

Development and Validation of a RP-HPLC Method for the Determination of Ellagic Acid in *Terminalia bellirica* Extract and Single Herb Capsule of *Terminalia bellirica*

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ABSTRACT

Objective: The present work describes development and validation of a simple, specific, sensitive, precise, reproducible and robust high performance liquid chromatographic method of analysis of Ellagic acid, as in *Terminalia bellirica* extract and from capsule formulation. A validated rapid HPLC–PDA method was developed for identification and quantification of Ellagic acid (EA) in the extracts prepared from the fruits of *Terminalia bellirica* available in India. **Methodology:** The separation was achieved on a Waters HPLC system coupled with a PDA detector. The separation was achieved by using mobile phase in composition of n-Hexane sulfonic acid (20 mM) and 100% Methanol (Gradient programme) at flow rate of 0.7 ml/min was used. **Results:** The retention time for Ellagic acid was found to be 21 min. The system suitability parameters were calculated and were found to be within limits. Linear relationship was obtained between response and amount of drug with high correlation coefficient (R²) in the range 250-750 ppm for Ellagic acid (R²=0.9998). The results of formulation analysis were validated as per International Council of Harmonization (ICH) guidelines indicating high degree of accuracy. **Conclusion:** The % R.S.D. value which is less than to 2 for robustness study suggests that the developed HPLC methods are unaffected by small changes in process parameters.

Key words: *Terminalia bellirica*, Ellagic acid, RP-HPLC, Validation, ICH Guidelines.

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INTRODUCTION

Natural products have been a highly productive source of leadbioactive molecules.^{1,2} Fruits and barks of *Terminalia bellirica* species have been used since the Vedic period for the treatment of various diseases.³ Many preparations from *Terminalia bellirica* species are used in traditional medicine as a cardiac tonic and diuretic.⁴ *Terminalia bellirica* is a large genus of deciduous trees of the flowering plant family Combretaceae comprising of 250 species distributed in the tropical regions of the world.⁵ About 16 species

occur in India. The medicinal properties of *Terminalia bellirica* species depend on the species and its organ; however, the presence of tannins in the fruits and barks is almost characteristic of this genus. *Terminalia bellirica* species represents a rich source of phenolic acids, tannins, cyclic triterpenoids and flavonoids. Their exact chemical classes and levels vary in different *Terminalia* species.⁶ *T. bellirica* grows wild up to 1,000 m, all over India except dry and marshy areas. The fruits of *T. bellirica* contain β-sitosterol,



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gallic acid, ethyl gallate, chebulagic acid, mannitol, galactose, fructose and rhamnose.⁷ Fruits of *T. bellirica* exhibited hypotensive, purgative and cholerectic activities. In combination with extract of *Emblica officinalis* it had protective effect in the experimental rats against myocardial necrosis. The bark may be used as an astringent for dressing wounds and sore throat.^{8,9}

EA (Figure 1) is the major tannin-related constituents of *Terminalia* species present in about two-third proportions.¹⁰ EA is a potent antioxidant marketed as a nutritional supplement.¹¹ It is dimeric derivative of gallic acid.¹² It is 2,3,7,8-tetrahydroxy-benzopyrano-5,10-dione. Its molecular weight is 302.19 g/mol and the empirical formula is $C_{14}H_6O_8$. EA, a dilactone of hexahydroxydiphenic acid, is found in raspberries, strawberries, cranberries, walnuts, pecans and other plant foods.¹³ EA has aroused great interest owing to its antioxidant, anti-inflammatory, and anti-neoplastic properties.¹⁴ EA is a highly thermodynamically stable molecule (Figure 1), with four rings representing the lipophilic domain and four phenolic and two lactone groups (which can act as hydrogen bond donor and acceptor, respectively) representing the hydrophilic part. Keeping in view of the above points in consideration, a rapid and simple HPLC method was developed for the identification and quantification of EA.

The aim of present work is to develop an accurate, specific, and reproducible HPLC method for determination of EA in *Terminalia bellirica* extracts and formulation respectively.

However, one of the impediments in the acceptance of the Ayurvedic or Herbal formulations is the lack of standard quality control profiles. The quality control of herbal medicine that is, the profile of the constituents in the final product has implications in efficacy and safety.¹⁵ 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 is expected to help in circumventing this problem. Hence, in the present paper we report the development of an optimized, validated and simple HPLC method for the estimation of EA from *Terminalia bellirica* and capsule formulation respectively.

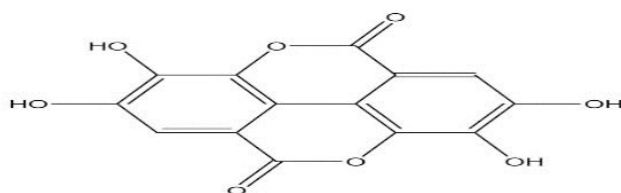


Figure 1: Structure of Ellagic acid.

MATERIALS AND METHODS

Instruments

HPLC instrument (waters 2998) PDA Detector Liquid chromatography with (Software "Empower"). It consists of Column Inertsil ODS 5 μ (4.6 X 250 mm).

Ultrasonicator water bath (Labtex Instruments) and Sartorius sensitive balance model SBT-224SCW were used. Merck Millipore Direct Q₃ Water Purification System was used to obtain Type I water.

Plant Material Extracts

The plant material extract of *Terminalia bellirica* is used for analysis is supplied by Kislaya Herbals of batch number (KBH/804) and the fruits part is used which is a hydroalcoholic extract.

Chemicals and Reagents

Ellagic acid: It was kindly supplied by Yucca Enterprises Pvt. Ltd. (Mumbai), its potency was found to be 98% according to the reported method.

All chemicals used throughout the work were of analytical grade and solvents for HPLC were of HPLC grade. These included Methanol, n-hexane-Sulfonic acid and Water (Merck specialities Pvt. Ltd).

Standard and test solutions

Preparation of the standard solution

10 mg of EA was accurately weighed and transferred into 10 mL of volumetric flask. Add about 5 mL of diluent methanol and sonicate in ultrasonic water bath for 30 min. Cool the solution and make up the volume with methanol to obtain stock solution of 1000 ppm. Use the resulting solution as standard solution.

Preparation of the test solution for extract

50 mg of *Terminalia bellirica* extract was weighed and transferred into a 20 mL of volumetric flask. Add about 15 mL of diluent methanol and then sonicate in ultrasonic water bath for 30 min. Cool the solution and make up the volume with diluent methanol. Then filter through 0.45 μ syringe filter. Use resulting solution as test solution.

Preparation of test solution for Baheda SH capsule formulation

250 mg of Baheda SH Capsule powder was weighed and transferred into a 20 mL volumetric flask. To this 15 mL of diluent methanol was added, sonicated in ultrasonic water bath for 30 min. Cool the solution and make up the volume with diluent methanol. Then filter through 0.45 μ syringe filter. Use resulting solution as test solution.

Chromatographic conditions

The method was carried out at ambient temperature on Inertsil ODS 5 μ (4.6 X 250 mm) column. The mobile phase consisted of n-hexane-sulfonic acid having pH = 2.5 \pm 0.1 adjusted with orthophosphoric acid and 100 % Methanol (70:30, v/v). The mobile phase was filtered using 0.45 mm membrane filters and degassed by ultrasonic vibrations for 30 min prior to use. It was delivered at a flow rate of 0.7 mL/min and the injection volume was 5.0 μ L. The column was conditioned for at least 35 min of gradient elution. PDA detection was achieved at 254 nm.

Method Validation

The proposed method was validated by studying several parameters such as linearity, specificity, accuracy, precision, robustness and system suitability parameters as per the ICH Guidelines

Preparation of Standard Calibration Curve

The stock solution of EA was diluted to seven different concentrations between 250-750 ppm of working concentration. The calibration graph was plotted by using the concentrations versus average peak area at 254 nm. The linearity of the detector response for the standards was determined by means of linear regression.

Linearity

The linearity of peak area response for EA was determined from 250-750 ppm of working concentration for EA. The stock solution of Ellagic acid was diluted to seven different known concentrations.

Range

The range of EA was determined by calculating precision at 50%, 100% and 150% level of the working standard concentration.

System Suitability Parameters

The certain resolution and reproducibility of proposed chromatographic system for estimation of EA in *Terminalia bellirica* extract and capsule formulation, system suitability parameters like retention time, tailing factor and column efficiency (number of theoretical plates, N) were studied. Five replicate solution of standard and test solution in duplicate were injected. The system suitability parameters from standard chromatogram were calculated.

Specificity

The specificity of the method for Assay was demonstrated by injection of following solutions into the HPLC system.

- Diluent as a Blank
- Standard Solution
- Test Solution

Precision

Six replicate area of test solution was measured at 254 nm as per the proposed method. System, method and intermediate precision of the developed method were expressed in terms of relative standard deviation (RSD) of the peak area.

Accuracy

The accuracy was determined from recovery studies. A known but varying amount of EA was spiked into pre-analysed extract test solution and placebo of formulation at 80%, 100% and 120% recovery levels of working standard in triplicate. The spiked test solution was analyzed according to the proposed procedure.

Robustness

To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of change in flow rate, temperature of column and wavelength on theoretical plates, tailing factor and % content were studied. The standard solution containing EA was injected (in triplicate) into the HPLC system under the varied conditions.

RESULT AND DISCUSSION

In any extract, there are hundreds of unknown components and many of them are in low amount. Moreover, there usually exists variability within the same herbal materials. Consequently, to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic components is not an easy or trivial work. Fortunately, HPLC offers very powerful separation ability, such that the complex chemical components in Extract can be separated into many relatively simple sub fractions. Furthermore, the recent approaches of applying hyphenated chromatography and spectrometry such as HPLC PDA detection could provide the additional spectral information, which will be very helpful for the qualitative analysis and even for the on-line structural elucidation. With the help of the spectral information the hyphenated instruments show greatly improved performances in terms of the elimination of instrumental interferences, retention time shift correction, selectivity, chromatographic separation abilities, and measurement precision.

Method development and optimization

To obtain the best overall chromatographic conditions, different mobile phase combination were studied for

Table 1: System suitability parameters of the proposed HPLC method.

Parameters	Ellagic acid	References value
Retention time (t_R)	21.15 min	-
Tailing factor (T)	1.39	T=1, for a typical symmetrical peak
Number of theoretical plates	12697	Increase with the efficiency of the separation

Table 2: Summary of linear regression data of Ellagic Acid for HPLC.

Parametrs	Ellagic Acid
Linearity range	250-750 ppm
Linear regression equation	$y = 54869.4x - 3300638.7$
Slope \pm SD	54869.4x
Intercept \pm SD	3300638.7
Correlation coefficient (r^2)	0.9998

Table 3: Specificity Parameters for HPLC.

Standard	Purity Angle	Purity Threshold	Purity Flag
Ellagic Acid	0.182	0.263	No Flag Found

Table 4: Result of Assay validation parameters for the proposed HPLC method for determination of Ellagic acid in *Terminalia bellirica* extract and Capsule formulation.

Parameters	Extract	Formulation
Accuracy (% mean)	80%	99.79
	100%	98.17
	120%	98.17
% Assay (w/w \pm SD)	0.468 \pm 0.054	0.42 \pm 0.006
Precision (%RSD)		
Method Precision	1.46	1.58
Intermediate Precision	1.23	1.50

Table 5: System Precision parameters for HPLC.

Name of Analyte	USP Tailing	USP Plate Count
Ellagic Acid	1.77	12897

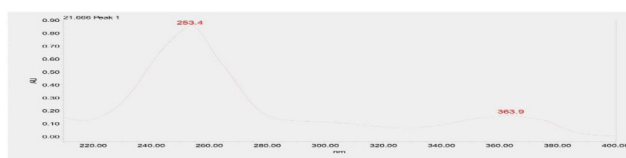


Figure 2: HPLC Spectra for Ellagic Acid.

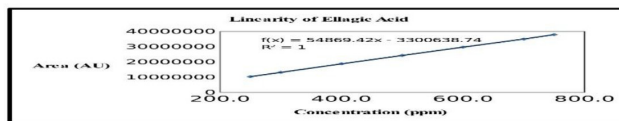


Figure 3: Linearity Graph for Standard Ellagic Acid by HPLC.

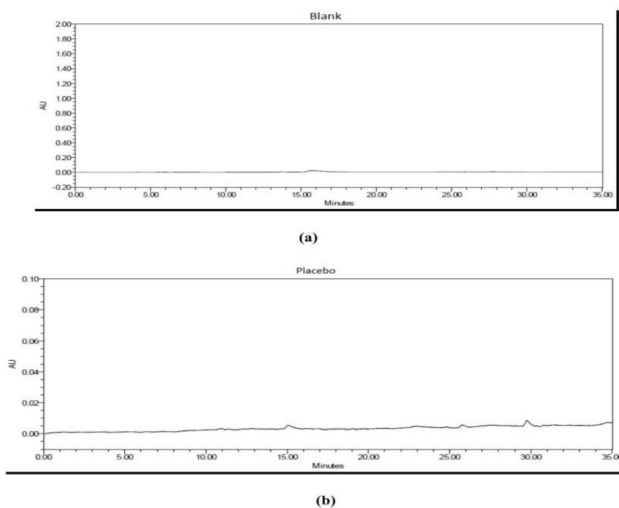


Figure 4: HPLC chromatogram of Blank solution (a) and Placebo (b).

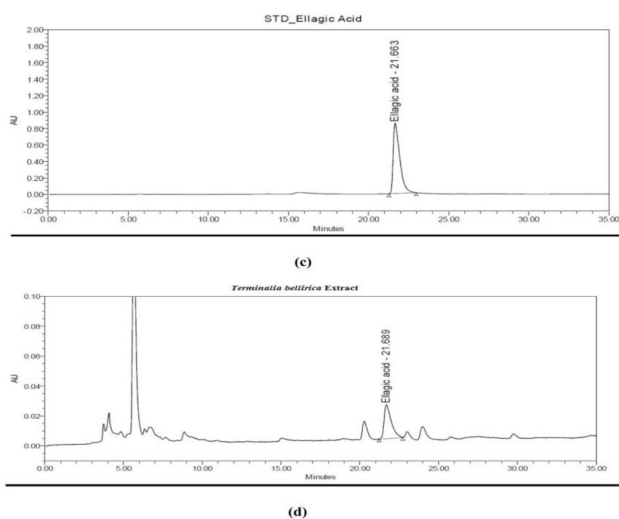


Figure 4: HPLC chromatogram of Standard Ellagic acid (c) and Determination of Ellagic acid in Extract (d).

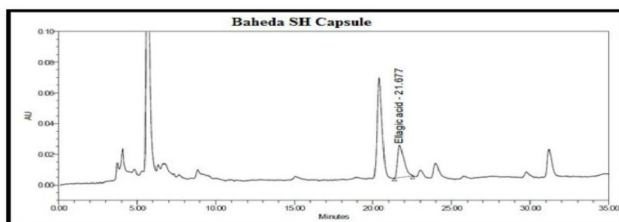


Figure 4: HPLC chromatogram of Determination of Ellagic acid in Formulation (e).

Table 6: Robustness parameters for proposed HPLC method.

Robust parameters	% RSD	Peak Tailing	Theoretical Plates	Remark
Ellagic Acid				
Scanning wavelength: -5 nm	0.40	1.77	12762	Pass
Scanning wavelength: +5 nm	0.36	1.75	12704	Pass
Column temperature: -5° C	0.41	1.79	14489	Pass
Column temperature: +5° C	0.17	1.80	14631	Pass
Flow rate: -0.1%	0.08	1.79	17129	Pass
Flow rate: +0.1%	0.12	1.80	16135	Pass

separation of Ellagic acid from *Terminalia bellirica* extract. The composition of solvent system for HPLC analysis was optimized by testing different solvent systems of varying polarity and the best results were obtained n-hexane sulfonic acid and Methanol (70:30) as Gradient elution. The mobile phase was optimized by examining the effect of pH and content of Methanol. Other chromatographic variables were investigated including column type and flow rate. The retention time for EA is found to be 21 min.

METHOD VALIDATION

Linearity and Range

Peak areas were found to have good linear relationship with the concentration as shown in Figure 3. The linearity graph shown coefficient of correlation (R^2) was found to be 0.9998 for EA. Calibration graphs were constructed in the ranges of 250-750 ppm for EA. The correlation coefficients, y-intercepts and slopes of the regression lines of the compounds were calculated and presented in Table 2.

Precision

The results showed that the system, method and intermediate variation of the results for estimation of EA was within the acceptable range. The sample was also analyzed by two different analysts within the same day and the results revealed that there is good intermediate precision. The area was measured and the % RSD found to be less than 2 are observed for extract and formulation respectively which is within acceptable limit indicates the precision of the system was shown in Table 4 and Table 5.

Quantification of Ellagic acid in the extract and in single herbal formulation containing *Terminalia bellirica*

5 μ L of the extract and 10 μ L of formulation were injected to in sixlet and the amount of EA in samples were determined by using Method precision. The results were shown in % Assay in Table 2. This method

shows the presence of 0.468 ± 0.054 % w/w in extract, $0.42\% \pm 0.006$ w/w in formulation respectively.

Accuracy

Accuracy was also assessed by standard addition technique and the percentage recoveries were calculated and found to be in the range of 98% to 99% for extract and formulation against respective levels as mentioned in Table 4 respectively. The results showed that the suggested method is reliable and reproducible.

Specificity

Specificity of the methods was tested by how accurately and specifically the analytes of interest are determined in presence of other components. Peak purity testing was done to ensure purity of the peaks in HPLC method. This is evident from HPLC chromatogram shown in Figure 4 proved the specificity of the proposed method and the results obtained confirmed the purity of EA peak shown in Table 3.

Robustness

Robustness of an analytical procedure is the capacity of the method to remain unaffected with small deliberate variations in method parameters and provides an indication of its reliability during normal usage. e.g.: flow rate ± 0.1 ml/min in HPLC method and changing the scanning wavelength ± 5 nm and column temperature $\pm 5^\circ\text{C}$ for the proposed methods. Robustness data is shown in Table 6. The value of % RSD which is less than 2 shows that the methods are robust and that the deliberate small change in the studied factors did not lead to significant changes in retention time values, area or symmetry of the peaks.

System suitability

An overall system suitability testing was done to determine if the operating system were performed properly. Parameters including retention time, tailing factor and number of theoretical plates were calculated where good results were obtained and peak information is

given in Table 1. Typical chromatogram obtained is shown in Figure 2.

CONCLUSION

A simple and efficient reverse-phase HPLC method was developed for the identification and quantification of EA in fruit extracts of *T. bellirica* and capsule formulation. The developed HPLC method is simple, precise, time saving, economic and accurate. Further, it was also validated as per ICH guidelines. The developed HPLC method analysis is rapid, therefore, suitable for quantitative analysis as well as quality control of extracts and herbal formulations from *Terminalia bellirica* species.

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CONFLICT OF INTEREST

There are no conflicts of interest.

ABBREVIATIONS

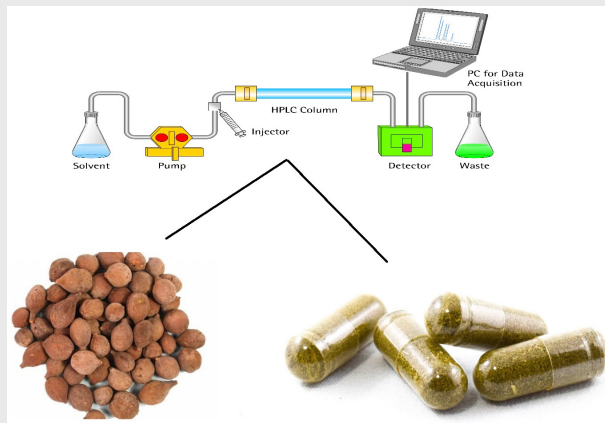
HPLC-PDA: High performance liquid chromatography-Photodiode array detector; **EA:** Ellagic acid; **ICH:** International Council of Harmonisation.

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PICTORIAL ABSTRACT



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SUMMARY

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