

DETERMINATION AND EVALUATION OF ELLAGIC ACID IN TERMINALIA CHEBULA FRUIT EXTRACT BY RP-HPLC

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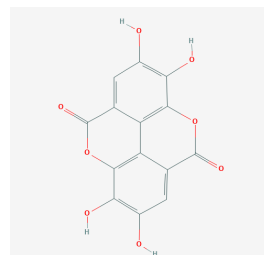
Abstract : Ayurveda, the Terminalia chebula (TC Terminalia Chebula Fruit Extract) herb, and especially its fruits dried pulp, are well-known. It is one of the important ingredients used in triphala or “three fruits”—the most frequently prescribed herbal drug remedy in ayurveda. Among Indian , The biochemical activities previously reported for water-soluble TC fruit extracts appear to be due to the presence of hydrolysable tannins; more specifically Ellagic acid , chebulinic and chebulagic acids. Ellagic acid has cytoprotective and cancer protective effect against various carcinogenic induced cancers cell . Some study on laboratory animals have pointed that periodical oral administration of ellagic acid in rats can circumvent the carbon tetrachloride (CCl₄) toxicity and formation of fibrosis in the liver, **METHOD** Ellagic acid compound was purchased from Sigma-Aldrich Co. LLC, USA, Product Number : E2250. All other reagents were of HPLC grade and purchased from SDFCL. **RESULTS:** In this analytical study, a simple, precise, accurate and rapid reverse phase HPLC method has been established and validated for the determination of ellagic acid in herbal formulation. The percentage recoveries for the ellagic acid was 98.96%, 100.1%, 100.14%. **DISCUSSION:** The High-performance liquid chromatography method was validated to estimate the precision, repeatability and accuracy. The relationship of the mean concentration of standard solution and the average peak response was slightly linear with the solution concentration range variables of 20 µg/ml to 120 µg/ml with a desirable correlation coefficient of 0.999. The $y = 43278x$ precision was found by continued repeated delivery of injections of the same concentration five times (% CV = 0.0572). The Accuracy level was determined at three levels (80%, 100% and 120%). The specific recoveries was found 98.96%, 100.1% and 100.14%. The phase detection of ellagic acid was calculated from its standard deviation of the linear curve and slope found 3.744 µg/mL.

Keyword : Ellagic acid, Terminalia chebula, RP-HPLC, anti-cancer, chebulagic acids

Introduction : Ellagic acid is known for its anti-oxidant, anti-mutagenic and anti-cancer properties. Many research analytical study show anti-cancer activity on metastatic cells of the colon , breast, oesophagus, skin, prostate and pancreas.¹ Precisely ellagic acid has got the tendency to prevents the destruction of specific P53 gene by oncogenic cells. Ellagic acid can form the binding with oncogenic mediators , and inhibit and inactivated . In Ayurveda, the Terminalia chebula (TC Terminalia Chebula Fruit Extract) herb, and especially its fruits dried pulp, are well-known.^{2,3} It is one of the important ingredients used in triphala or “three fruits”—the most frequently prescribed herbal drug remedy in ayurveda. Among Indians , TC is highly recognised for its purifying attributes activities; in fact, its fruit is depicted in the hands of the medicine ancient Buddha in sacred paintings.^{4,5} These small, round and lite brownish -colored fruits were historically used to stabilised the general balance of the body. Multiple reviews of TC’s pharmace-utical, medicinal chemistry and biochemical effects recently were published, In relation, this paper describes several biochemical tests to examine the skin benefits of a hydrolyzable, tannin-enriched Terminalia chebula fruit extract.^{6,7} The biochemical activities previously reported for water-soluble TC fruit extracts appear to be due to the presence of hydrolyzable tannins; more specifically Ellagic acid , chebulinic and chebulagic acids . Commercially available fruit extracts of TC typically contain ~35% of total hydrolyzable tannins; however, their content may vary as low as 21%. The TC fruit extract used for the described studies was therefore standardized for total hydrolyzable tannins (> 50%) as well as Ellagic acid , chebulinic and chebulagic acids.⁸ The results shown that a defined, water-soluble past of TC fruit extract can be used topically for preventive and restorative anti-aging and skin tone-evening effects. In some study the effects of dietary herbal crude ellagic acid shown on rat hepatic cells and gastrointestinal lining of mucosal cytochromes P450 and phase II enzymes. Study also shown ellagic acid along with gallic acid can causes a reduction in hepatic mucosal cytochrome P450 and an increase in some liver enzyme phase II activities, so enhancing the ability of the inflamed target tissues to detoxify the reactive chemical

intermediates. Ellagic acid has cancer chemoprotective effect against various chemically carcinogens induced cancers.⁹ . Some study on laboratory animals have pointed that periodical oral administration of ellagic acid in rats can circumvent the carbon tetrachloride (CCl₄) toxicity and formation of fibrosis in the liver. The main mechanism by which crude Ellagic acid is act as scavenger of carcinogen and cancer-causing mediators and making them completely inactive provide the cytoprotecting against mutations in bacteria. Ellagic Acid prevents binding to carcinogens through promoter gene to DNA, and systemically reduces the chance of cancer in the cultured human cells exposed to carcinogens and carcinogenic chemicals ^{10,11} The present study aims to development the new chemical evaluation by RP-HPLC method for the screening of ellagic acid present in the terminalia Chebula fruit pulp formulation.

Chemistry of ELLAGIC ACID



Molecular formula: C₁₄H₆O₈

IUPAC name: 4, 4', 5, 5', 6, 6'-hexahydrodiphenic acid 2, 6, 2', 6'-diulactone

Molecular weight: 302.197.

Purity (HPLC): 96.1% min

Description: Ellagic acid appears as cream-colored needles (from pyridine) or yellow powder.

Density: 1.67 g/cm³

Melting point: > 350 °C

Solubility: Soluble in methanol, and slightly soluble in water.

Stability: Stable under normal conditions.

Chemical and Physiological Properties

Methods :

Chemicals: Ellagic acid compound was purchased from Sigma-Aldrich Co. LLC, USA, Product Number : E2250. All other reagents were of HPLC grade and purchased from SDFCL. Terminalia Chebula tablets

dried fruit (500g) in five pack of 100g were procured from Clouddtail , amazon India, India Private Limited *GMR Airport City, Survey No. 99/1, Mamidipally Village, Shamshabad Hyderabad, Telangana, 500108, The chromatographic system (HPLC) consisted of Shimadzu, Prominence injector with a 20 μ L fixed loop separation was by Phenomenex C18 ODS column (250 mm x 4.6 mm I.D., 5 μ m) at normal room temperature with a UV Visible detector.

1. Chromatographical condition :

Chromatographic determination was performed by using a C18 reverse phase column at room temperature with the injection volume of 20 μ L fixed loop at a flow rate of 1 mL/min. The mobile phase composition was optimized with solution of acetonitrile and water containing 0.01% v/v orthophosphoric acid having ratio of 80: 20 at detection wavelength of 270 nm. Ellagic acid . In the fixed chromatographic conditions was kept for the evaluation of ellagic acid in formulation by using RP-HPLC method. (Figure :1)

Mobile phase Preparation:

The mobile phase is prepared by using 1 ml of orthophosphoric acid , dissolved in 1000 ml of milli-Q-water. The Filter and discard by using sonication followed by mixed up with Acetonitrile and buffer in the ratio of 80: 20 v/v.

2. Standard solution preparation

The standard solution containing 10 mg of fresh ellagic acid taken into 10 ml flask and dissolved into 5mL methanol alcohol . The volume standard up by 10 ml with methanol to give the concentration of 1mg/ml. Further dilutions was prepared and quantify to 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml of working standard solution of fresh ellagic acid and add the volume to 10 ml to form the mobile phase, gives the divided concentrations of 20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml, 100 μ g/ml and 120 μ g/ml of ellagic acid solution respectively.

3. Formulation solution :

The dose form of terminalia chebula 20 tab, powdered weigh 5.19 gm weighed mixed into a 10 ml extracted mixed methanol 10 ml allow the sonication for 15 minutes. Finally 0.8 ml was diluted to 10 ml with mobile phase.

4. Recordings:

The standard solutions steady baseline was observed , chromatograms were recorded and the peak areas under curve of standard chromatograms recorded . The virtual calibration graph was plotted , peak area vs concⁿ . Then terminalia chebula prepared solution was injected and then amount of the ellagic acid present in the test formulation solution was calculated using calibration curve. The amount of alkaloid chemical ellagic acid is found in each tablet formulation was 0.955 mg.

5. Chromatograms Recording:

Stock standard solution fragmented the dilutions prepared in strength of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml of effective work standard solution of compound ellagic acid and volume to 10 ml with mobile phase in container . This gives the 20 μ g/ml, 40 μ g/ml, 60 μ g/ml, 80 μ g/ml, 100 μ g/ml and 120 μ g/ml of ellagic acid solution. standard solutions injected after obtaining a steady baseline. Then peak areas of standard chromatograms was plotted. After that a calibration graph were plotted using peak area Vs concentration soon after the test formulation solution was injected to get ellagic acid present in the formulation was evaluated from the calibration curve. Once again amount of ellagic acid present in each tablet terminalia chebula formulation 0.955 mg.

Application to Herbal formulation (Terminalia Chebula tablet) Sample preparation

6. Selection of Mobile phase

The Acetonitrile having a UV cut off < 200 nm, RF (refractive index) – 1.3441, viscosity- 0.38, boiling point- 82°C, polarity value- 5.8 and solvent miscibility number of 11 as it should not be more than 17.

7. Selection of buffer solution

The reverse phase (RP) chromatography most of the time mobile phase pH values are kept between 2 and 7. ellagic acid is very ionizable under reverse phase this is due to the carboxylic functional group -COOH as a functional group with pKa between 4.5 to 10

8. Selection of the column: In this adopted method Symmetry Shield TM RP 18, C 18 4.6 X 250 mm, 5 µm column. (Figure : 2)

Chromatogram of Terminalia chebula tablet

Results

In this analytical study, a simple, precise, accurate and rapid reverse phase HPLC method has been established and validated for the determination of ellagic acid in herbal formulation. The developed analytical method was validated as per ICH method validation guidelines. The validation parameters are LOD, LOQ, linearity, accuracy, precision, robustness, ruggedness and specificity

Accuracy

To obtain the accuracy the graph plotted b/w the area under curve on y-axis and concentration of standard solutions on x-axis with the sample area under the curve, which is obtained by injecting the sample solution to HPLC. The percentage recoveries rate for ellagic acid was 98.96%, 100.1% and 100.14%. (Table : 1)

A study to establish the interferences effecting the elution of peak of the Ellagic acid alkaloid. Using injecting blank of standard solution and test sample solutions to HPLC, the resultant chromatograms were not shown any interference to elution of gallic acid peak at 2.82 min. This may be possible indication of other ingredients present in the formulation which do not interfere in estimation of ellagic acid by this procedure

Linearity

The standard stock solution was diluted further in order to get concentration in the range between 20 µg/ml to 120 µg/ml of ellagic acid. Each concentration was injected in triplicate and the average area was calculated. A calibration curve was plotted using peak area Vs concentration. The calibration plot is shown in figure. The correlation coefficient was found to be 0.999.

Precision

Precision of an analytical procedure is the degree of agreement among individual sample test results when this method is applied randomly and repeatedly. The intraday and inter-day precisions of the designed method were determined by estimation of the corresponding response three times on the same day and on six different days over a period of 1 week for three different concentrations of ellagic acid. The results are found in terms of relative standard deviation (RSD) in (Table 2).

Method validation parameters for the estimation of ellagic acid by the HPLC method

Robustness

For demonstration of the robustness, the following optimized conditions were slightly changed. By changing the temperature (28^o C) ±3 units (Table :3).

Ruggedness

For demonstrating ruggedness of the method, the standard drug solution was injected in triplicate by two different analysts (Table : 4).

Discussion

The High-performance liquid chromatography method was validated in terms of precision, repeatability and accuracy. This method is specific for well-resolved ellagic acid with a retention time (Rt) value of 4.6 min in the presence of some other

components in the sample of terminalia chebula tab formulation.

The relationship between the mean concentration of standard solution and the average peak response was linear with the concentration range variables of 20µg/ml to 120µg/ml with a correlation coefficient of 0.999. The $y = 43298x$ precision was studied by repeated delivery injections of the same concentration five times (% CV = 0.0571). The Accuracy level developed method was determined at three levels (80%, 100% and 120%). The specific recoveries was found 98.95%, 100.2% and 100.15%. The limit of phase detection of ellagic acid was calculated from the standard deviation of the linear curve and slope found to be 3.744µg/mL. The limit of quantification of the ellagic acid was calculated from the standard deviation of the linear curve and slope, was found to be 16.84µg/ml. There is permissible variation in temperature, which shown the method is robust enough. The low RSD value (0.0178) for percent assay of the test preparation revealed in this proposed method is rugged. The graph of chromatogram of sample indicate a single peak at the retention time of the ellagic acid which further indicating that there is no interference of the fluctuation change in the persons for injecting the sample to the instrument. The amount of ellagic acid present in the terminalia chebula tab formulation of was found to be 0.955 mg. to conclude One of the challenging task to achieve such testing is the natural variation in quantitative levels of the chemical alkaloid compounds in herbs, which gets even more complicated when three herbs are blended together compounding and analysis difficult . as we have chosen TC with single compound so approach has been provided to test for the uniformity of mixing/composition during blending/manufacturing of Terminalia Chebula , with the knowledge of each marker in the raw herb used for blending. the above application and approach has potential to add variation data generation for GLP for product like TC with up to for single herbs forming its composition. The developed HPLC method analysis is rapid, therefore, sophisticated suitable for quantitative analysis as well as quality control of extracts and herbal formulations from Terminalia Chebula species.

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Table 1.

Solution Conc (µg/ml)	Peak area	Average	Recovery (µg)	Recovery (%)
80	3426765	3428409	79.15	98.93
	3429187			
	3427691			
100	4345684	4336757	100.2	100.2
	4343285			
	4331503			
120	5204638	5204347	120.2	100.1
	5204629			
	5202306			

Table : 2

s.no	Validation Parameter	Ellagic acid
1.	System suitability (asymmetry %CV)	2.6
2.	Specificity	Specific
3.	Linearity (correlation coefficient R ²)	0.999
4.	Precision (% CV) (n = 5)	0.0572
5.	Limit Of Detection (LOD)	3.743 µg
6.	Limit Of Quantitation (LOQ)	16.83 µg
7.	Robustness (variation in temperature)	Robust
8.	Ruggedness (% CV)	0.0178

Table : 3

Temp(°C)	RT (min)	Peak area
25	5.426	5538434
28	4.524	4727345
30	3.916	4103773

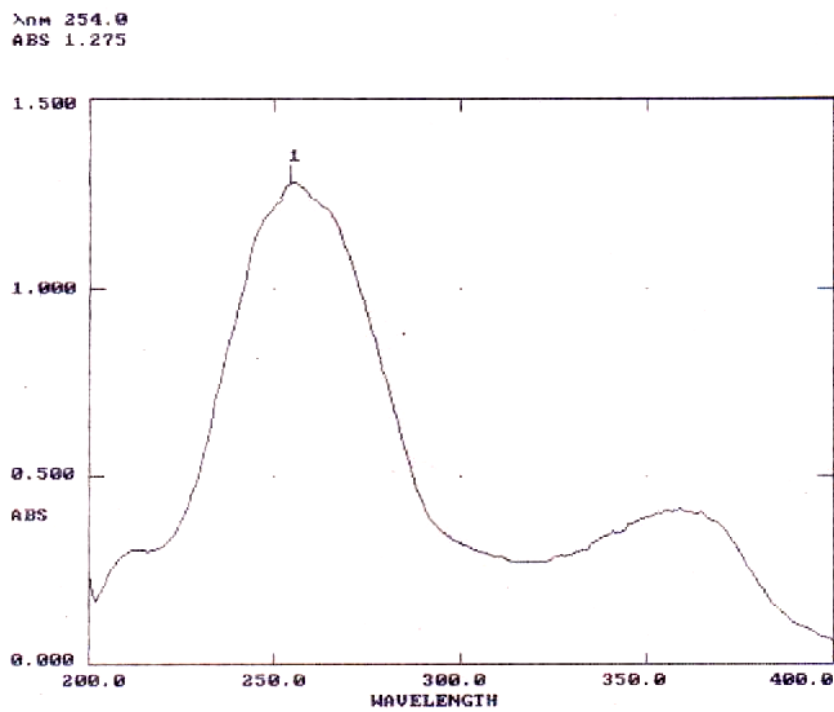
Table : 4

Analyst	Peak	Average	%RSD
1	8809193	8807878	0.0178
2	8806764		

Table : 5

Parameters	Results
Linearity	
Range	20-120 µg/ml
Liner equation	Y = mx + C
Slope (m)	43.30
Intercept (C)	-5.891
Correlation coefficients(r^2)	0.999
Standard deviation(SD)	0.0179
Precision(%RSD)	
Intraday Precision(n=3)	% RSD = 0.124
Inter day Precision(n=3)	% RSD = 0.0307
Limit of detection(LOD)	3.744 µg
Limit of Quantification (LOQ)	16.84 µg
Ruggedness	0.0178
Robustness	Robust
Specificity	Specific
System Suitability	2.519
Quantification	0.955 / tablet

Figure 1.



Chromatogram : Ellagic acids component

Figure : 2

