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HPTLC Method for estimation of Gallic acid and Rutin in Haritaki -An Ayurvedic Formulation

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Abstract: Standardization of Herbal Formulation is essential to assess the quality of drugs based on the concentration of their active principle. Haritaki possess a wide variety of activities like antimicrobial, antioxidant, antispasmodic and antipurgative. In the present study, an attempt has been made to develop simple, precise and accurate HPTLC method for Haritaki on Laboratory Formulation (LF) and it is compared with different Marketed Formulation (MF_1, MF_2, MF_3, MF_4) by estimating Gallic acid and Rutin as a marker compound. The Five different Formulations were subjected to methanolic extract by Maceration process for 7 days. The detection and quantification of Gallic acid and Rutin were performed at 280 nm and 254 nm respectively. The linearity of Gallic acid and Rutin were found to be 100-500 ng/spot and 1000-5000 ng/spot. The correlation coefficient of Gallic acid and Rutin was found to be 0.9967 and 0.9974 respectively. The Percentage recovery of Gallic acid and Rutin were found to be 99.12%w/w and 97.85%w/w respectively which is highly satisfactory. The LOD and LOQ of Gallic acid were found to be 70.96 ng/spot and 212.89 ng/spot respectively. The LOD and LOQ of Rutin were found to be 63.19 ng/spot and 189.57 ng/spot respectively. The overlay spectrums of standard Gallic acid with sample (LF and MF) were found to be similar and the method was found to be very specific. Since this proposed validated method resolves and quantifies Gallic acid and Rutin effectively, it can be used routinely to quantify the concentration of both the active principles in the herbal formulation
Keywords: HPTLC, Haritaki, Rutin, Gallic acid, Laboratory Formulation and Marketed Formulation.

1. Introduction:

Terminalia Chebula has been extensively used in ayurveda, unani & homoeopathic medicine and has become cynosure of modern medicine. The Sanskrit name 'Haritaki' is rich with meaning, reffering to the yellowish dye (harita) that contains the god Siva (Hari, i.e. the Himalayas) and that it cures (harayet) all the disease. Its other commonly used Sanskrit name, Abhaya, refers to the 'fearlessness' it 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[1, 2]. *T. Chebula* possesses a wide variety of activities like antimicrobial, antioxidant, antiviral, anticarcinogenic, hypocholesterolemic, radio - protective, antispasmodic & antipurgative[3]. Gallic acid is phenyl propanoid, chemically it is 3, 4, 5,- Trihydroxybenzoic acid, and possess astringent activity. Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. To date about 300 varieties of flavonoids are known[4]. Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity. Rutin, 5, 7, 3', 4', tetrahydroxy flavonol -3-rhamnoglucoside and quercetin 5, 7, 3', 4', - tetrahydroxy flavonol exhibit anti-inflammatory, anti hepatotoxic, antiulcer, antiallergic and antiviral actions and some of them provides protection against cardiovascular mortality. Both possess antioxidant activity and reduce low density lipoproteins (LDL) oxidation[5]. Phytochemical evaluation is one of the tools for the quality

assessment, which includes preliminary phytochemical screening, chemoprofiling 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 phytochemical analysis of herbal drugs and formulations[6].

A suitable, sensitive, and reliable quantitative high-performance thin-layer chromatographic (HPTLC) method has been developed for the first time for quality control determinations of gallic acid and Rutin in Terminallia chebula. Presently, this technique is being used extensively for quantification of these compounds in plant extracts and marketed formulation because of its simplicity, accuracy, cost effectiveness, and rapidity.

2. Experimental Methods

2.1. Instrumentation

Analysis was performed on a Camag HPTLC system equipped with a sample applicator Linomat V, twin trough development chamber (10x10) size, TLC Scanner III, Wincats integration software was used.

2. 2. Reagents and Chemicals

Analytical grade Toluene, Ethyl acetate, Methanol, Formic acid, Chloroform, glacial acetic acid was obtained from SD fine chem. Ltd, Mumbai. Pure Gallic acid and Rutin were obtained from the Natural Remedies Ltd, Bangalore. Precoated TLC aluminium sheets silica gel $60F_{254}$ (10 x10 cm, 0.2 mm thick) were obtained from E. Merck Ltd, Mumbai.

2.3. Collection of Raw Material

Terminalia chebula (Haritaki) were collected from the local market and physically authenticated with herbarium specimens present in Pharmacognosy laboratory for its genuine. The Marketed Formulations Haritaki (MF_1, MF_2, MF_3, MF_4) were obtained from local Ayurvedic pharmacy, Coimbatore, Tamilnadu.

2.4. Preparation of Standard Gallic acid and Rutin Solution

10 mg of Gallic acid and Rutin were accurately weighed into 10 ml volumetric flask, and then the solution was made up to 10ml with methanol (1mg mL^{-1})[7]. From the Stock solution of gallic acid 1ml was pipette out and further diluted upto 10 ml to obtain the final concentration of 100 µg/ml.

2.5. Preparation of Extracts

The dried fruit pulp of Terminalia chebula the Laboratory Formulation (5 gm) and different Marketed formulation sample (5 gm) of Haritaki were extracted by Maceration process in 100ml of methanol for 7 days. The methanolic extract was filtered through Whatmann Filter paper No.1 and then concentrated at low temperature. The stock solutions were further diluted to produce a uniform concentration of 10 mg mL^{-1} for both the samples.

2.6. Chromatographic Conditions

Samples of methanolic extracts of LF and CF of Haritaki and standard Gallic acid and Rutin were spotted on a Precoated TLC aluminium sheets silica gel 60 F_{254} (10x10cm, 0.2mm thickness) as 8mm wide band width by using automatic TLC applicator Linomat V,10mm from the bottom. The Mobile phase used was Toluene: Ethyl acetate: Formic acid (3:6:1v/v) for Gallic acid and Chloroform: Ethyl acetate: Methanol: Formic acid (3.5:5:0.5:1 v/v) for Rutin.The plates were kept for saturation in twin trough chamber for 15min. After development the plates were dried in air and scanned at 280 nm for Gallic acid and 254 nm for Rutin by using CAMAG Scanner III. The plates were photographed at 254 nm by using CAMAG Reprostar instrument and shown in **(Fig.1, 2).**

2.8. Calibration Curve for Standard Gallic acid

The standard solutions (100-500 ng/spot) were applied on TLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above. The peak areas were recorded. Calibration curve of gallic acid was prepared by plotting peak area against concentration of gallic acid applied.

2.9. Calibration Curve for Standard Rutin

The standard solutions (1000-5000 ng/spot) were applied on TLC plate and further it was developed and scanned as per the chromatographic conditions mentioned above. The peak areas were recorded. Calibration curve of rutin was prepared by plotting peak area against concentration of rutin applied.

Table 1. Quantification of Gallic acid and Rutin in Haritaki Formulations.

Sample	Amount Present	
	Gallic acid (%w/w)	Rutin (%w/w)
LF	0.9936	0.9452
MF1	0.9732	0.9154
MF2	0.9689	0.8907
MF3	0.9849	0.9281
MF4	0.9901	0.9361

Table 2. Recovery studies for Gallic acid and Rutin in Haritaki.

Compound	Contents (mg)	Amount added (mg)	Recorded amount (mg)	Recovery (%)
Gallic acid		2	6.149	97.99
	4.128	4	8.269	99.29
		6	10.421	99.76
Rutin		2	4.721	97.81
	2.889	4	6.291	96.42
		6	8.194	99.32

Table 3.Summary of Validation parameter

PARAMETERS	GALLIC ACID	RUTIN
Linearity		
i) Range	100-500 ng/spot	1-5 µg/spot
ii)Correlation Coefficient	0.9967	0.9973
iii) Rf Value	0.41	0.13
Precision (%RSD)		
i) Intra-day	0.9013	0.4729
ii) Inter-day	0.604	0.7254
iii) Repeatability	0.9769	0.5959
LOD	70.96 ng/spot	63.19 ng/spot
LOQ	212.89 ng/spot	189.57 ng/spot
Ruggedness (%RSD)	0.9416	0.8114
Specificity	Specific	Specific
Robustness	Robust	Robust
Plate Efficiency	24.88 mm	24.88 mm
Flow constant	4.9 mm^2/s	6.125 mm^2/s

Fig.1. Photo documentation of Gallic acid at 254 nm

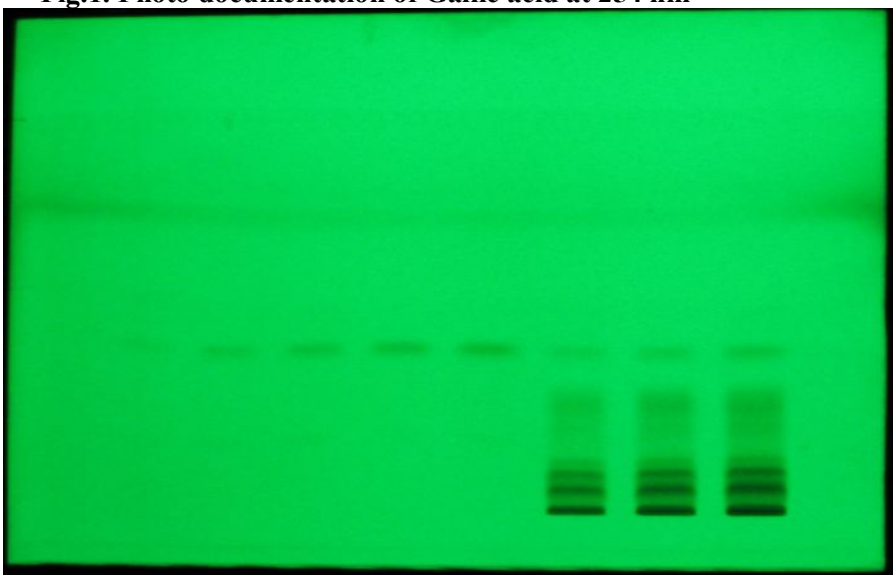


Fig.2. Photo documentation of Rutin at 254 nm

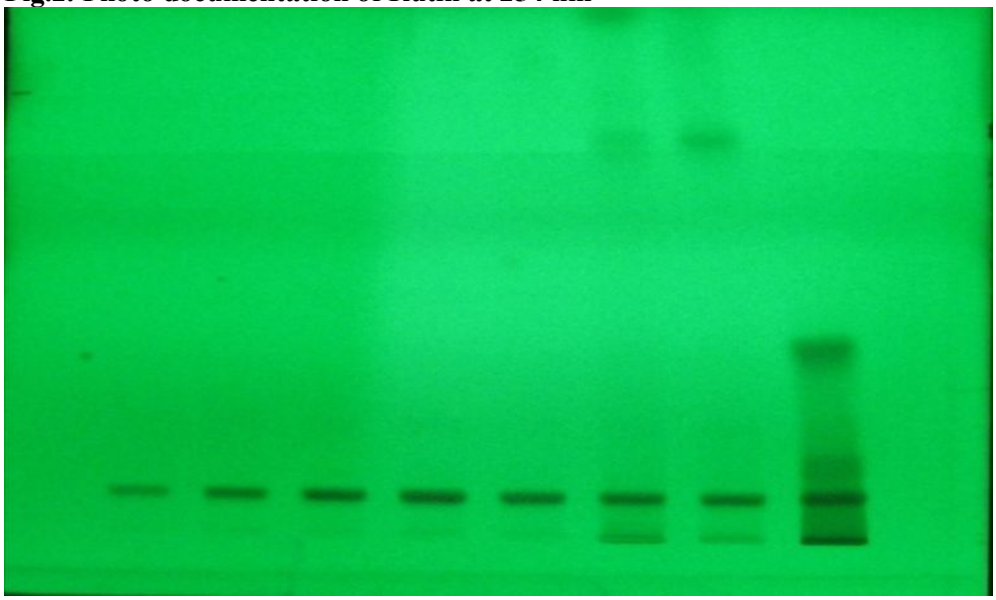


Fig.3. Calibration curve for gallic acid

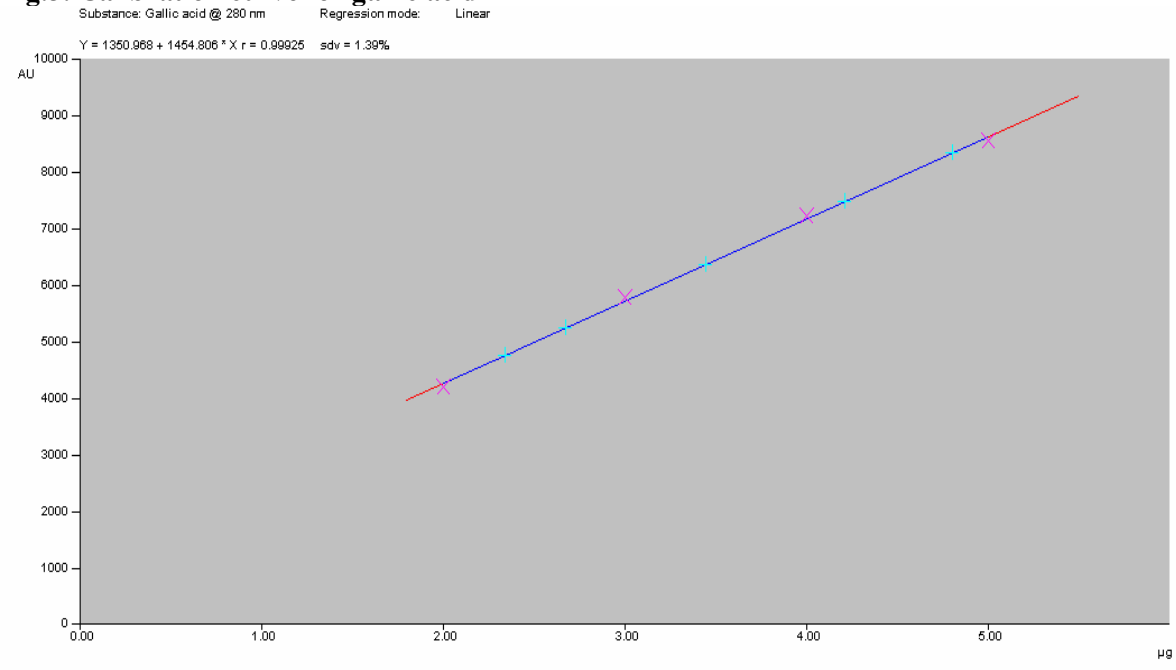


Fig.4. Calibration curve for Rutin

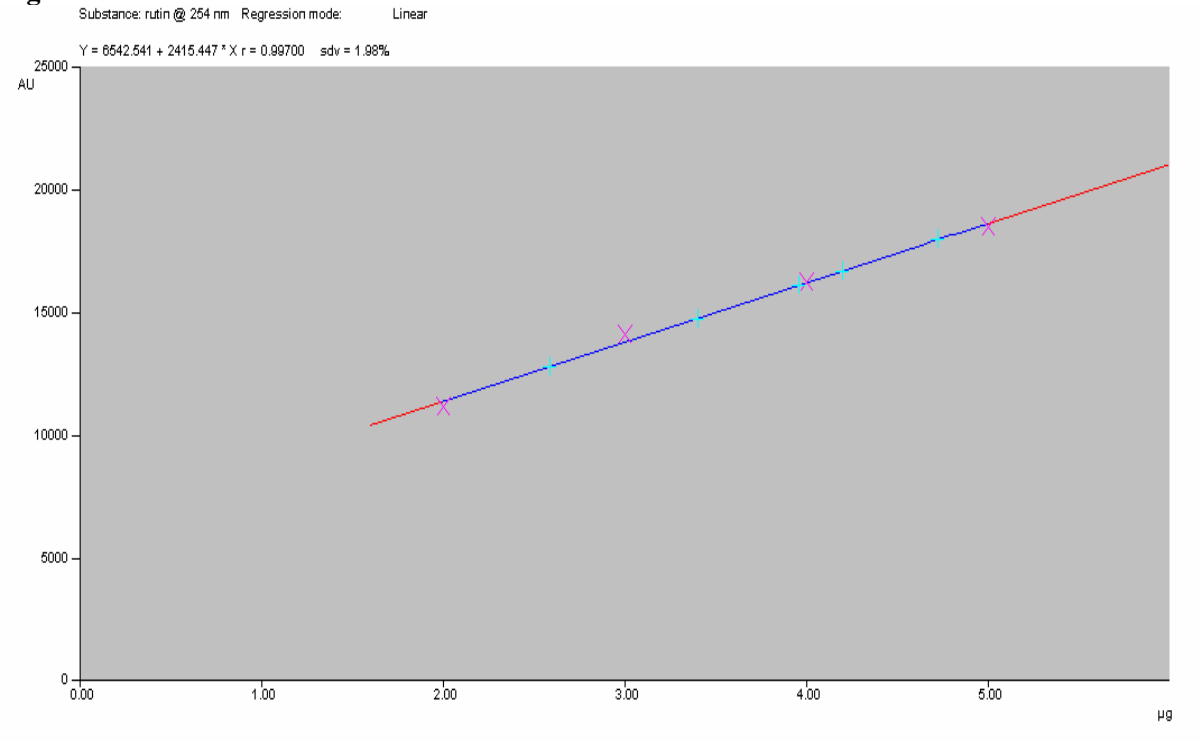


Fig.5. Chromatogram of standard gallic acid

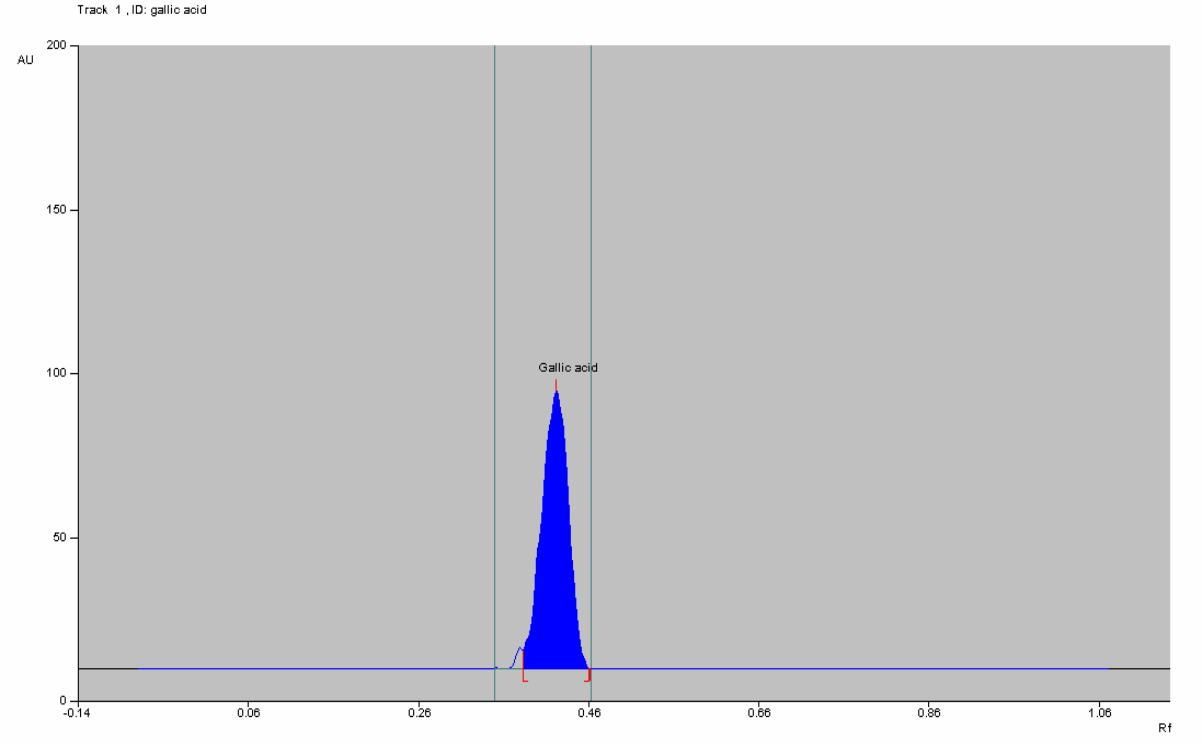


Fig.6. Chromatogram of standard Rutin

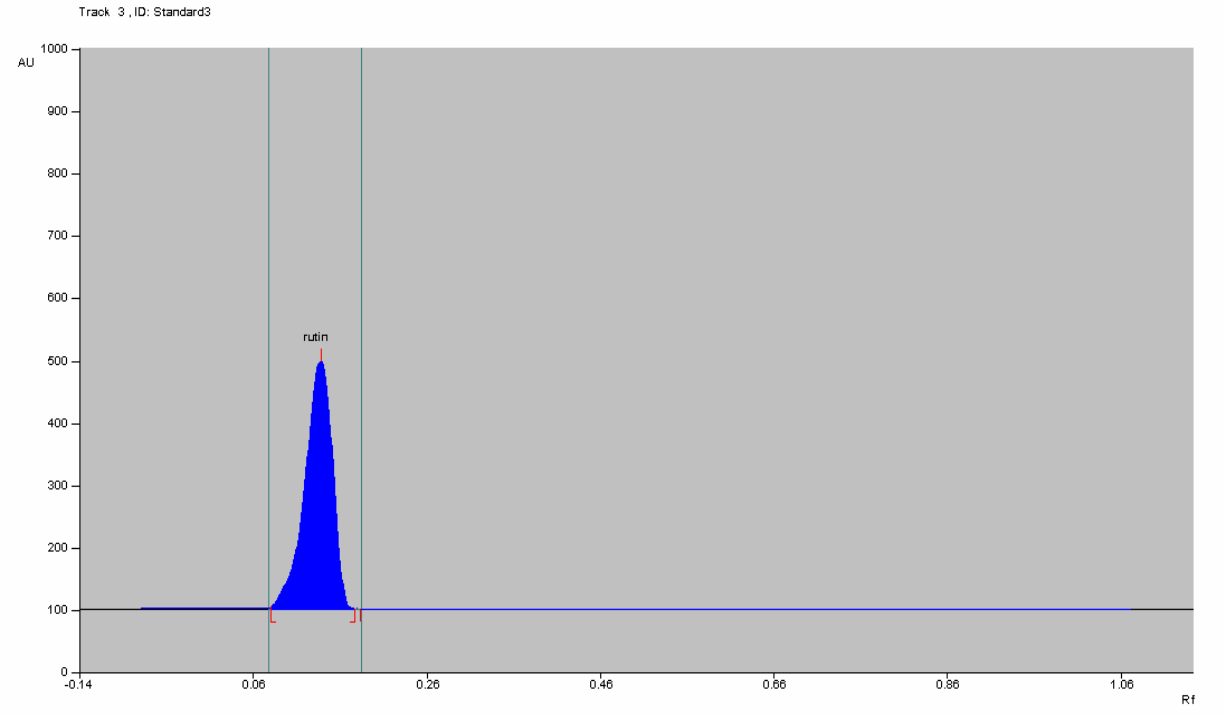


Fig.7. Chromatogram of Laboratory Formulation Haritaki (Gallic acid)

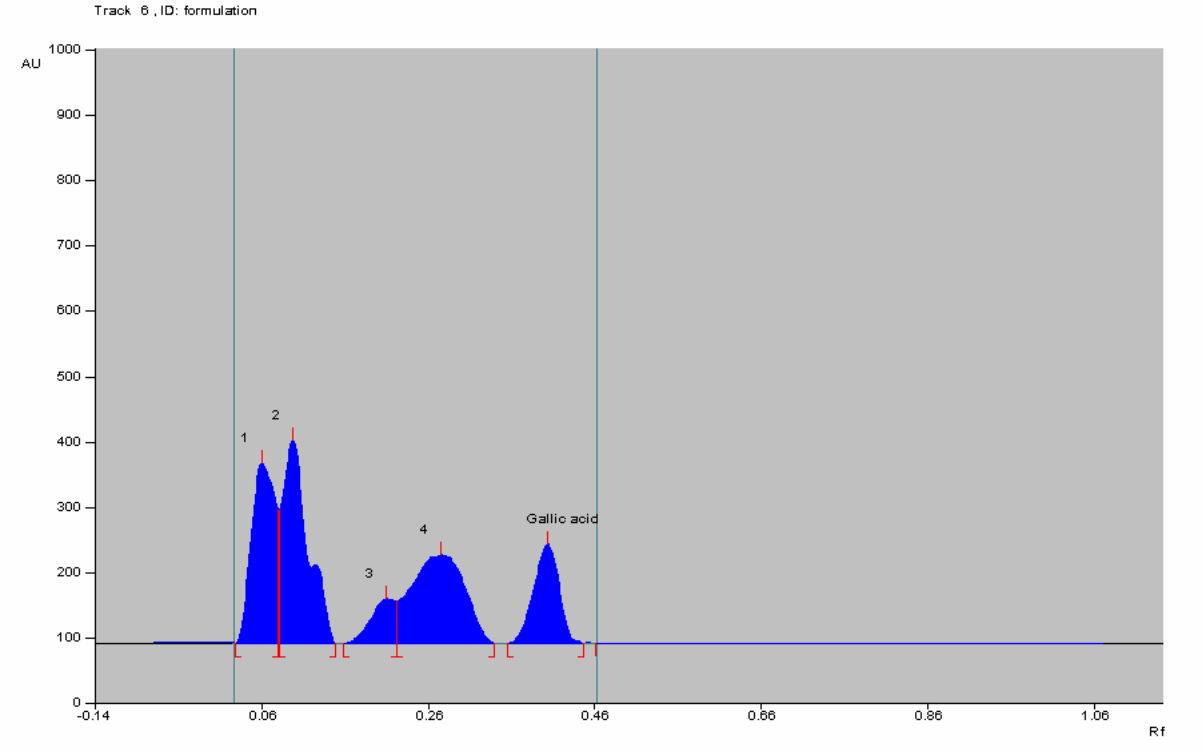


Fig.8. Chromatogram of Marketed Formulation Haritaki 1 (Gallic acid)

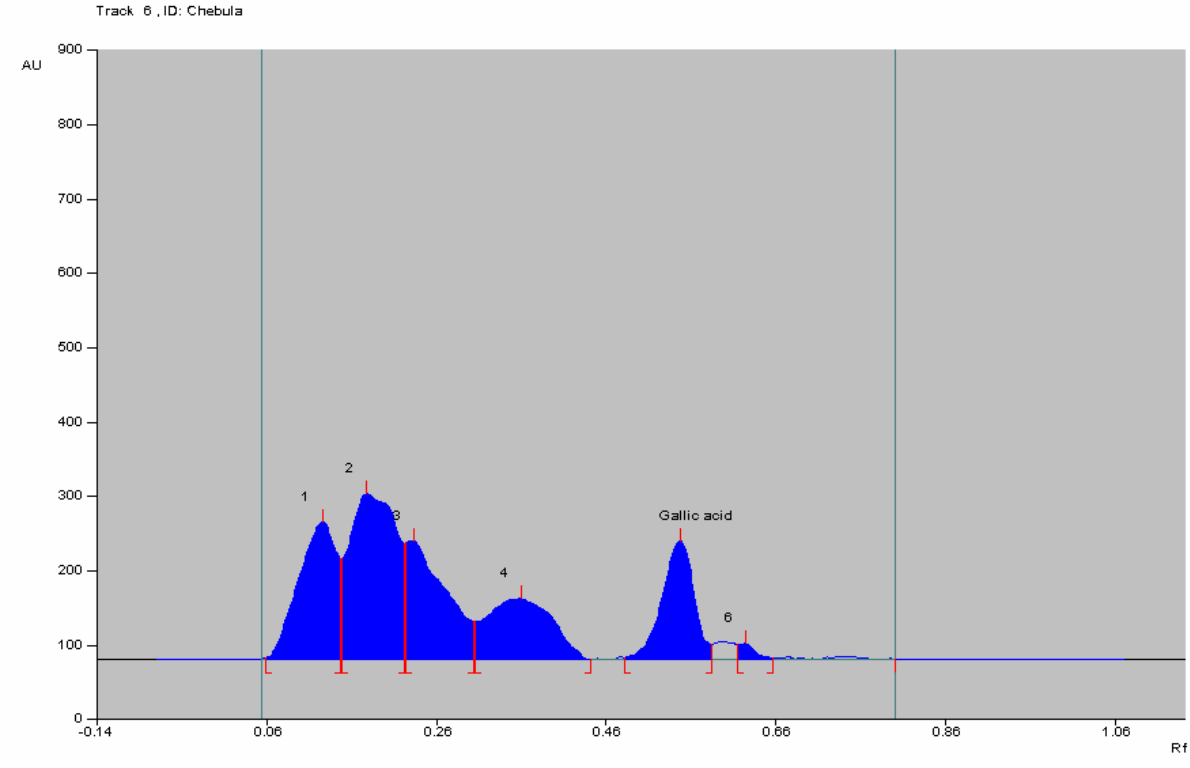


Fig.9. Chromatogram of Marketed Formulation Haritaki 2 (Gallic acid)

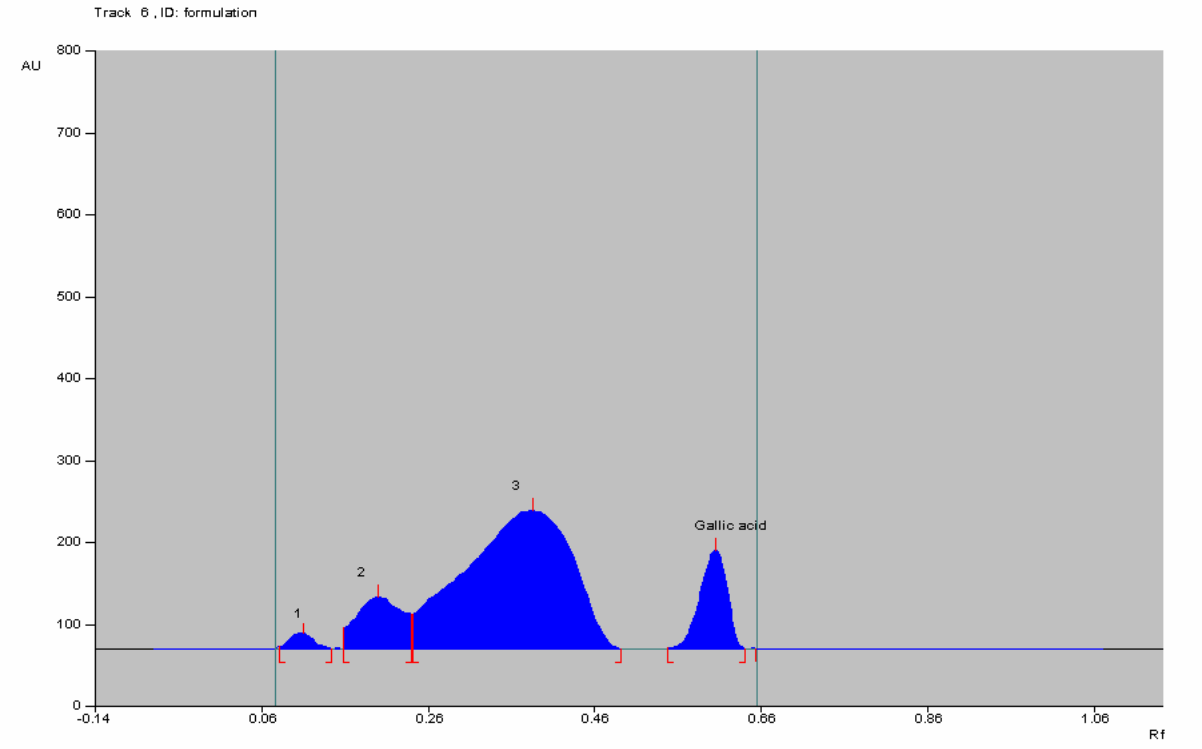


Fig.10. Chromatogram of Marketed Formulation Haritaki 3 (Gallic acid)

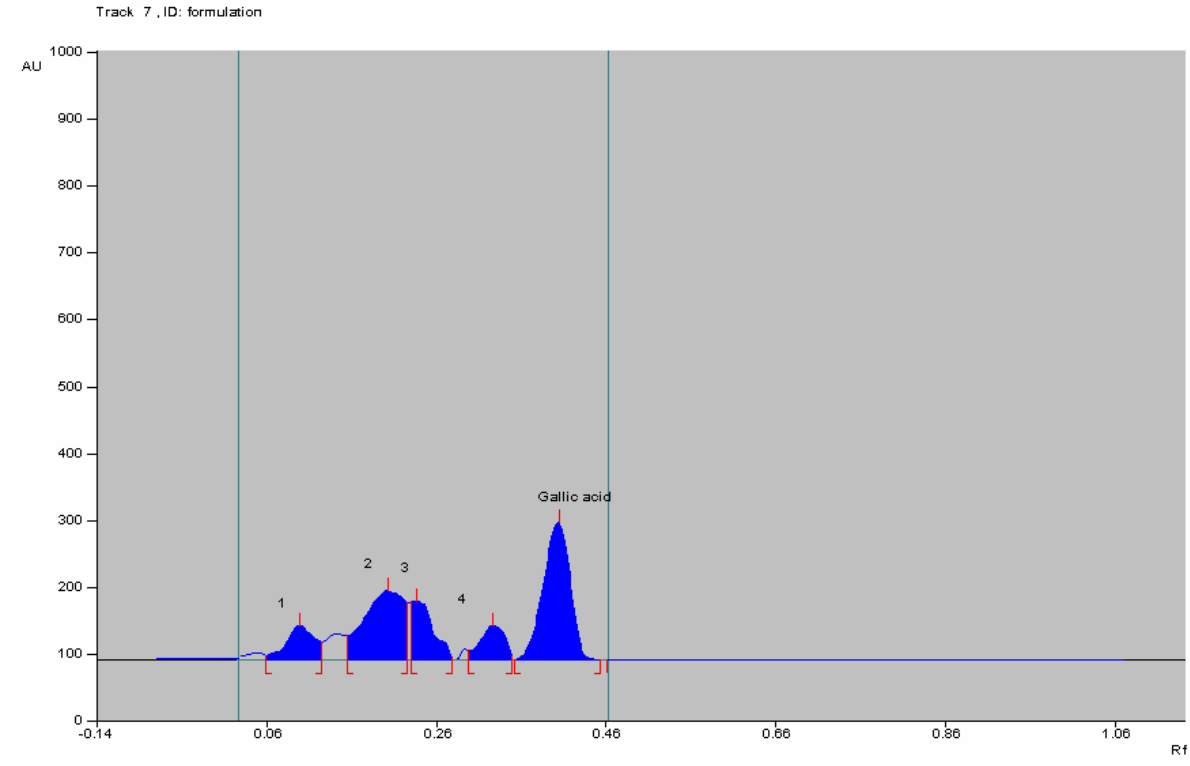


Fig.11. Chromatogram of Marketed Formulation Haritaki 4 (Gallic acid

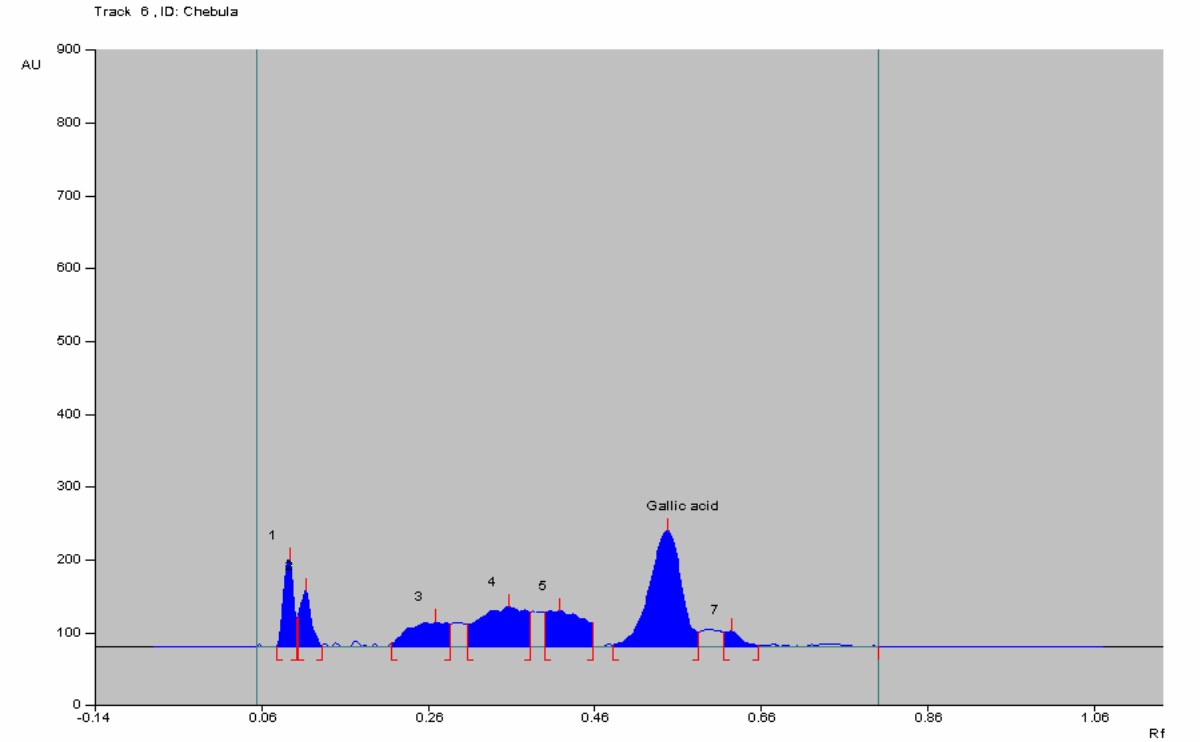


Fig.12. Chromatogram of Laboratory formulation Haritaki (Rutin)

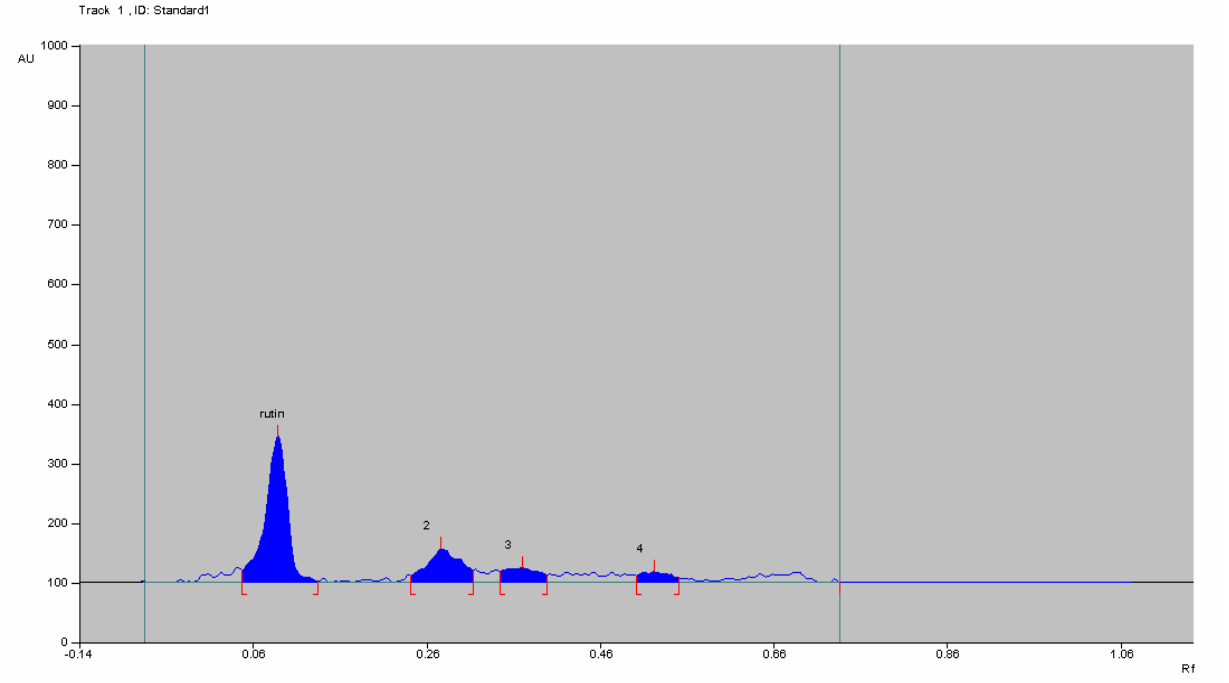


Fig.13. Chromatogram of Marketed Formulation Haritaki 1(Rutin)

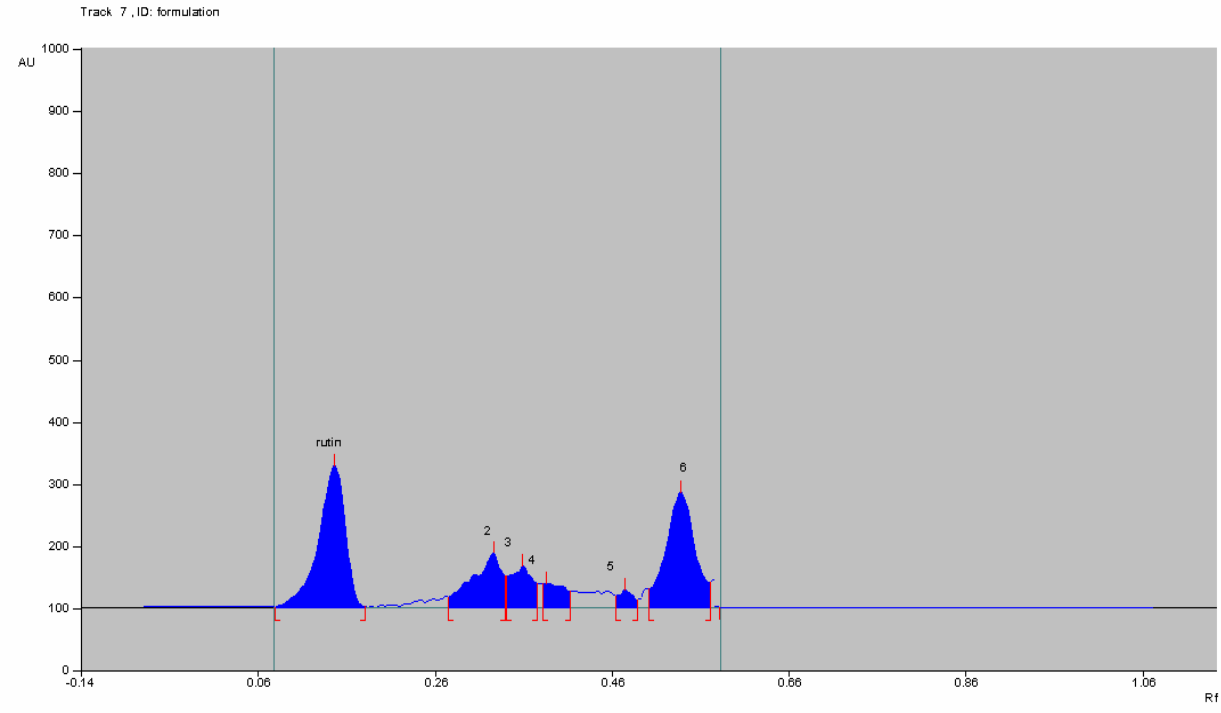


Fig.14. Chromatogram of Marketed Formulation Haritaki 2 (Rutin)

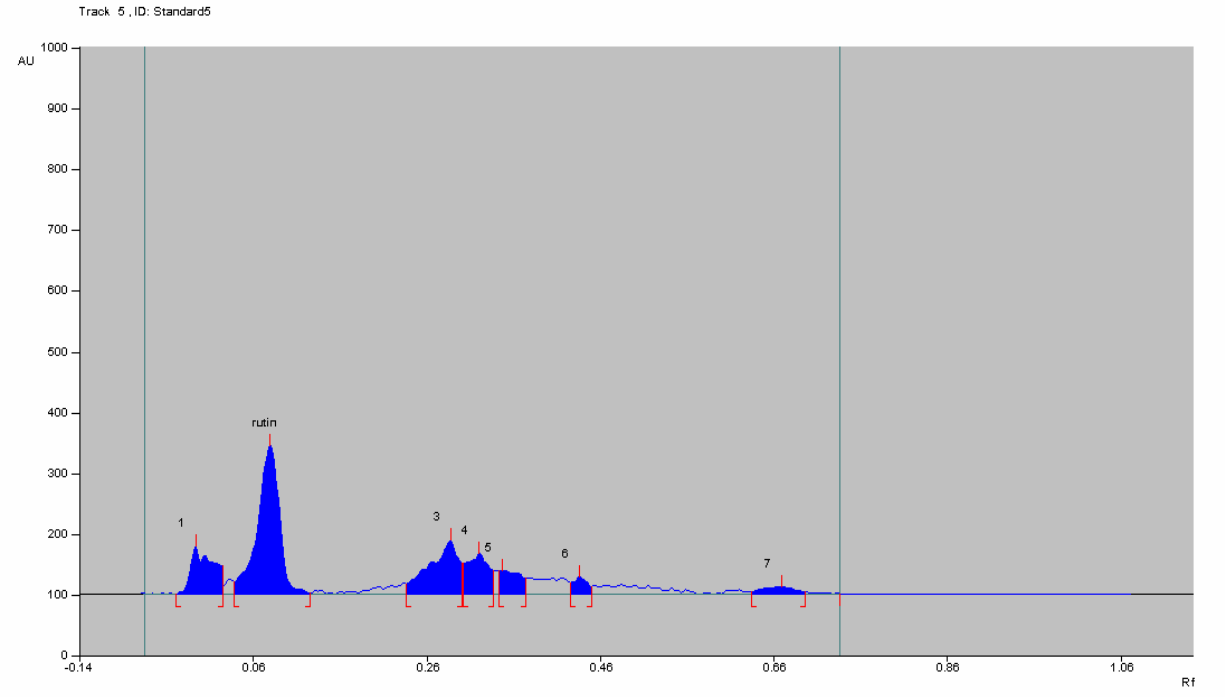


Fig.15. Chromatogram of Marketed Formulation Haritaki 3 (Rutin)

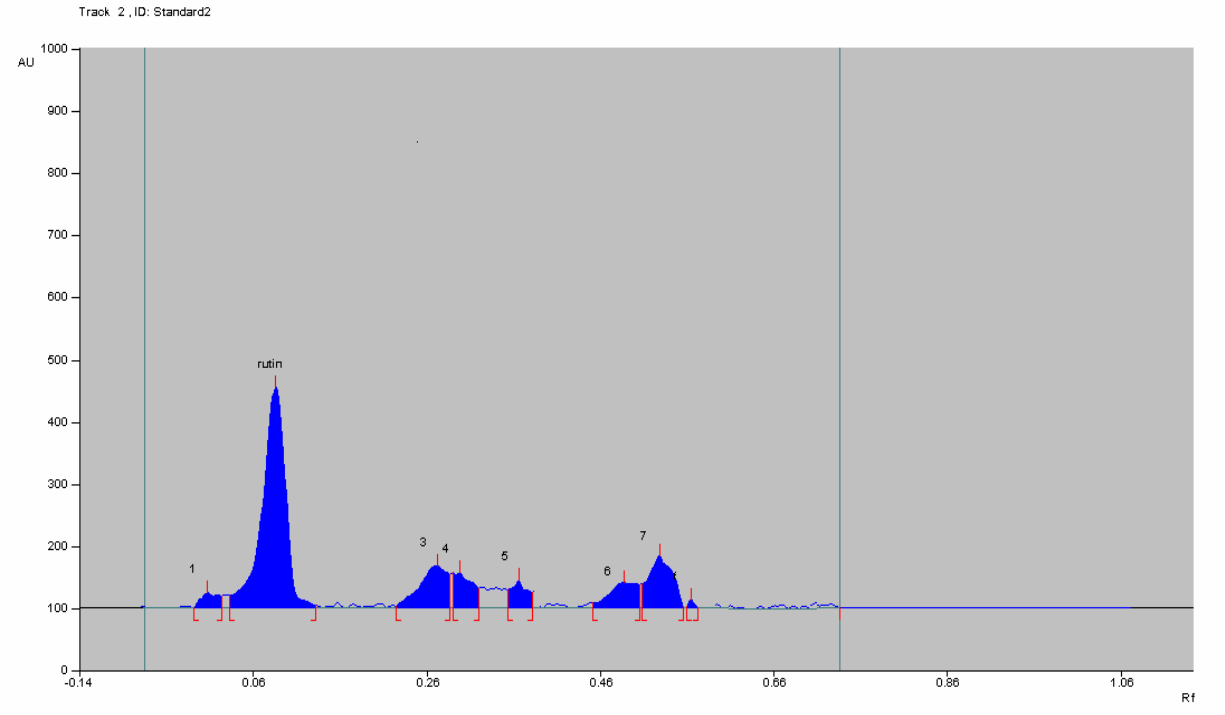


Fig.16. Chromatogram of Marketed Formulation Haritaki 4 (Rutin)

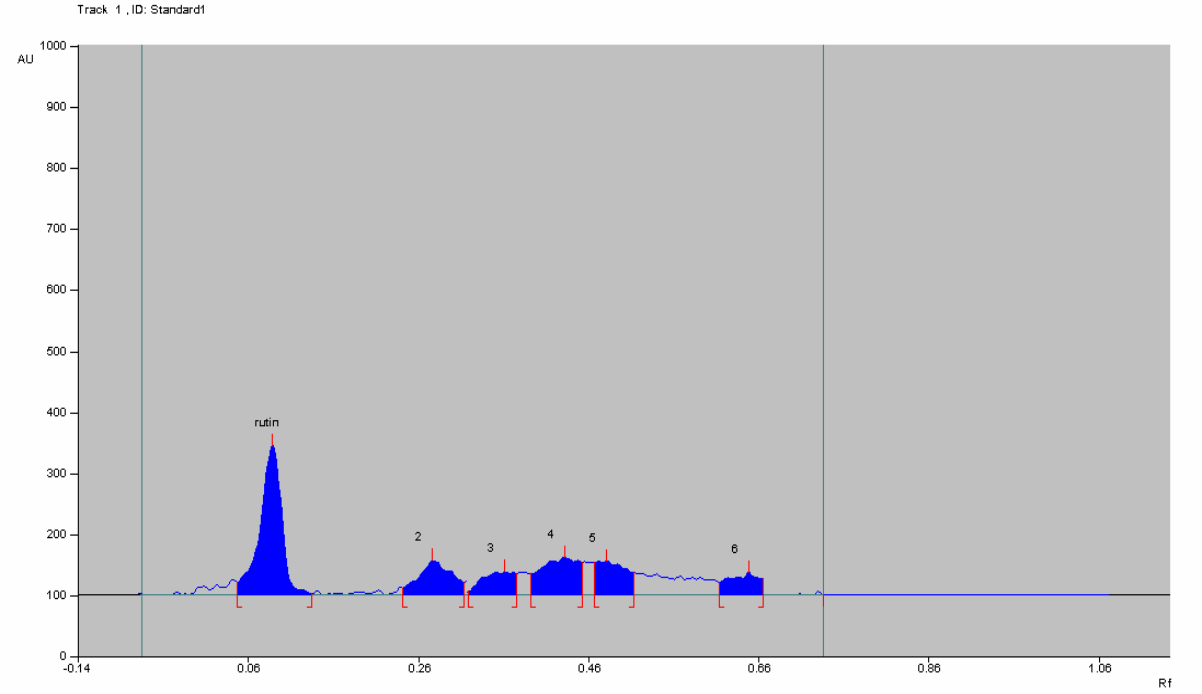


Fig.17. Overlay Spectrum of Gallic acid

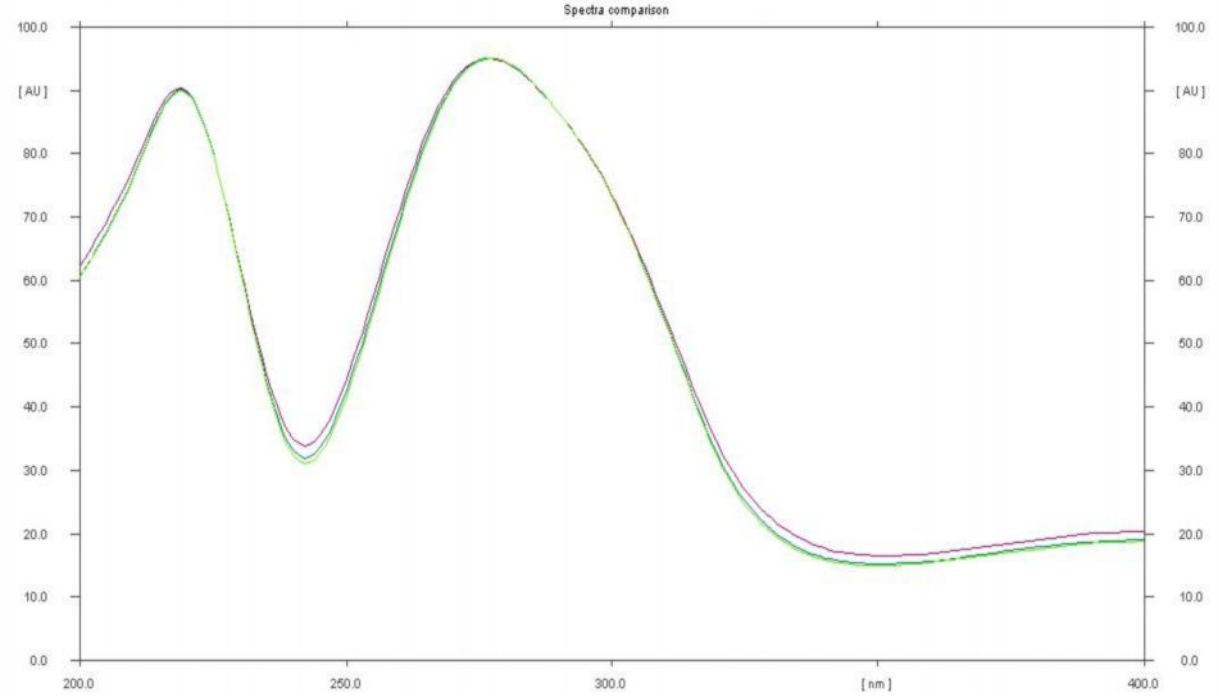
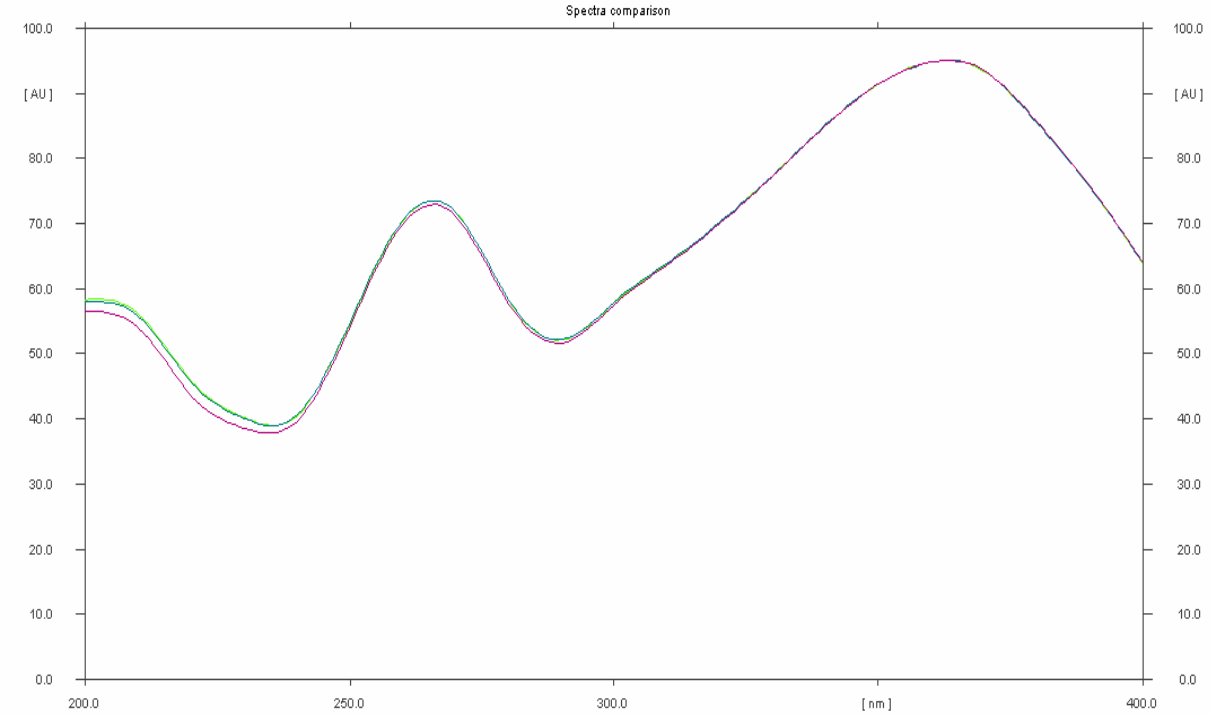


Fig.18. Overlay Spectrum of Rutin



3. Results and Discussion

3.1. Optimization of HPTLC Chromatographic Conditions:

Standard Gallic acid (RF: 0.41) and Rutin (RF: 0.13) showed single peaks in HPTLC chromatogram. The amount of Gallic acid and Rutin present in the above formulation were computed from the above calibration curve. The linearity of Gallic acid and Rutin were found to be 100-500 ng/spot and 1-5 µg/spot. The correlation coefficient of Gallic acid and Rutin was found to be 0.9967 and 0.9974 respectively. The Percentage recovery of Gallic acid and Rutin were found to be 99.42%w/w and 97.14%w/w respectively which is highly satisfactory. The LOD and LOQ of Gallic acid were found to be 70.96 ng/spot and 212.89 ng/spot respectively. The LOD and LOQ of Rutin were found to be 63.19 ng/spot and 189.57 ng/spot respectively. The method was found to be very specific. Since the overlay spectrums of standard Gallic acid with sample (LF and MF) were found to be similar.

3.2. Quantification of Markers present in Haritaki Formulations:

The contents of the active compounds were quantified using calibration curve (Fig.3, 4) of each marker individually (Fig.5, 6). The two markers used for quantification were found and well separated by proposed method in Haritaki formulations (Fig.7-16). The Result obtained was shown in (Table1).

3.3. Validation of the Proposed Method

ICH guidelines were followed for the validation of the analytical methods developed (precision, repeatability, accuracy, Ruggedness, Robustness, Plate efficiency, Flow constant[8]. Summary of validation parameters was listed out in (Table 3).

3.3.1. Linearity:

A representative calibration curve of gallic acid and rutin were obtained by plotting the peak area of gallic acid and rutin against the concentration of gallic acid (100-500 ng/spot) and Rutin (1-5 µg/spot), respectively. The correlation coefficient of gallic acid and rutin were found to be 0.9967 and 0.9973, respectively and thus exhibits the good linearity between concentration and peak area.

3.3.2. Inter-day and intra-day precision:

The inter-day precision (RSD) was determined by analyzing standard solution of Gallic acid and Rutin over the entire calibration range for three different days. The intra-day precision (RSD) was determined by analyzing standard solution of Gallic acid and Rutin over the entire calibration range for three times on the same day

3.3.3. Repeatability:

Repeatability of measurement of peak area and peak height: Standard Gallic acid and Rutin solution was spotted on a TLC plate, developed and dried. The separated spot was scanned for six times without

changing plate position and RSD for measurement of peak area was computed.

3.3.4. Limit of Detection:
The minimum detectable limit was found to be 70.96 ng/spot for gallic acid and 63.19 ng/spot for rutin.

3.3.5. Limit of Quantification:
The minimum quantified limit was found to be 212.89 ng/spot for gallic acid and 189.57 ng/spot for rutin.

3.3.6. Specificity:
It was observe that other constituents presents in the formulation did not interfere either with the peak of gallic acid and rutin. Therefore the method was specific. The overlay spectrum of standard gallic acid and rutin spots and gallic acid and rutin spots present in the samples were found to be similar or overlap. The peak purity of the gallic acid was assessed by comparing the spectra at three different levels, viz. peak start, peak apex and peak end positions of the spot. The overlay spectrum was shown in (Fig 17, 18).

3.3.7. Robustness of the method:
By introducing small changes in the mobile phase composition, mobile phase volume and duration of mobile phase saturation, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 2µg/spot and the %R.S.D. of peak area was calculated.

3.3.8. Ruggedness of the method:
It expresses the precision within laboratories variations like different days, different analyst, and different equipment. Ruggedness of the method was assessed by spiking the standard 6 times in two different days with different analyst.

3.3.9. Plate efficiency (N):
Plate efficiency, also known as number of theoretical plates was calculated for the described method by the following equation:

$$N = \frac{16 \times l \times z}{w^2}$$

Where, l is the distance (in mm) traveled by solvent front, z is the distance (in mm) traveled by the target spot from application point and w is the width of spot (in mm) in the direction of mobile phase ascending. The plate efficiency was calculated for gallic acid and rutin and it was found to be 24.88 mm respectively.

3.3.10. Flow constant:
The Flow constant or velocity constant (k) is a measure of the migration rate of the solvent front. It is an important parameter for TLC users and can be used to calculate, for example, development times with different separation distances, provided that the sorbent, solvent system, chamber type and temperature remain constant. The flow constant is given by the following equation:

$$k = \frac{Z_F^2}{t}$$

Where, k is flow constant [mm^2/s], Z_F is distance between the solvent front and the solvent level [mm] and t is the development time [s]. The flow constant as calculated by this method was found to be 4.9 $mm^2\ s^{-1}$ and 6.12 $mm^2\ s^{-1}$.

3.3.11. Accuracy: The accuracy was determined by standard addition method. To a fixed amount of pre-analyzed sample of Haritaki, increasing amount of standard Gallic acid and Rutin were added in all the levels of calibration curve. The Percentage recovery of Gallic acid and
Rutin were calculated at each level (n = 3) and shown in (Table 2).

4. Conclusion:

Standardization of Haritaki as well as raw materials is important as per analysis of formulation is concerned. The results indicate that Haritaki contains a number of markers that may be responsible for its therapeutic activity. The developed HPTLC method will assist in the standardization of Haritaki using biologically active chemical markers. The marker content of laboratory Haritaki was found higher than that of market samples of Haritaki by HPTLC method, may be due to use of inferior quality of materials in the preparation of tablets. The proposed Validated HPTLC method for Gallic acid and rutin from Haritaki seems to be accurate, precise, reproducible and repeatable. Haritaki also contained a number of other constitute, which are currently the subject of further investigation, apart from those standards studied. Also profiles of the individual components in Haritaki have been recorded as a standardization tool. With the growing demand for herbal drugs and with increased belief in the usage of herbal medicine, this standardization tool will help in maintaining the quality and batch to batch consistency of this important Ayurvedic preparation.

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