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Research Article

Simultaneous quantification of gallic acid and ellagic acid in Triphala using reverse-phase high-performance liquid chromatography (RP-HPLC)

Runjhun Pallavi*, Shivesh Jha, Swadesh Pattanik[1]

Department of Pharmaceutical Sciences, Birla Institute of Technology (Mesra) Ranchi, Jharkhand, India.

*Corresponding author: r.pallavi975@gmail.com

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Abstract: An Ayurvedic formulation, Triphala consists of three dried fruits of *Phyllanthus emblica, Terminalia belerica, and Terminalia chebula* in equal proportion was found to be potentially effective and used as an antioxidant, anti-inflammatory, antibacterial, and antimutagenic, antineoplastic, and incorporated in prevention and treatment of many Gastrointestinal diseases like constipation, chronic ulcer, jaundice anaemia, asthma. The polyphenols present in the formulation is responsible for their therapeutic efficacy. The proposed method of reverse-phase high-performance liquid chromatography RP-HPLC method was found to be simple, sensitive, precise, and accurate and validation of the method was performed to demonstrate its selectivity, linearity, precision, accuracy, and robustness. The chromatographic conditions used for the separation were mobile phase comprised of O- phosphoric acid (0.3%) OPA in Water: Acetonitrile (80:20% v/v) and pH was maintained at 4.5. The flow rate was 1.0mL/min with detection at 270nm. The linearity was found to be in the range of 20-100µg/mL and the correlation coefficient of Gallic acid (GA) and Ellagic acid (EA) was 0.998 and 0.995 respectively. The retention time was found to be 5.40 and 10.01 for GA and EA respectively in the in-inhouse preparation of Triphala (Ti) and 5.42 and 10.04 in Triphala marketed (Tm). The result indicates that Triphala contains several markers that are responsible for its therapeutic activity. The developed method of RP-HPLC assists in the standardization of Triphala as the marker contents of in-house Triphala were found to have higher than that of marketed Triphala which may be due to the use of inferior quality material used in the preparation of Triphala.

Keywords: triphala, gallic acid, ellagic acid, reverse-phase-HPLC

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INTRODUCTION

Triphala is a well-known ayurvedic preparation made of three myrobalans, *Emblica officinalis* Geartn., *Terminalia chebula* Retz. and *Terminalia belerica* Roxb. blended in equal proportion (Ayurvedic Pharmacopoeia of India 2003). Triphala is used to promote immunity, improve digestion and act as a cardio-tonic that controls bp and reduces cholesterol level, immunomodulatory, (Jagetia *et al.* 2002), and antioxidant activity (Vani *et al.* 2008). The multidirectional action of Triphala is due to the active ingredients like gallic acid, ellagic acid, ascorbic acid, chebulinic acid, chebulagic acid, tannins and polyphenols present in the formulation which are responsible for its therapeutic efficacy (Bahulikar *et al.* 2003). Gallic acid and ellagic acid have antioxidant, anticancer, antimutagenic, hepatoprotective, and neuroprotective effects. Chebulinic acid and chebulagic acid have antitumour activities of Triphala (Peterson *et al.* 2017), It is therefore essential to assess the quality, purity, efficacy, and safety of the formulation of Triphala. In the present work, the Reverse-Phase High-performance liquid chromatography RP-HPLC method has been used for the quantification of two marker compounds Gallic acid and Ellagic acid and validated using ICH guidelines.

METHODS

Collection and authentication: The plants of Amla, Baheda and Harad were collected from the forest of Chaibasa, Jharkhand, India. The herbs were authenticated by a Central National Herbarium, Botanical Survey of India, and specimens were preserved. Each herb was dried and powdered using a grinder. Then it was passed through the sieve of mesh size #60. Triphala was prepared by mixing the powders of *Phyllanthus emblica, Terminalia belerica, and Terminalia chebula* powders in a 1:1:1 proportion.

Reagents and standards: Pure Gallic acid (99.5%) was purchased from Loba chemicals Pvt. Ltd. and ellagic acid (97%) was purchased from Alfa Caesar, marketed by Thermo Fisher. The peak purity of these markers was checked before analysis (peak purity minimum 90%), and HPLC-grade acetonitrile was obtained from Merck. Potassium dihydrogen phosphate (GR grade) and Orthophosphoric acid (LR grade) were obtained from Merck. Ultra-pure water was obtained using a Millipore (MilliQ apparatus)

Preparation of sample: Triphala powder weighing 100mg was extracted with 100ml of methanol three times and the extracts were combined and concentrated at a reduced temperature of 50°C. The extract was filtered on a vacuum pump and the filtrate obtained was used for analysis.

Chromatographic conditions: Chromatographic separation was performed on Knauer scientific instruments, Germany, equipped with a PDA detector with software Eurochrome version 3.05 column parameter 250 x 4.6 mm id particle size 5micron Mobile phase A was 0.136 g of potassium dihydrogen phosphate dissolved in 1litre of water. Orthophosphoric acid (OPA) 0.5 ml was added and shaken to get a homogenous solution. Mobile phase B was HPLC-grade acetonitrile. The mobile phase was filtered through a 0.45-micron membrane filter and degassed. Analysis was performed at 40°C. The injection volume was kept at 20 µl with a total flow rate of 1ml/min for elution. Detection was done at 270 nm.

Standard and calibration curve: Calibration curve was generated to quantify Gallic acid, Ellagic acid in samples. Five dilutions of each standard, at concentrations ranging from 20 to 100 μg/ml were prepared to generate the calibration curve. Each standard was run in triplicate. The corresponding peak areas were plotted against the concentration of each of the markers under study.

Accuracy and precession: Accuracy and precision were determined by recovery experiments. Before extraction, recovery studies introduced three different doses of Triphala into the samples. The spiked samples were extracted three times and then analyzed using the methodology outlined above. The relative standard deviation of recovery and the percentage recovery was determined from the data. The study of intra-day and inter-day fluctuation showed how precise the procedure was. Six replicate injections were used in the intra-day experiments, and the percentage RSD was calculated. Injections of the standard sample solutions were made in six replicates for the inter-day investigations, and the response factor of the standard compounds and the percentage RSD were computed. (ICH,1996)

Linearity and range: Linearity of the method was determined at five concentration levels of each standard ranging from 20 to 100 μg/ml. Each standard was run in triplicate the linearity of the detector response for the prepared standards was assessed using linear regression concerning the amounts of each standard, measured in μg, and the area of the corresponding peak from the chromatogram. (ICH,1996)

Robustness: The robustness of the method was determined by making slight changes in the chromatographic condition (flow rates, column dimensions, and gradient variation). It was observed that there was no marked change in the chromatograms.
The ruggedness of the method was determined by repeating the experiments on the HPLC system by different operators. (ICH,1996)

Selectivity and peak purity: A diode array detector was applied for the selectivity test. The test was carried out on standard compounds and Triphala extracts. The principle of peak purity judgment is to obtain three spectra, one at 50% upslope, one at the apex, and one at 66% downslope of individual peaks. These three spectra are compared, aiming for a similarity index as close to 1.00 as possible. The peak purity was studied in the major peaks. (ICH,1996)

Limit of detection (LOD) and limit of quantification (LOQ): The limit of detection (LOD) and limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solution using the developed RP-HPLC method (signal to noise ratio of 3) the LOD and LOQ of gallic acid and ellagic acid were determined by formula 3.3x**σ/slope and**

10xσ/slope where σ is the standard deviation: The content of the markers was determined using a calibration curve established with six dilutions of each standard at concentrations ranging from 20 to 100 μl/ml. each concentration was measured in triplicate. The corresponding peak area was plotted against the concentrations of markers injected Peak identification was achieved by comparison of both the retention time.(ICH,1996)

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RESULT AND DISCUSSION

The method of RP-HPLC was used to analyse the samples of Triphala in-house and Triphala marketed which was validated using guidelines on validation of analytical procedure-methodology, International Conference on Harmonization(ICH) The calibration curve of the marker compounds gallic acid and ellagic acid shown in figure1 and figure2 respectively. The linearity was in the range of 20-100µg/mL with a correlation coefficient of 0.998 and 0.995 respectively. The validation parameter is summarised in table1. HPLC profile of the mixture of standard gallic acid and standard ellagic acid is shown in figure 3.The retention factor was found to be 5.38 and 9.98 for GA and EA respectively in the in-inhouse preparation of Triphala as shown in fig.3 and 5.44 and 10.02 in Triphala marketed as shown in figure 4.

Conc	Area
20	13.144
40	30.6348
60	46.2171
80	59.7349
100	77.3304

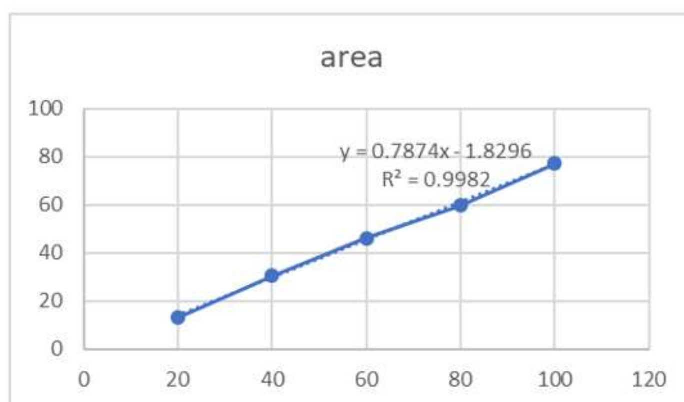

Fig 1: Calibration curve of Gallic acid

Conc	Area
2	19.4399
4	37.3476
6	58.3521
8	72.6908
10	97.2102

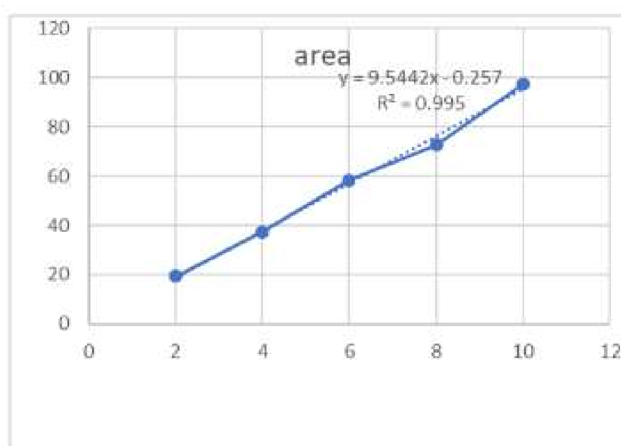

Fig 2: Calibration curve of Ellagic acid

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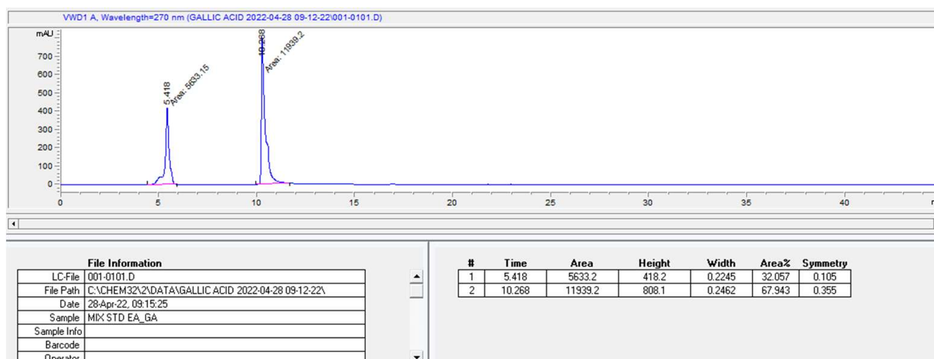

Fig 3: HPLC profile of the mixture of standard Gallic acid and standard Ellagic acid

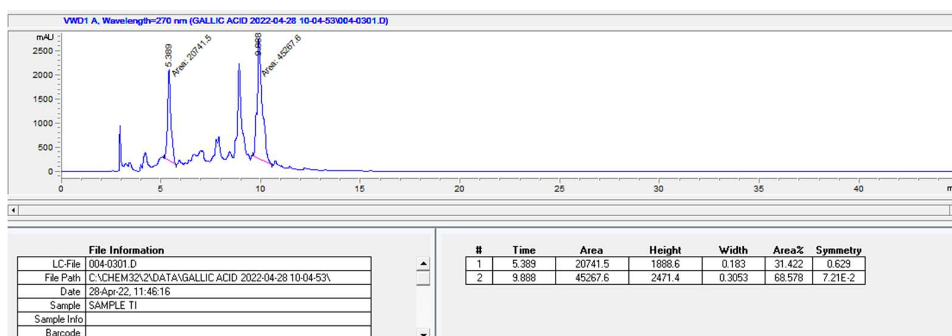

Fig 4: HPLC profile of the methanolic extract of Triphala –In-House and its constituents

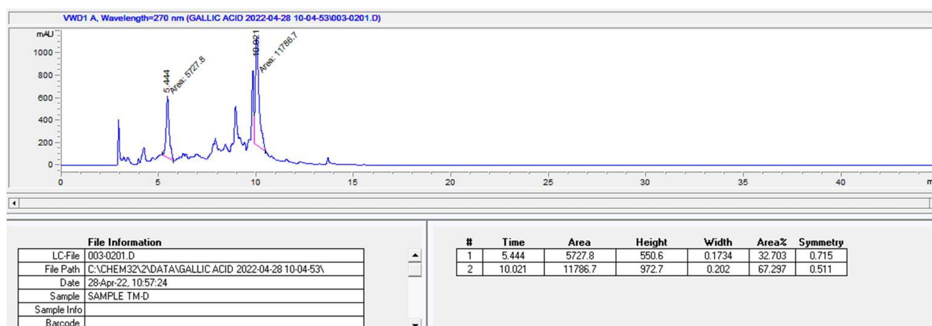

Fig 5: HPLC Profile of the Methanolic extract of Triphala Marketed and its constituents

A simple reverse-phase high-performance liquid chromatographic method has been developed for the analysis of gallic acid and ellagic acid in Triphala in-house and marketed. Good separation was achieved by employing an isocratic system using acetonitrile and 0.01 % phosphate buffer, pH adjusted to 4.5 and the ratio of 80:20 at a constant flow rate of 1 ml/min. The detection was carried out at the analytical wavelength of 270 nm. Thus the developed RP-HPLC method is simple, accurate, and reproducible which uses a simple symmetry shield C-18 column under less mobile phase consumption consisting of acetonitrile and phosphate

buffer (pH 4.5) and also less run time. So this method can be used as a routine analytical tool for the analysis of gallic acid and ellagic acid in other herbal formulations.

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Parameters	Gallic acid	Ellagic acid
Range	2-10 µg/mL	20-100 µg/mL
Retention factor	5.44	10.02
Linear expression	y = 0.7874x + 1.8296	y = 9.5442x + 0.257
Correlation coefficient (R^2)	0.998	0.995
Limit of Detection (LOD)	54.59	65.42
Limit of Quantification (LOQ)	168.11	199.25
Recovery	97.49%	92.88%

Table1: Validation parameters of standard Gallic acid and standard Ellagic acid

CONCLUSION

The present work provides an opportunity for the comparative study between Triphala in-house formulation and commercialized Triphala. The developed method of RP-HPLC will assist in the standardization of Triphala, as the marker contents of gallic acid and ellagic acid of in-house Triphala were found to have better quality than that of marketed-Triphala, the reason being the use of inferior quality material. This method of quantification of Triphala seems to be accurate, reproducible, and repeatable and can be used as an important tool for the standardization and validation of gallic acid, ellagic acid, and other marker compounds which can be evaluated for its applicability to test variation in Triphala preparations and in other polyherbal Ayurvedic medicines.

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DECLARATION OF CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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