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Research Article

ESTIMATION OF GALLIC ACID IN *TERMINELLIA CHEBULA* LINN. FRUIT BY VALIDATED HPTLC METHOD

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ABSTRACT

The current study describes development and validation of new, rapid, simple, sensitive, accurate, robust and precise high-performance thin-layer chromatographic (HPTLC) method for quantitative determination of Gallic acid in *Terminalia chebula* fruit with densitometric detection. The chromatographic separation was carried out on Merck Silica gel $60F_{254}$ (10x20 cm, 0.2 mm thick) HPTLC pre-coated plates using toluene: ethyl acetate: formic acid (7:2.5.:0.5 v/v) as mobile phase and scanned at 540 nm. The aforesaid mobile phase gave well defined peaks at Rf value of 0.27 for Gallic acid. The linear regression analysis data for the calibration plots showed good linear relationship with regression coefficient $r^2 = 0.99931$ in the concentration range of 100-800 ng $spot^{-1}$, with respect to the peak area. The method was validated for linearity, precision, specificity, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per guidelines of the International Conference on Harmonization (ICH). The overlay spectrums of standard Gallic acid with sample were found to be similar and the method was found to be specific, reproducible and selective. Since this proposed validation method resolves and quantifies gallic acid effectively, it can be applied for identification and quantification of gallic acid in the any herbal formulations with Haritaki.

Keywords: *Terminellia chebula*, Haritaki, Gallic acid, HPTLC, Validation, Densitometry.

INTRODUCTION

Haritaki consist of pericarp of mature fruits of *Terminellia chebula* Retz. (Fam. Combretaceae), a moderate sized or large tree found throughout India, chiefly in deciduous forest[1]. It has been extensively used in folklore medicine and traditional medical system like Ayurveda, Unani & Homoeopathic medicine and has become cynosure of modern medicine. The Sanskrit name "Haritaki" refers to the yellowish dye (Harita) that contains the God Shiva (Hari, i.e. the Himalayas) and that it cures (Harayet) all the disease. Its other synonym Abhaya, refers to the "fearlessness" provides in the face of the disease. According to Indian mythology, this plant originated from the drops of ambrosia (Amrita) which fell on the earth when Indra was drinking it[2,3]. *Terminellia chebula* fruit contains chebulin, palmitic, steoric, oleic, linoleic, arochidic and belenic acids, chebulnic acid, tannic acid, gallic acid, resin, glycosides of anthroquinons derivative etc. Tannins are one of the most widely occurring groups of natural substances in different families of higher plants. These secondary metabolites are present in solution form in the cell sap and also in distinct vacuoles and having the capacity to combine with tissue proteins and precipitate them. They are, therefore used in medicines for allied purposes. Hydrolysable tannins are hydrolyzed by acids or enzymes quickly and the products of hydrolysis are gallic acid or ellagic acid. Gallic acid is phenyl propanoid, chemically it is 3, 4, 5,- Trihydroxybenzoic acid. *T. chebula* possesses a wide variety of activities like antioxident[4], antimicrobial[5], antiviral, astringent[6] and carcinogenic, hypocholesterolemic, radio-protective, antispasmodic, antipurgative, stomachic, tonic and laxative[7]. Researches shown significant effect of gallic acid on lipid profile[8,9]**.** The ethanol extract from the fruit of *T. chebula* exhibit significant inhibitory activity on oxidative stress and the age-independent shortening of the telomeric DNA strenth[10].

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. However, one of the impediments in the acceptance of the traditional medicines is the lack of standard quality control profiles[11]. Standardization of plant drugs is essential to assess the quality of drugs based on the concentration of their active principle. Phytochemical evaluation is one of the tools for the quality control assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis

using modern analytical techniques. In the last two decades high performance thin layer chromatography (HPTLC) method has emerged as an important tool for the qualitative and quantitative analysis of herbal drugs and formulations[12-13]. The goal of the present article is to validate and determine the content of gallic acid in the dried fruit extract of *Terminellia chebula* . For this purpose, a new, simple, sensitive, precise, and robust HPTLC method was developed and validated for linearity, precision, specificity, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per guidelines of the International Conference on Harmonization (ICH).

EXPERIMENTAL

Materials and Chemicals

The plant drug Terminellia chebula, purchased from local market in Chitrakoot and physically authenticated with the help of herbarium specimens present in pharmacognosy laboratory for its genuine**.** Analytical grade solvents viz. toluene, ethyl acetate, methanol, formic acid, were used and obtained from SD fine chemicals, Mumbai, India. Standard marker Gallic acid (98% pure) was procured from Sigma Aldrich Chime (Steinheim, Germany). Silica gel $60F_{254}$ (10x20 cm, 0.2 mm thick) HPTLC pre-coated plates were procured from Merck (Darmstadt, Germany).

Preparation of standard solution

A stock solution of Gallic acid (1000 μg /ml) was prepared by dissolving 10 mg of accurately weighed gallic acid in 10 ml with methanol[13, 14]. The stock solution was further diluted with 10 ml methanol to give the final concentration of .1 mg /ml (1μg/μl). This concentration was used as the working standard for the HPTLC method.

Preparation of extract and Sample solution

The dried fruit powder of *Terminellia chebula* (5gm) was extracted in 100 ml of methanol by maceration process. The methanolic extract was filtered through Whatman filter paper No.1 and then concentrated at low temperature. Accurately weighed 500 mg of dried extract and added 10 ml of methanol to it. Pipette out 1 ml of this solution in a 10 ml volumetric flask and dilute up to the mark with methanol. The stock solution of the sample, having concentration of 5 μg /ml is prepared thus. This concentration is used for the estimation of Gallic acid from the dried fruit powder of the plant material. All samples were filtered through a 0.45 μ membrane filter (Millipore).

Instrumentation and Chromatographic conditions

HPTLC aluminum plate pre-coated with silica gel $F60_{254}$ (10 × 20 cm) with 200 µm thickness (E.Merck, Germany) was used as stationary phase. The plates were pre-washed with methanol and activated at 110°C for 10 minutes prior to chromatography. The samples were spotted in the form of bands, of 8.0 mm, with the help of a Camag syringe (100 µl) using a Camag Linomat V (Switzerland) sample applicator. The slit dimension was kept at 6 mm × 0.45 mm, with a scanning speed of 20 mm/second, and a data resolution of 100 μm/step was employed. The composition of the mobile phase was toluene: ethyl acetate: formic acid (7:2.5.:0.5 v/v). Linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 20 min at room temperature (28 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate was allowed to dry in air. The separated bands on the HPTLC plates were scanned over the wavelength of 540 nm by using Camag Scanner 3. The source of radiation utilized was the tungsten (w) lamp. The plates were photographed at visible light by using Camag Reprostar 3 with Wincats integration software.

Calibration curve

A stock solution of gallic acid (1μg/μl) was prepared in methanol. Serial dilutions were made to get concentration in the range of 100- 800 ug/ml. of Gallic acid. Aliquot of above solutions were applied (20μl) on TLC plate and peak area of each band was recorded. Calibration curve was obtained by plotting peak area vs. concentration of standard.

Method Validation

The developed densitometry method was optimized and validated as per the International Conference on Harmonization ICH Q2 (R1) guidelines[15-19.]

Linearity and Range

Linearity was determined by applying standard solution on HPTLC plate in the range of 100- 800 ng band $^{-1}$ for Gallic acid. Peak area versus concentration was subjected to least square linear regression analysis to obtain correlation coefficient (r^2) and equation of the line.

Sensitivity

The sensitivity of measurement of gallic acid by use of the proposed method was estimated in terms of limit of quantification (LOQ) and the lowest concentration detected under the chromatographic conditions as the limit of detection (LOD). The LOD and LOQ were calculated by the use of the equations LOD= 3x σ/s and LOQ= 10x σ/s where σ is the standard deviation of the peak areas of the Gallic acid (n=6) and s is the slope of the corresponding calibration plot.

Precision

Precision was measured by using standard solutions containing gallic acid at concentrations covering the entire calibration range. Repeatability of the sample application and measurement of the peak area were carried out using six replicates of the same spot (200 ng spot^{-1}) and were expressed in terms of percent relative standard deviation (% R.S.D.) The intra- and inter-day variation for the determination of gallic acid was carried out at three different concentration levels of 200, 500, and 700 ng spot^{-1} on the same day and three deferent days respectively.

Accuracy and Recovery

The accuracy of the method was determined by the addition of standard compounds in the samples at three different levels (50, 100, and 150 %), and the mixture was analyzed under optimized conditions. The accuracy was calculated from the test result as the percentage of analytes recovered by the assay.

Specificity

This is very important factor for quantitative determination and the baseline resolution (R_s above 1.25) of the targeted compound must be validated. Specificity can be enhanced by densitometric evaluation at a wavelength unique for the absorption of the compound. The mobile phase designed for the method resolved the sample components very efficiently. The specificity of the method was ascertained by analyzing the standard drug and sample. The band of gallic acid in the

sample was identified by comparing its R_f value and its absorbance/reflectance spectrum with those of standard. the peak purity of gallic acid was tested by comparison of spectra of markers at peak start, peak apex, and peak end position of the band, that is, r (S,M) and r (M,E).

Robustness of the method

Robustness is a measure of the capacity of a method to remain Unaffected by small but deliberate variations in the method conditions, and is an indication of the reliability of the method. By introducing small changes in the mobile phase composition, mobile phase volume, duration of mobile phase saturation, and activation of the pre-washed TLC plates with methanol, the effects on the results were examined. Robustness of the method was performed in triplicate at a concentration level of 100 ng $spot^{-1}$ and 200 ng $spot^{-1}$ and the % R.S.D and S.E. of peak areas was calculated.

RESULTS AND DISCUSSION

Lack in standardization and validation of suitable analytical methods for simultaneous estimation of active constituents in the drug are problems for acceptability of traditional medicine. In case of natural product analysis, HPTLC is more widely used than other chromatographic methods. In the present article, an attempt has been made to develop and validate new, precise, accurate, and robust HPTLC method for concurrent quantification of gallic acid in formulation with Haritaki. Results obtained indicate the reliability of the proposed densitometric method**.**

Optimization of HPTLC Method

To obtain the desired Rf value range, minimum resolution, different individual solvents as well as a combination of solvents were tried. Initially the solvent system used was butanol, glacial acetic acid, and water, in the ratio 4:1:1, but the plate was not well resolved. Next, toluene, ethyl acetate, formic acid, and water in varying ratios were tried. The mobile phase toluene: ethyl acetate: formic acid (5:4:1) was selected for obtaining well separated peaks and gave a good resolution with retention factors (R_f) = 0.27 for Gallic acid. The wavelength used for detection and quantification was 540 nm [Figure 1].

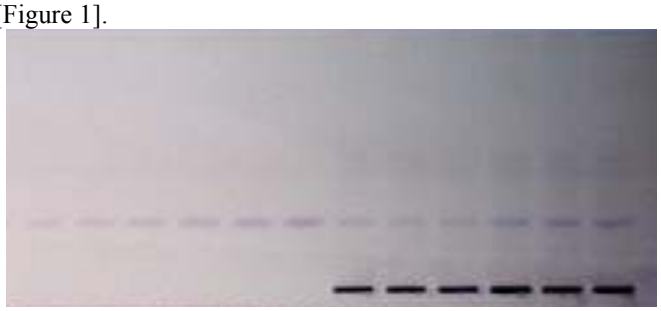

Figure 1: Photo documentation of Gallic acid at visible light
Tracks: 1-6 Standards, 7-8 Haritaki sample
Mobile phase: toluene: ethyl acetate: formic acid (5:4:1)
Derivatising reagent: Anesaldehyde- Sulphuric acid
Detection: Visible light

Quantification of Marker present in Haritaki

The content of active compounds was quantified using calibration curve of Gallic acid. The gallic acid used for quantification was found well separated by proposed method in Haritaki samples. The percent content of gallic acid in Haritaki sample was found to be 1.95% and the calibration results per analysis obtained were shown in Table-1.

Table 1: Quantification of Gallic acid (Calibration results per analysis)

Substance	Rf	X(average)	CV (%)	n	Regression	%w/w GA
Gallic acid	0.27	488.85 ng	17.816	6	Linear	1.95%

Linearity

Linear relationship was observed by plotting marker concentration against peak area obtained. The present HPTLC method has shown a calibration curve in the concentration range of 100-800 ng/spot for Gallic acid. The correlation coefficient of Gallic acid was found to be 0.99931 .the peak area (y) is proportional to the concentration of Gallic acid following the regression equation Y= -117.4 +3.91*X. The standard deviation of intercept has been found to be 2.56%. No significant difference has been observed in the slopes of the standard curves [Fig-2].

Sensitivity

The minimum detectable limit was found to be 94.96ng/spot for gallic acid and minimum limit of quantification was found to be 426.47ng, indicating good sensitivity of the HPTLC method.

Precision

As recommended by ICH guideline, both intra and interday precision studies showed RSD<2%, indicating good precision [Table-2].

Accuracy and Recovery

Recovery of 99.86% for Gallic acid indicates that the proposed densitometric method is reliable for the estimation of Gallic acid in the formulation with same drug [Table-3].

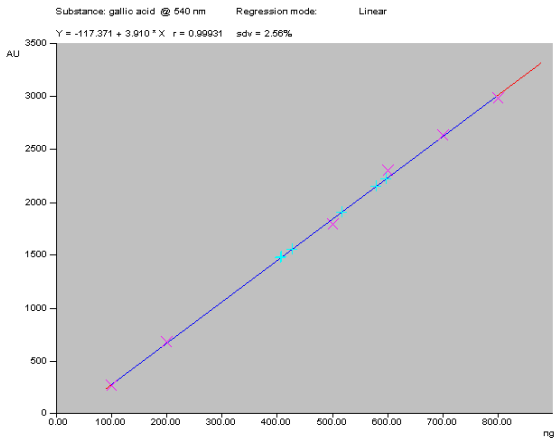

Figure 2: Calibration curve for Gallic acid

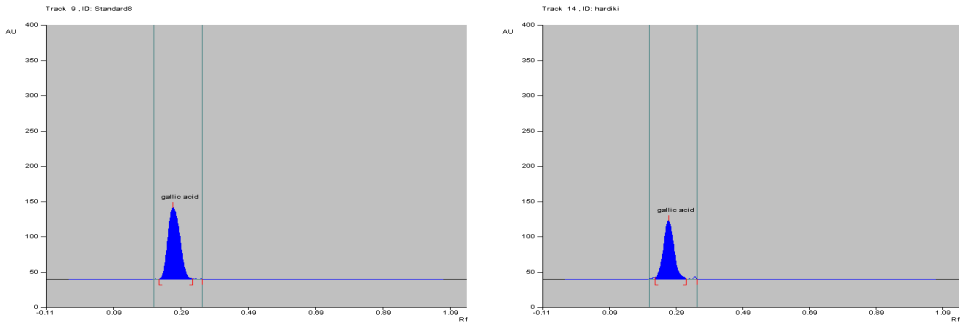

Figure 3: Chromatograms of standard Gallic acid and Haritaki

Specificity

It was observed that other constituent's presents in the drug did not interfere with the peak of Gallic acid. The overlay spectrum of standard Gallic acid corresponds with sample. Therefore the method was specific [Fig-4].

Robustness of the method

Robustness of the densitometric method was checked after deliberate alternations of the analytical parameters [Table-4]. It showed that area of peak of interest remained unaffected by small changes of operational parameters (%RSD<2) which indicate robustness of the method.

Table 2: Inter and intraday precision of HPTLC method (n=6) for Gallic acid

Amount ng/spot	Inter day precision	%RSD	Intraday precision	%RSD
200	672.323	0.29297	671.7967	0.71430
500	1791.807	0.28746	1790.897	0.77313
700	2629.557	0.03214	2629.373	0.55320

RSD= Relative Standard Deviation, each result is an average of three measurements, all values are mean ±SD

Table 3: Recovery studies for Gallic acid in Haritaki (n=6)

Sample	Amount of marker added (%)	Amount recovered (ng)	Recovery (%)	RSD (%)	Average recovery (%)
Haritaki	100	99.92	99.90	0.145	99.86
	200	199.32	99.76	0.189	
	300	301.34	100.10	0.576	

RSD= Relative Standard Deviation, each result is an average of three measurements, all values are mean ±SD

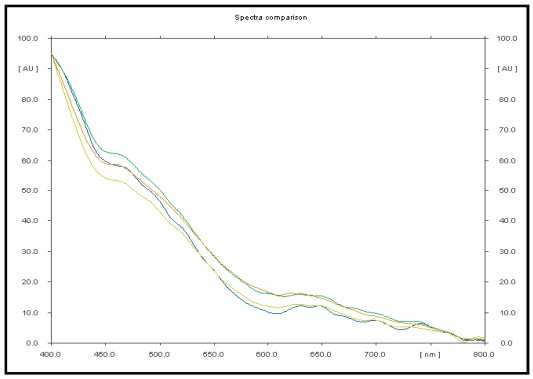

Figure 4: Overlay spectrum of standard Gallic acid and Haritaki

Table 4: Results from Robustness testing

Amount ng/spot	Change in mobile phase (% RSD)	
	Toluene: ethyl acetate: formic acid (7: 2.5.: 0.5 v/v).	Toluene: ethyl acetate: formic acid (7:2:1 v/v).
100	0.96	1.10
200	0.97	0.99
	Change in saturation time (% RSD)	
	20 min	30 min
100	0.96	1.10
200	0.97	0.99

Each result is an average of three measurements. All values are mean ±SD

CONCLUSION

Herbal medicine being generally harmless in prescribed doses are becoming popular all over the world and WHO also currently encourages, recommends and promotes the inclusion of herbal drugs in national health care programs. Standardization of Haritaki as raw material is important as per analysis is concerned. The interest in TLC has increased with the improvements in TLC instrumentation and methods and further in the last few years with the development of new MS methods for detection. The developed HPTLC method will assist in the standardization of Haritaki using chemical markers. The overlay spectrums of standard gallic acid with sample were found to be similar and the method was found to be rapid, simple, precise, specific, reproducible, selective and accurate. Since this proposed validated method resolves and quantifies gallic acid effectively so it can be used routinely to quantify the concentration of the active principle in any herbal formulations with Haritaki. There are significant data in the literature which proves that this drug contains a number of markers that may be responsible for its therapeutic activity and a number of other constitute, which are currently the subject of further investigation.

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