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Development and Validation of HPLC Method for Quantification of Phytoconstituents in *Haritaki Churna*

Anil D. Mahajan*, Nandini R. Pai

Department of Chemistry, D.G.Ruparel College, Mahim, Mumbai - 400 016, India.

Corres. author: anilmahajan243@gmail.com
Cell No : +919920243614

Abstract: A high performance liquid chromatography method coupled with diode array detection was developed to simultaneously determine seven different marker compounds in *Haritaki churna,* an ayurvedic formulation. These markers are gallic acid (1), methyl gallate (2), ethyl gallate (3), ellagic acid (4), chebulagic acid (5), chebulinic acid (6) penta-*O*-galloyl-*β*-D-glucose (7). HPLC analysis was carried out at wavelength 272nm. The developed method was able to determine the marker compounds with excellent resolution, precision and recovery. The chromatographic separation was performed on Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (100mm×4.6mm, 3µm). The mobile phase was consisted of 0.02% triethyl amine aqueous pH 3.0 with ortho-phosphoric acid (A) and acetonitrile (B) at a flow rate of 1.0 ml/min gradient mode. Regression equations showed good linear relationships ($R^2 > 0.998$) between the peak area of each marker and concentration. The assay was reproducible with overall intra- and inter-day variation of less than 3.4%. The recoveries, measured at three concentration levels, varied from 97.8% to 101.1%. The method was applied to determine the amounts of the marker compounds in three different commercial market samples, and significant variations in phytoconstituents were observed.
Keywords: Haritaki churna, RP-HPLC, Method validation, Chebulagic acid,Chebulinic acid,Gallic acid.

Introduction

Medicinal plants are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for synthesis of drugs or as models for pharmacologically active compound.[1]The dried fruit of *Terminalia chebula* is regarded as the "king of medicines" and has been used by Tibetans, Chinese. Indian and many Asian and African countries and is also held in high esteem by other folk medicinal practitioners.[2] *Terminalia chebula* also popularly known as *Haritaki* in Sanskrit named for god *Shiva* (*hara*), who brings fearlessness in the face of death and disease. *Haritaki* enjoys the prime place among medicinal herbs in India since ancient times. *Vagbhata* has praised it to be the drug of choice in the therapy of vata-kapha diseases and one of its famous preparations – Triphala containing equal proportions of *Haritaki* , *Vibhitaka* (*Terminalia bellerica*) and *Amalaki* (*Emblica officinalis*).[3] It is also used in powder form as *Haritaki churna*. In *T. chebula*, 33% of the total phytoconstituents are hydrolysable tannins (which may vary from 20-50%) and are responsible for pharmacological activity. These tannins contain phenolic carboxylic acid like gallic acid, ellagic acid, chebulic acid and gallotannins such as 1,6 di-*O*-galloyl-β-D-glucose, 3,4,6 tri-*O*-galloyl-β-D-glucose, 2,3,4,6 tetra-*O*-galloyl-β-D-glucose, 1,2,3,4,6 penta-*O*-galloyl-β-D-glucose. Ellagitannin such as punacalagin, casurarinin, corilagin and terchebulin and others such as chebulanin, neochebulinic acid, chebulagic acid and chebulinic acid reported in literature.[4] *T.chebula* reported as one of the potent antioxidant and a probable radioprotector.[5] It is also reported to exhibit

variety of biological activity including, anticancer,[2] antidiabetic,[6] antimutagenic,[7] antibacterial,[8] purgative[9] etc.

Despite of many reports on the pharmacological activity of phytoconstituents only limited number of papers have been published on method development and validation of *T.chebula* or its formulation *Haritaki churna* using gallic acid and ellagic acid as a marker.[10-12] In general, one or two markers or pharmacologically active components in herbs and or herbal mixtures were currently employed for evaluating the quality and authenticity of herbal medicines, in the identification of the single herb or herbal preparations, and in assessing the quantitative herbal composition of an herbal formulation. This kind of the determination, however, does not give a complete picture of herbal formulation, because multiple constituents are usually responsible for its therapeutic effects. Recently determination of hydrolysable tannins in the fruits of *T Chebula* by HPLC and capillary electrophoresis was reported[4]. But there is no report on method development and validation of Haritaki churana and comparative study of commercial marketed formulation using phytoconstituents present which are responsible for therapeutic activities. The present manuscript describes a simple, rapid, precise and accurate gradient reversed-phase method for the simultaneous determination of seven phytoconstituents in *Haritaki churna*.

Materials and Methods

Chemicals and materials:

The chemicals used were analytical or HPLC-grade. HPLC-grade acetonitrile, ortho-phosphoric acid (AR grade) and triethyl amine (AR grade) were purchased from Merck specialty India Pvt. Ltd. Ultra pure water, generated by use of a Milli-Q System (Millipore), was used for sample preparation and preparation of mobile phases for HPLC analysis. Dried fruits of *Terminalia chebula Retizus* (Combrataceae) was procured from local market in Mumbai, India and authenticated at Agharkar Research Institute Pune, India, with a voucher specimen (F-138) which deposited in the herbarium. Standard compounds gallic acid (1) and ellagic acid (4) were purchased from Sigma Aldrich. Other standard compounds such as methyl gallate (2), ethyl gallate (3), chebulagic acid (5), chebulinic acid (6) and penta-*O*-galloyl-*β*-D-glucose (7) were isolated from the dried fruit of the *Terminalia chebula* Retz. (Combrataceae).

Sample preparation

The samples were passed through a 60-mesh sieve. The accurately weighed powder (0.2 g) was transferred in 50ml volumetric flask contains 30ml extraction solvent (methanol–water (70:30, v/v) and sonicated for 20 min at 27±3°C in ultra sonicator water bath and diluted up to mark. The solution was filtered through a 0.45-μm membrane prior to injection into the HPLC system.

Chromatographic condition

HPLC Analysis was performed on a Waters chromatographic system consisting Waters 2695 separation module (quaternary pump) equipped with an auto injector and Waters 2998 photodiode array detector. Data acquisition was made with Waters Empower Pro software. Separation was achieved on Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (100mm×4.6mm, 3μm). The mobile phase was consisted of 0.02% triethyl amine aqueous pH 3.0 with ortho-phosphoric acid (A) and acetonitrile (B). The gradient program was as follows: 0–15 min, linear gradient 10–33% B; 15–20 min, linear 33–33% B; 20–21 min linear gradient 33–10% B; 22–27 min linear gradient 10–10% B. The flow rate was 1.0 ml/min and aliquots of 10μl were injected. The UV detection wavelength was set at 272 nm. Absorption spectra of compounds were recorded between 200 and 400 nm. The compounds were identified by comparing their retention times and UV spectra with those of the markers.

Results and discussion

Extraction procedure

Various extraction methods, solvents and times were evaluated to obtain the best extraction efficiency. The results revealed that ultrasonic bath extraction was better than other extraction methods, so the further experiments were carried out with ultrasonic bath extraction. Various solvents including water, methanol–water (50:50 v/v; 60:40 v/v; 70:30 v/v; 80:20 v/v) and methanol were screened. Methanol–water (70:30 v/v) exhibited complete extraction of all the major constituents. No second extraction step was found necessary.

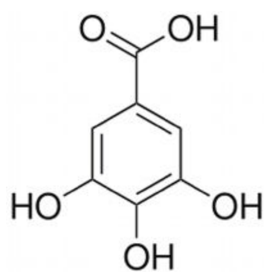


Fig.1.1 Gallic acid

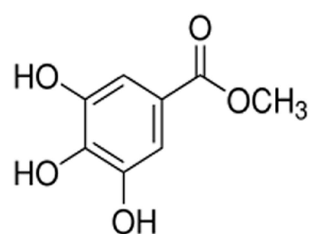


Fig.1.2 Methyl gallate

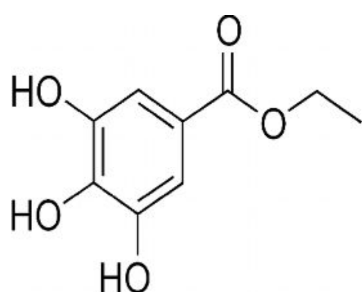


Fig.1.3 Ethyl gallate

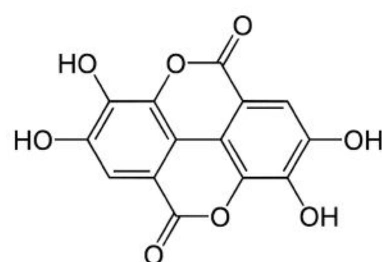


Fig.1.4 Ellagic acid

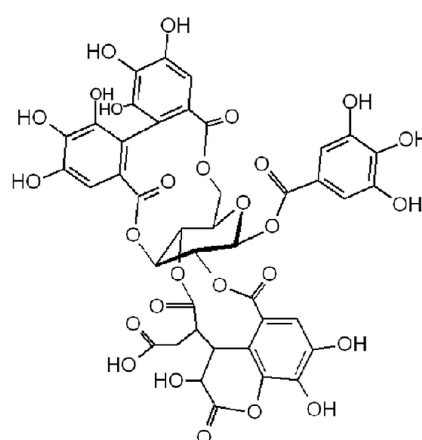


Fig.1.5 Chebulagic acid

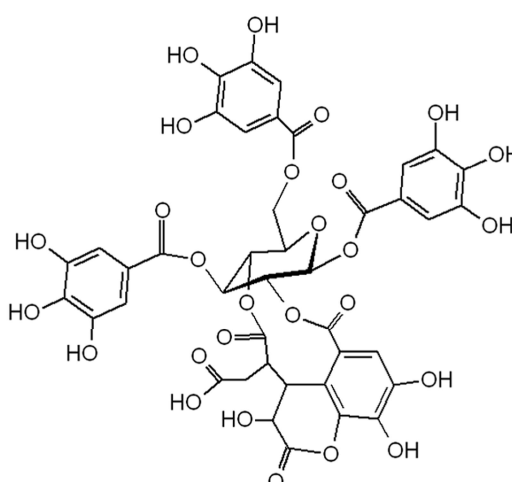


Fig.1.6 Chebulinic acid

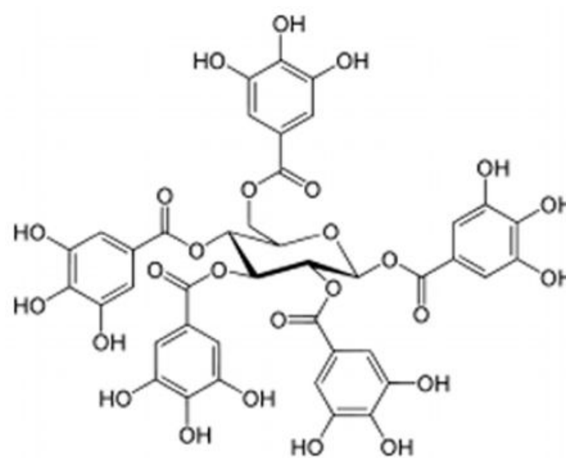


Fig.1.7 Penta-*O*-galloyl-*β*-D-glucose

Optimization of chromatographic conditions

During the optimization of method different columns of different selectivity like C18, Cyno and Phenyl columns were tried. When acetonitrile used as organic modifier achieved better separation with short retention time and sharp peak shape than methanol. After a series of screening experiments, it was concluded that 0.02% triethyl amine aqueous pH 3.0 with ortho-phosphoric acid and acetonitrile in gradient mode gave better separation with good resolution of adjacent peaks with acceptable tailing factors. The chromatographic separation was achieved on a Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (100mm×4.6mm, 3µm) The mobile phase was consisted of 0.02% triethyl amine aqueous pH 3.0 with ortho-phosphoric acid (A) and acetonitrile (B) in the gradient mode to keep short run time 27 min. As the maximum types of phytoconstituents show UV maxima between 270-278nm UV detector was set at 272nm to provide sufficient sensitivity for each analyte.

Fig.2.(a) standard mixture: gallic acid (1), methyl gallate (2), ethyl gallate (3), ellagic acid (4), chebulagic acid (5), chebulinic acid (6), penta-*O*-galloyl-*β*-D-glucose (7).

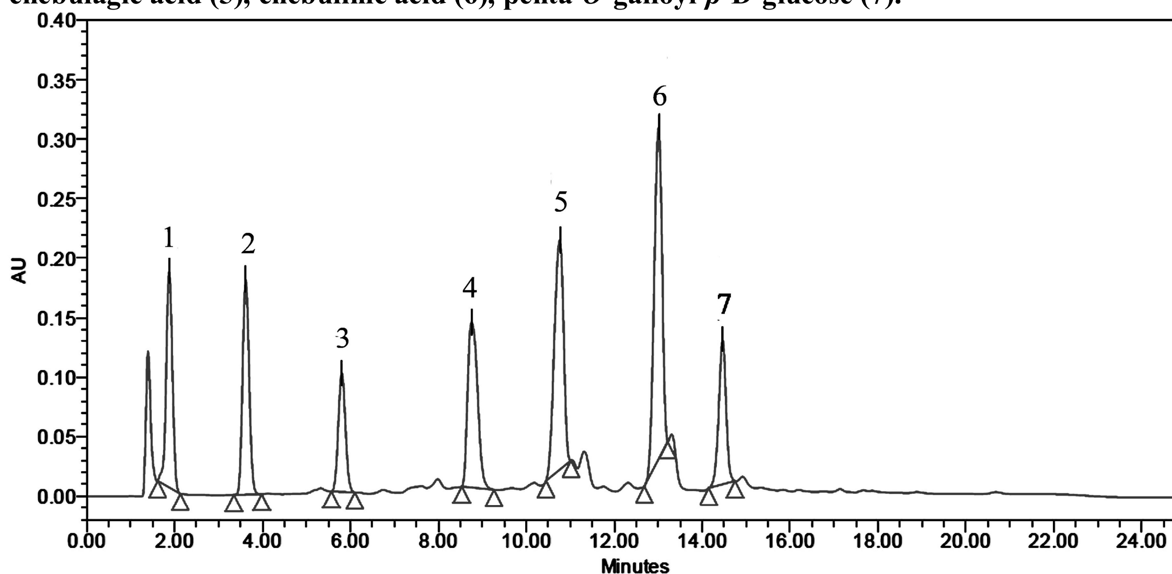


Fig.2.(b) Haritaki churna 1 (HC1)

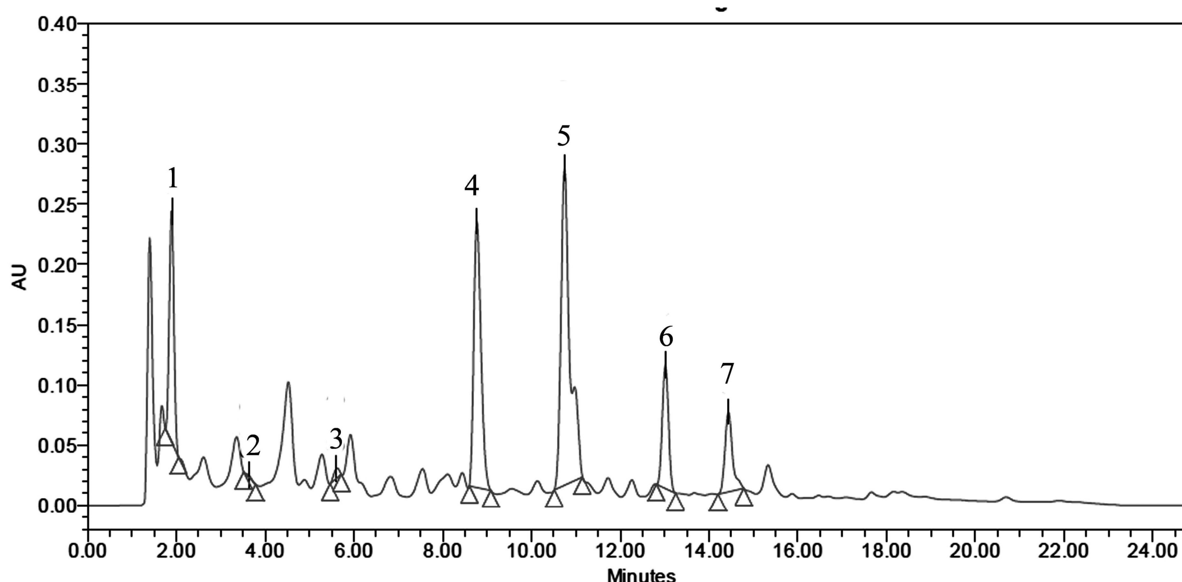


Fig.2.(c) Haritaki churna 2 (HC2)

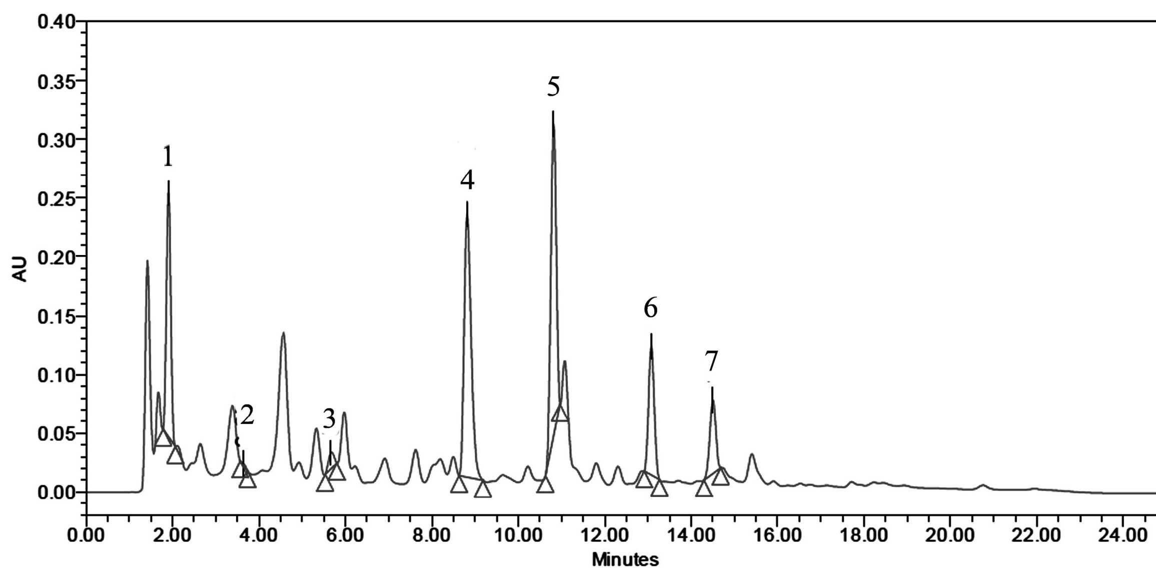


Fig.2.(d) Haritaki churna 3 (HC3)

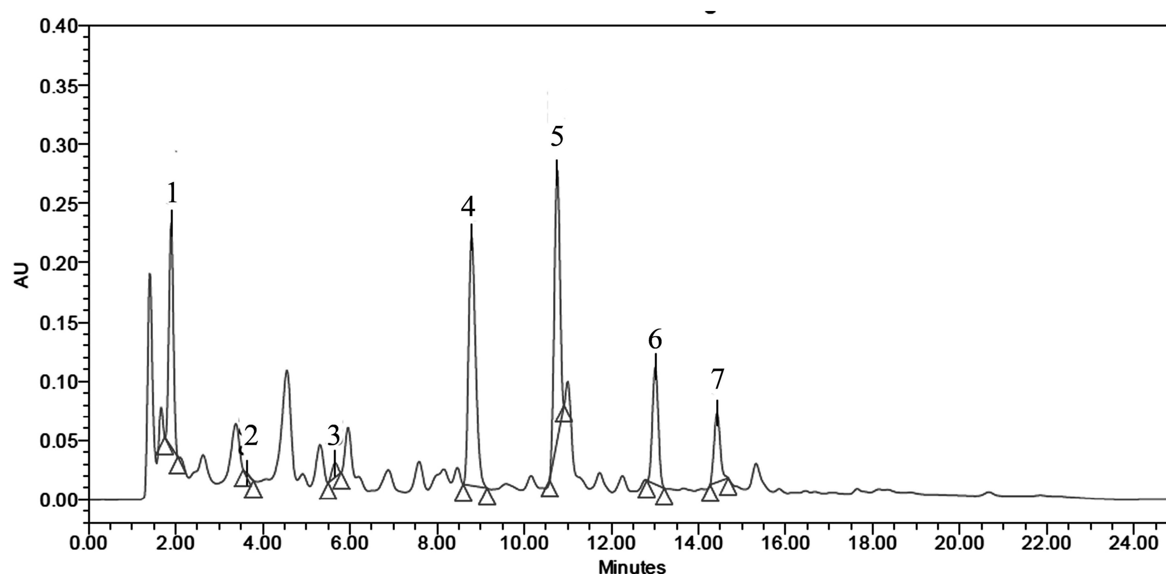


Table.1 Method validation data (Linearity, LOD & LOQ)

Phytoconstituent	RT (min)	R^2	Linear range (µg/ mL)	LOD (µg/ mL)	LOQ (µg/ mL)
Gallic acid	1.88	0.999	2-140	0.6	1.998
Methyl gallate	3.62	0.999	2-30	0.5	1.665
Ethyl gallate	5.8	0.998	2-30	0.5	1.665
Ellagic acid	8.77	0.999	2-80	0.6	1.998
Chebulagic acid	10.77	0.998	2-350	0.5	1.665
Chebulinic acid	13.02	0.999	2-45	0.55	1.8315
PGG	14.47	0.999	2-45	0.5	1.665

Table.2 Method validation data (Recovery and precision)

Phytoconstituent	Recovery [a] (%)	Precision (RSD%) Intra-day [b]	inter-day [c]
Gallic acid	96.9	1.2	1.3
Methyl gallate	99.2	2.2	2.4
Ethyl gallate	96.5	2.7	2.5
Ellagic acid	101.1	2.5	2.6
Chebulagic acid	100.9	3.1	2.9
Chebulinic acid	98.0	3.2	2.9
PGG	97.8	3.5	3.4

[a] mean of triplicate analysis of three different concentrations
[b] mean of samples were analysed on same day n=6
[c] mean of samples were analysed on two consuutive day day n=6

Sample analysis
The method was applied to simultaneous determination of phytoconstituents (1-7) in three commercial formulation samples of *Haritaki churna* (HC1, HC2, HC3) collected from market. Representative chromatograms are shown in Figure. 2. The contents of the seven compounds in the samples were quantified and the results are shown in Table 3 with the mean values of three replicate injections. Variations of the seven compounds content in three market samples (HC1, HC2, HC3) are shown in histogram Figure 3.

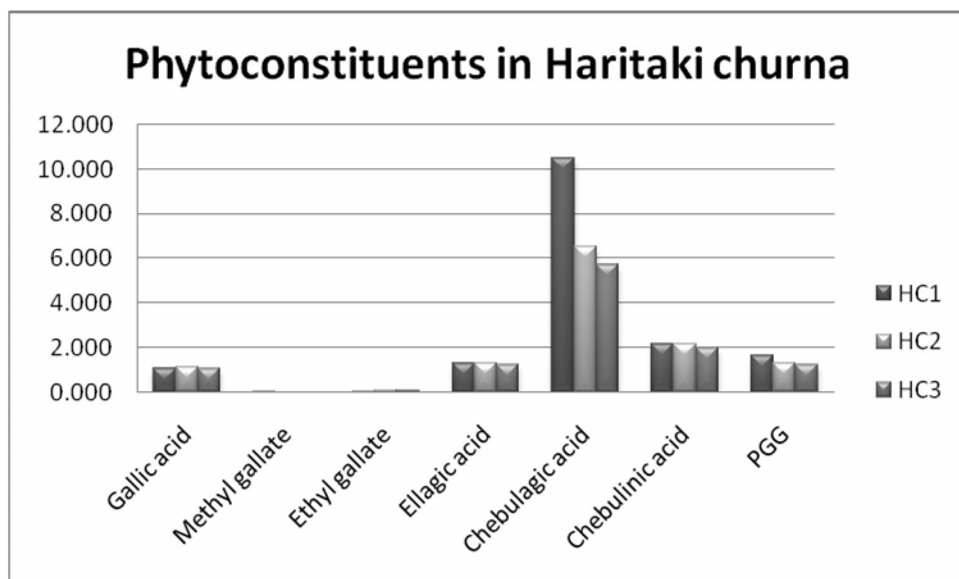
Accuracy
Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of *Haritaki churna* samples prior to extraction. The resultant samples were then extracted and analyzed with the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount *versus* added amount. The recovery of the method was in the range of 97.8–101.1%, as shown in Table 2. Considering the results, the method was deemed to be accurate.

Precision
The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The intra and inter-day precisions were determined by analyzing known concentrations of the seven analytes in six replicates during a single day and by duplicating the experiments on two successive days. In order to confirm the repeatability, six different working solutions prepared from the same sample obtained from different manufacturers were analyzed. The relative standard deviation (R.S.D.) was taken as a measure of precision and repeatability. The results are shown in Table 2, indicating that the intra-, inter-day and repeatability R.S.D. values of the eight compounds were all less than 3.5%, which showed good reproducibility of the developed method.

Table.3 Quantification of Phytoconstituents in Haritaki churna

Phytoconstituent	HC1 (%)	HC2(%)	HC3(%)
Gallic acid	1.088	1.138	1.043
Methyl gallate	0.009	0.006	0.004
Ethyl gallate	0.056	0.097	0.088
Ellagic acid	1.295	1.313	1.222
Chebulagic acid	10.463	6.538	5.701
Chebulinic acid	2.167	2.171	2.014
PGG	1.672	1.288	1.219

Fig.3. Histogram presenting phytoconstituents contents in Haritaki churna samples.



Linearity

Standard stock solutions containing gallic acid, methyl gallate, ethyl gallate, ellagic acid, chebulagic acid, chebulinic acid and penta-*O*-galloyl-*β*-D-glucose were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the analyte solutions were analyzed in triplicate, and then the calibration curves were constructed by plotting the mean peak areas *versus* the concentration of each analyte. The calculated results are given in Table 1. All the analytes showed good linearity ($R^2 > 0.998$) in a relatively wide concentration range.

Limits of detection and quantification

The working solutions of the analytes were further diluted with methanol to yield a series of appropriate concentrations. Limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method .The LOD and LOQ for each investigated compounds were calculated at signal to noise ratio of 3:1 and 10:1 respectively as shown in Table.1.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness of the method was investigated under a variety of conditions including changes of pH of the mobile phase, flow rate and gradient variation. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters has proven that the method is robust. The ruggedness of the method was determined by repeating the experiments on Jasco HPLC system by different operators in addition to Waters HPLC system.

Conclusion

In this study, an HPLC–DAD method for the qualification and quantification of phtyoconstituents in *Haritaki churna* has been developed and successfully applied for comparison of three marketed samples (HC1, HC2, HC3). Significant variation in phytochemical composition of these samples observed. The possible reasons for these variations may be due to quality of the source materials which depend on several factors like varietal selection, climatic and soil requirement, time of harvest, drying parameter and post drying storage condition. The quality of formulation also depends on how elements are handled in production processes i.e. improper and inadequate mixing, variation in particle size of the myrobalan and demixing during transportation. This method is validated for good accuracy, repeatability and precision, and can be used to evaluate the quality of the drug. This multi-phytoconstituents assay method will be helpful to quality control and stability studies of *Haritaki churna*.

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