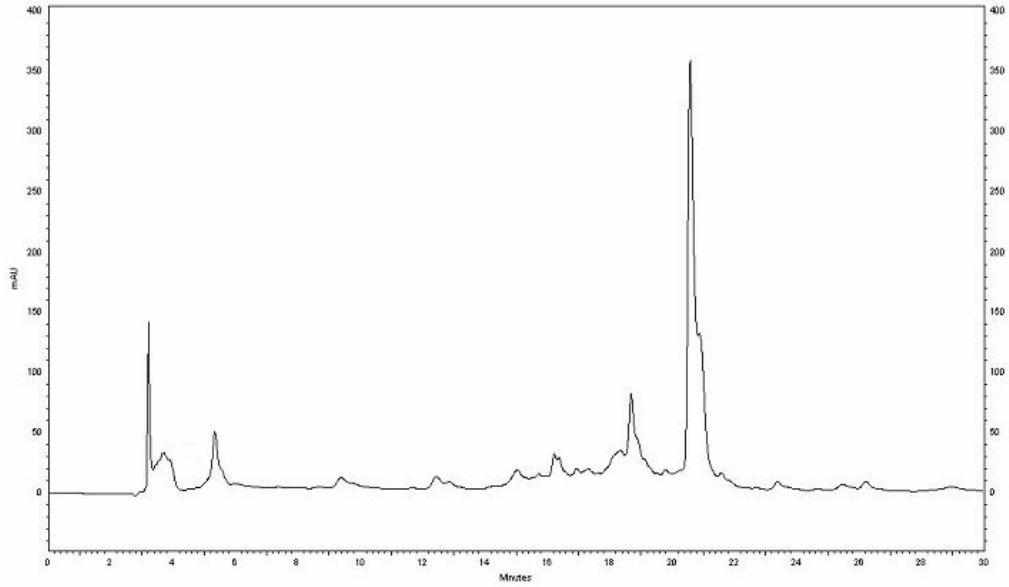


Figure 7: HPLC chromatogram of *Phyllanthus emblica.*

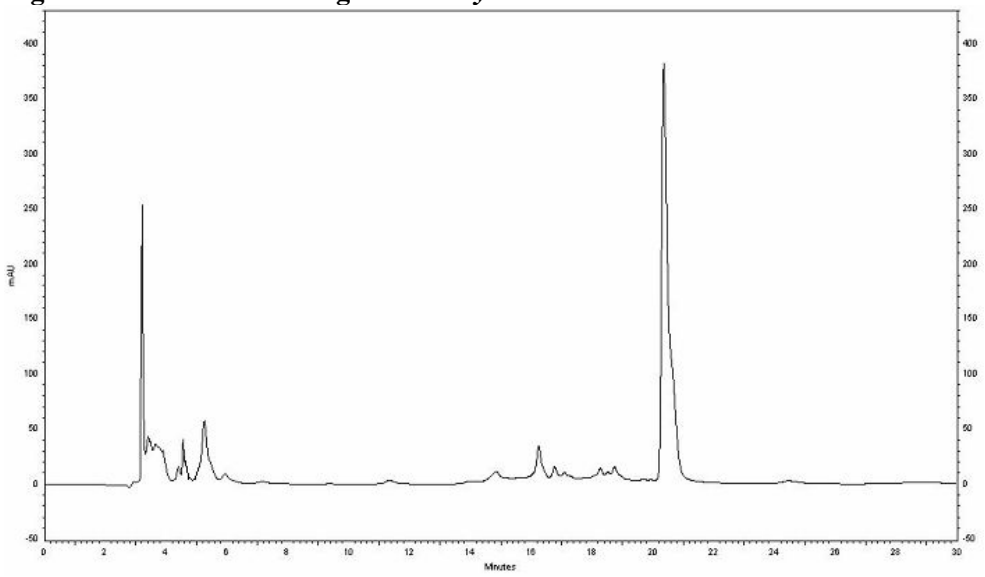


Figure 8: HPLC chromatogram of *Terminalia belerica.*

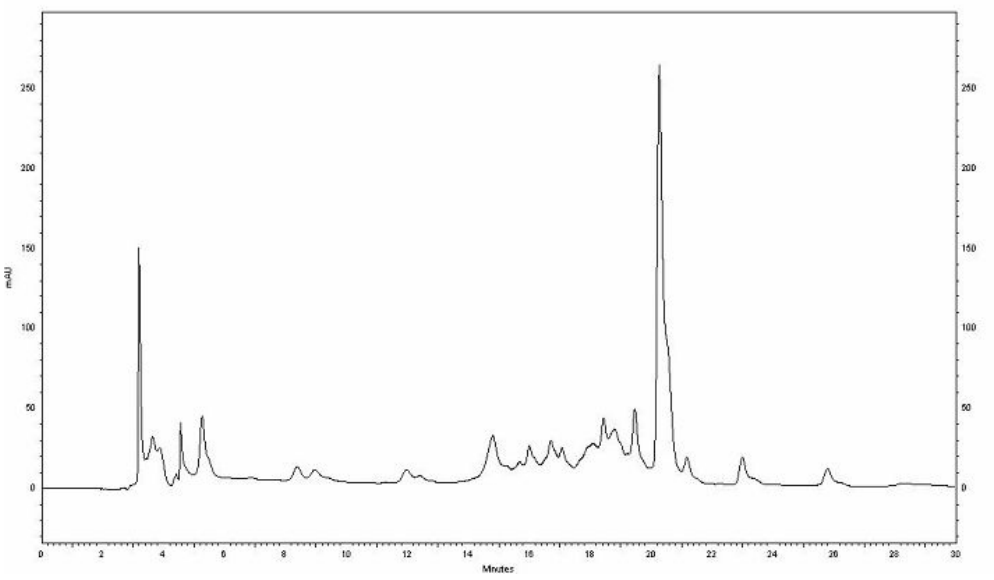
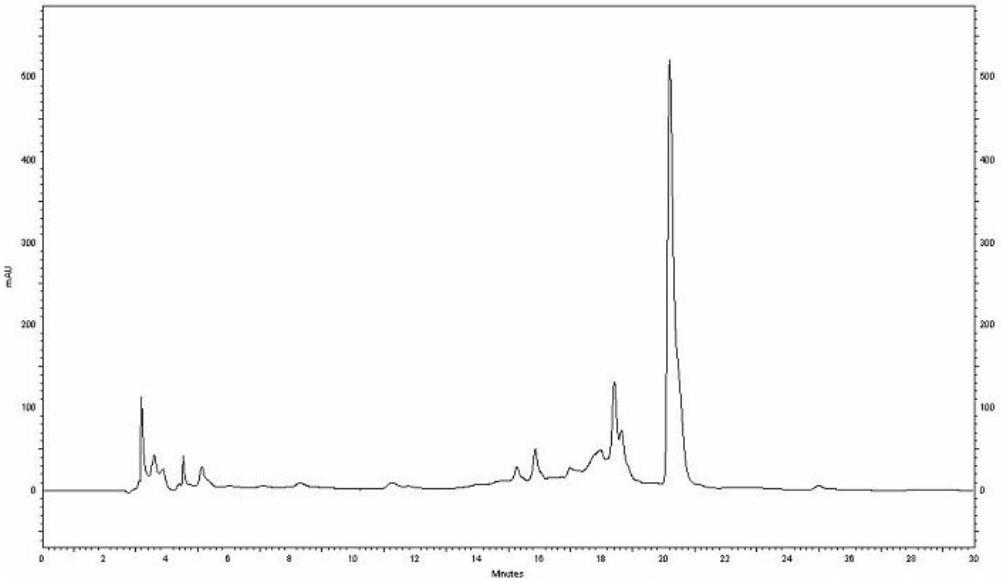


Figure 9: HPLC chromatogram of *Terminalia chebula.*



Conclusion

Standardization of Triphala churna as well as raw materials is important as per analysis of formulation is concerned. The results indicate that Triphala churna contains a number of markers that may be responsible for its therapeutic activity. The developed RP-HPLC method will assist in the standardization of Triphala churna using biologically active chemical markers. The marker content of laboratory Triphala churna was found higher than that of market samples of Triphala churna by HPLC method, may be due to use of inferior quality of materials in the preparation of churna. The proposed HPLC methods for simultaneous estimation of Gallic acid and Ellagic acid from Triphala churna seems to be accurate, precise, reproducible and repeatable. Triphala churna also contained a number of other constitute, which are currently the subject of further investigation, apart from those standards studied. Also profiles of the individual components in Triphala churna have been recorded as a standardization tool. With the growing demand for herbal drugs and with increased belief in the usage of herbal medicine, this standardization tool will help in maintaining the quality and batch to batch consistency of this important Ayurvedic preparation.

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