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HPTLC Method for Estimation of Ellagic Acid and Gallic Acid in *Triphala churanam* Formulations

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Abstract: Churanams are important group of formulations used by traditional physicians to treat various types of diseases. *Triphala churanam*, as per Siddha literature is used for the treatment of wounds and local ulcers. In the present study, an attempt has been made to develop a HPTLC (High Performance Thin Layer Chromatography) method of quantitative estimation of marker compounds, ellagic acid and gallic acid in laboratory prepared authentic formulation and a commercial formulation of *Triphala churanam*. The two formulations were subjected to methanol and ethyl acetate extractions by using Soxhlet apparatus. Ellagic acid and gallic acid were quantified in the above two extracts by using HPTLC. The detection and quantification were performed at a wavelength of 280 nm. The laboratory formulation was found to contain 0.201% w/w of ellagic acid and 0.656% w/w of gallic acid in methanol extract while it shows 0.573% w/w of ellagic acid and 2.664% w/w of gallic acid in the ethyl acetate extract. The commercial formulation shows 0.058% w/w of ellagic acid and 0.573% w/w of gallic acid in methanol extract and 0.422% w/w of ellagic acid and 1.637% w/w of gallic acid in ethyl acetate extract. Linearity studies indicated that ellagic acid and gallic acid were in the linear range of 125-500 ng and 1.25-5.00 μg, respectively, while the % recovery studies revealed a recovery of 99.2% w/w of ellagic acid and 98.13% w/w of gallic acid, thus proving the accuracy and precision of the analysis. Since this method resolves and quantifies ellagic acid and gallic acid effectively, it can be used to quantify the concentration of both the active principles in the herbal formulations.

Key words: HPTLC, *Triphala churanam*, ellagic acid, gallic acid, laboratory formulation, commercial formulation

INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the Ayurvedic or Siddha formulations is the lack of standard quality control profiles (Bagul and Rajani, 2005). The quality of herbal medicine, that is, the profile of the constituents in the final product has implication in efficacy and safety. 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 are expected to help in circumventing this problem.

Triphala churanam (TPC) is an herbal formulation used extensively in Siddha system of Indian Medicine, treating wounds and local ulcers. Since it contains enormous amount of tannins such as Ellagic Acid (EA) and Gallic Acid (GA), it is extensively used as an astringent (Kokate *et al.*, 1997). Standardization of Ayurvedic or Siddha formulations is the need of the day. Many of them do not have standard identification tests or analytical procedures to maintain their quality and purity (Patel, 1996).

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Hence, modern methods can be used to set up certain standards for the herbal formulations. *Triphala churanam* formulation consists of one part each of Katukkay tol (*Terminalia chebula*), Nellikay (*Embelica officinalis*) and Thanrikay (*Terminalia belerica*). The pharmacopoeial standards in Ayurvedic or Siddha Pharmacopoeia are not adequate enough to ensure the quality of plant drugs or their formulations. No work has been carried out in the estimation of marker compounds in the Siddha formulation of TPC. However, in a case study of Prabakara vati an Ayurveda formulation, ellagic acid and gallic acid were estimated by using methnol for extraction (Bagul and Rajani, 2005). In the present study, an authentic TPC formulation is compared with the commercial formulation of TPC by estimating the marker compounds, ellagic acid and gallic acid in both the formulations by using two different solvents. Therefore, the formulations were subjected to HPTLC analysis by developing a method for the determination of ellagic acid and gallic acid in the methanol and ethyl acetate extracts of laboratory prepared Authentic Formulation (LF) and a Commercial Formulation (CF) of *Triphala churanam*, since ellagic acid and gallic acid are the marker compounds present in high concentration in the churanam. The developed method is also utilized to determine the purity and quality of the market sample by comparing with the authenticated formulation. The proposed method has been validated as per ICH guidelines (ICH Q2A, 1994; Q2B, 1996).

MATERIALS AND METHODS

Equipment

A Camag HPTLC system equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scauner III, Reprostar and Wincats 4.02, integration software. (Switzerland).

Chemicals

Analytical grade toluene, ethyl acetate, methanol and formic acid were obtained from SD Fine Chem Ltd. (Mumbai, India). Pure ellagic acid and gallic acid were obtained from Natural Remedies Ltd., (Bangalore, India) as gift samples. Pre-coated silica gel 60 F_{254} TLC aluminium plates (10×10 cm, 0.2 mm thick) were obtained from E. Merck Ltd. (Mumbai, India).

Drugs

Terminalia chebula (Chebulic myrobalan), *Emblica officinalis* (Embelic myrobalan) and *Terminalia belerica* (Beleric myrobalan) were collected from the local market and authenticated by the Department of Pharmacognosy, Annamalai University, Annamalai Nagar, Tamil Nadu, India. The commercial formulation-*Triphala churanam*-was obtained from Indian Medical Practitioners Co-operative Pharmacy (IMPCOPS), (Chennai, India).

Estimation of Ellagic Acid and Gallic Acid

Estimation of ellagic acid and gallic acid in two different extracts of LF and CF of TPC in done by Sethi (1996).

Preparation of Standard Ellagic Acid Solution

A stock solution of ellagic acid (100 µg mL^{-1}) was prepared by dissolving 10 mg of accurately weighed ellagic acid in methanol and making up the volume to 100 mL with methanol. The stock solution was further diluted with methanol to give a standard solution of ellagic acid (25 µg mL^{-1}).

Preparation of Standard Gallic Acid Solution

A stock solution of gallic acid (1 mg mL^{-1}) was prepared by dissolving 10 mg of accurately weighed gallic acid in methanol and making up the volume to 10 mL with methanol. The stock solution was further diluted with methanol to give a standard solution of gallic acid (250 µg mL^{-1}).

Chromatographic Conditions

Stationary phase : Pre-coated silica gel 60F$_{254}$ TLC plate (10×10 cm, 0.2 mm thickness).
Mobile phase : Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.8:0.2 v/v)
Saturation time : 15 minutes
Wavelength : 280 um
Lamp : Deuterium

Calibration Curve for Standard Ellagic Acid

The standard solutions (0.125 to 0.5 µg per respective spot) were applied in triplicate on TLC plate. The plate was developed and scanned as per the chromatographic conditions mentioned above. The peak areas were recorded. Calibration curve of ellagic acid was prepared by plotting peak areas vs. concentrations of ellagic acid applied.

Calibration Curve for Standard Gallic Acid

The standard solutions (1.25 to 5.00 µg per respective spot) were applied in triplicate on TLC plate. Calibration curve of gallic acid was prepared similar to that of ellagic acid.

Preparation of *Triphala churanam*

Triphala churanam was prepared in the laboratory as per the formulation and method described in the Siddha Formulary of India (Anonymous, 1992).

Formulation:

Siddha formulary name	Synonym	Botanical name	Quantity
Katukkay tol	Chebulic myrobalan	*Terminalia chebula*	1 part
Nellikay	Emblic myrobalan	*Emblica officinalis*	1 part
Thanrikay	Beleric myrobalan	*Terminalia belerica*	1 part

The individual drugs were powdered separately and sieved through a fine mesh. Then the required quantities by weight were taken and thoroughly mixed to uniformity.

Preparation of Extracts

The LF samples (10 g each) and the CF samples (10 g each) of TPC were extracted for six hours by using two different solvents, methanol and ethyl acetate in a Soxhlet apparatus. All the four extracts were then concentrated at a low temperature, filtered through Whatman filter paper No 1 and the final volumes were made up to 10 mL with more respective solvents (Stock solutions). The stock solutions were further diluted to produce an uniform concentration of 20 mg mL^{-1} for all the samples.

Analytical Procedure

Samples of methanol and ethyl acetate extracts of LF and CF of *Triphala churanam* and standards-ellagic acid and gallic acid were spotted on a 10×10 cm precoated TLC plates as 6 mm wide band by using automatic TLC applicator Linomat V, 8 mm from the bottom. The mobile phase used was as mentioned above. The plates were developed in a twin trough chamber by ascending mode to a distance of 8 cm under chamber saturation conditions. After development the plates were dried in
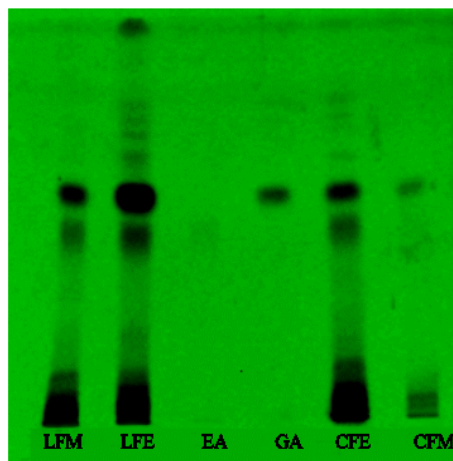
Fig. 1: TPC at 254 nm; LFM: Lab. Formulation-Methanol extract; LFE: Lab. Formulation-Ethyl acetate extract; EA: Ellagic Acid GA: Gallic Acid, CFM: Com. Formulation-Methanol extract; CFE: Com. Formulation-Ethyl acetate extract

air and scanned at 280 nm by using CAMAG Scanner 3. The plates were photographed at 254 and 366 nm by using CAMAG Reprostar instrument (Fig. 1). The contents of ellagic acid and gallic acid in the LF and CF of methanol and ethyl acetate extracts of the two formulations were determined by comparing the area of the chromatogram of the above formulations with the calibration curve of the working standards of ellagic acid and gallic acid.

RESULTS AND DISCUSSION

Standard ellagic acid (Rf:0.47) and gallic acid (Rf:0.56) showed single peaks in HPTLC chromatogram (Fig. 2 and 3). Calibration curve of ellagic acid was prepared by plotting concentrations of ellagic acid versus average area of the peak. Similarly, the calibration curve of gallic acid was prepared (Fig. 4 and 5). The formulation samples were analyzed by the proposed method. The amount of ellagic acid and gallic acid present in the above formulation samples were computed from the above calibration curves.

The LF was found to contain 0.201% w/w of ellagic acid and 0.656% w/w of gallic acid while the CF contained 0.058% w/w of ellagic acid and 0.573% w/w of gallic acid in methanol extracts. In the ethyl acetate extracts, the LF was found to contain 0.573% w/w of ellagic acid and 2.664% w/w of gallic acid, while the CF contained 0.422% w/w of ellagic acid and 1.637% w/w of gallic acid. It is also revealed from the data that ethyl acetate was a better solvent to extract ellagic acid and gallic acid from the formulations than that of methanol (Table 1). Further, the ethyl acetate extracts of both the formulations have shown maximum number of peaks in the chromatogram (Fig. 6-9). The quantity of ellagic acid and gallic acid in both the extracts of LF were much higher than that of the CF indicating the superiority of the authenticated LF sample. It may be due to varied factors like improper selection of the drug variety, incorrect identification of the drug, variation in the weight of the drug added to the formulation, addition of exhausted material and processing conditions (Patel *et al.*, 2006).

Validation of HPTLC Method
Linearity

A representative calibration curve of ellagic acid and gallic acid were obtained by plotting the peak area of ellagic acid and gallic acid against the concentration of ellagic acid (125-500 ng) and gallic acid
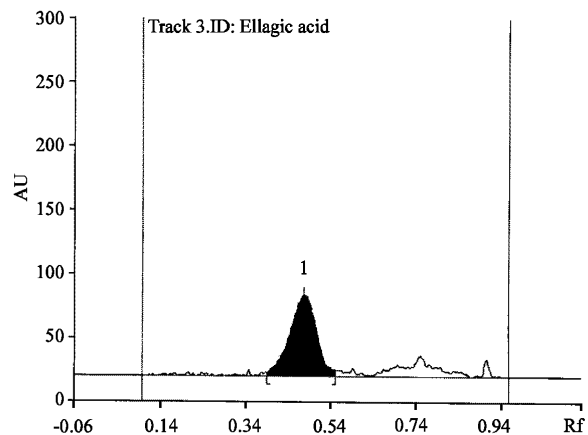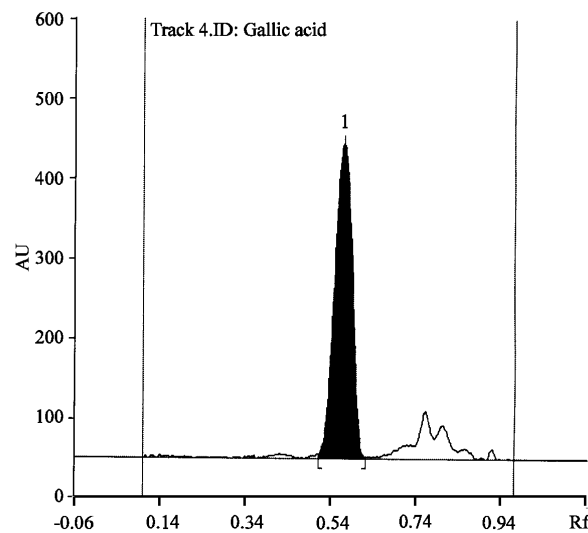
Fig. 2: Chromatogram of standard ellagic acid

Fig. 3: Calibration curve of gallic acid

Table 1: Percentage of ellagic acid and gallic acid in different formulation of TPC

TPC formulations	%w/w ellagic acid	%w/w gallic acid
LF-Methanol ext.	0.201	0.656
LF-Ethyl acetate ext.	0.573	2.664
CF-Methanol ext.	0.058	0.573
CF-Ethyl acetate ext.	0.422	1.637

(1.25-5.00 µg), respectively. The correlation coefficient for ellagic acid and gallic acid were found to be 0.981 and 0.996, respectively and thus exhibits good linearity between concentration and area (Table 4).

Accuracy (Recovery %)

The percentage recovery of ellagic acid and gallic acid were found to be 99.20 and 99.36, respectively which are highly satisfactory (Table 2 and 3).
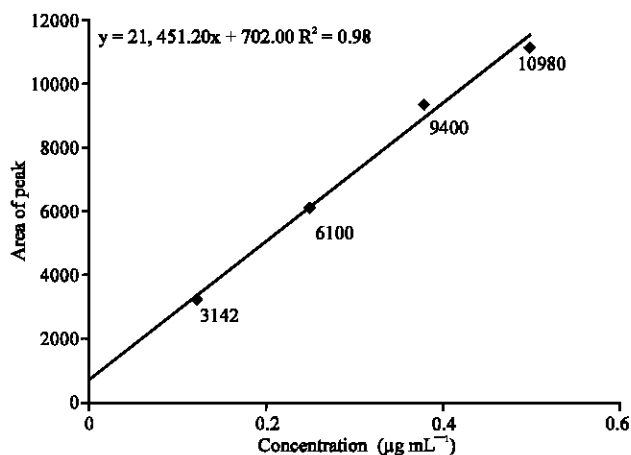
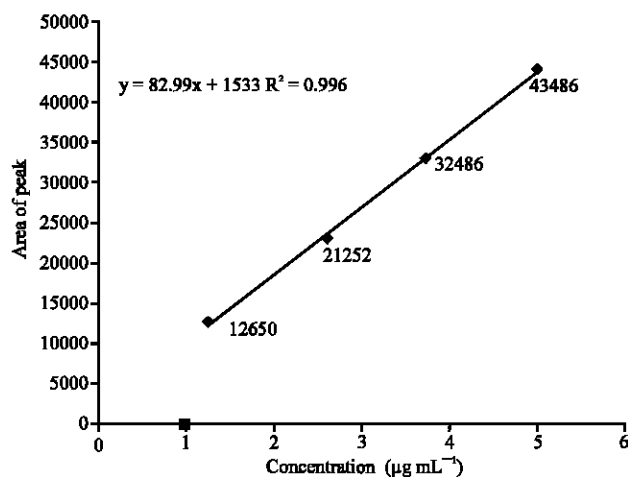5

Fig. 4: Calibration curve of ellagic acid

Fig. 5: Calibration curve of gallic acid

Table 2: Results of recovery study of the method for ellagic acid (EA)

Amount of samples taken (mg) A	Amount of EA in A (mg) B	Amount of EA added to A (mg) C	Amount of EA taken B+C (mg) D	Total EA found (Mean±SD, n = 5) (mg) E	Recovery E/D×100 (%)
1100	2.26	2	4.26	4.12±0.1318	96.7
1200	2.38	5	7.38	7.50±0.4564	101.6
1300	2.58	10	12.58	12.46±0.5432	99.3

Average recovery: 99.2%

Table 3: Results of Recovery study of the method for gallic acid (GA)

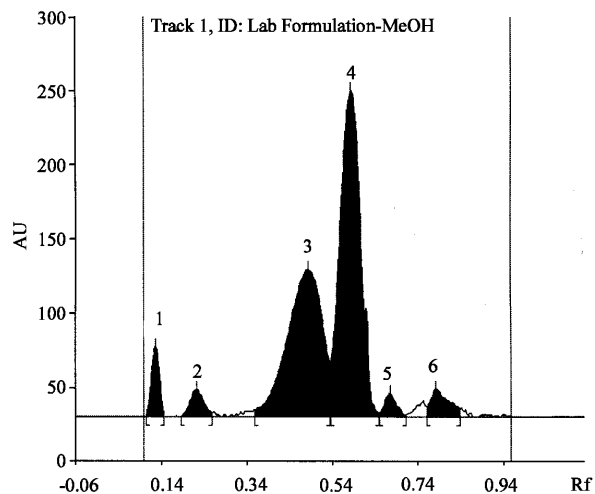| Amount of samples taken (mg) A	Amount of EA in A (mg) B	Amount of EA added to A (mg) C	Amount of EA taken B+C (mg) D	Total EA found (Mean±SD, n = 5) (mg) E	Recovery E/D×100 (%)
1100	7.16	10	17.16	17.00±0.8712	99.36
1200	7.85	15	22.85	22.20±0.7654	97.15
1300	8.50	20	28.60	28.06±0.4326	98.11

Average recovery: 98.13%

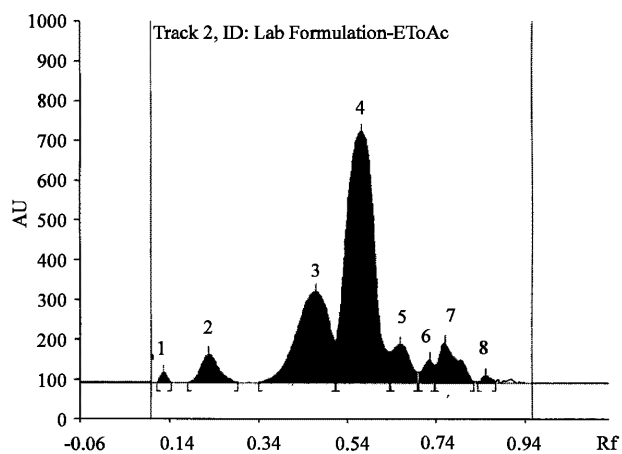Fig. 6: Chromatogram of methanol ext. of TPC (LF)

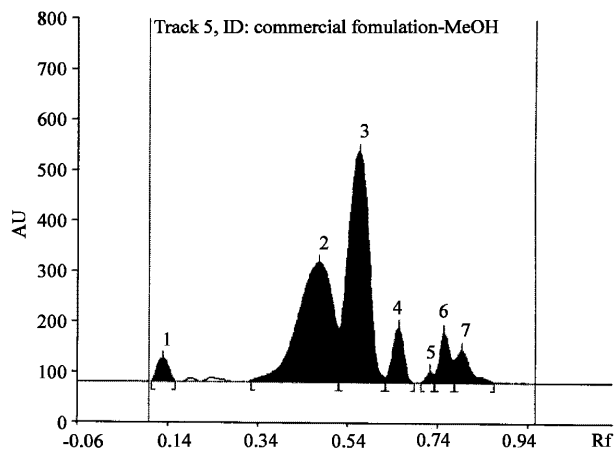Fig. 7: Chromatogram of ethyle acetate ext. of TPC (LF)

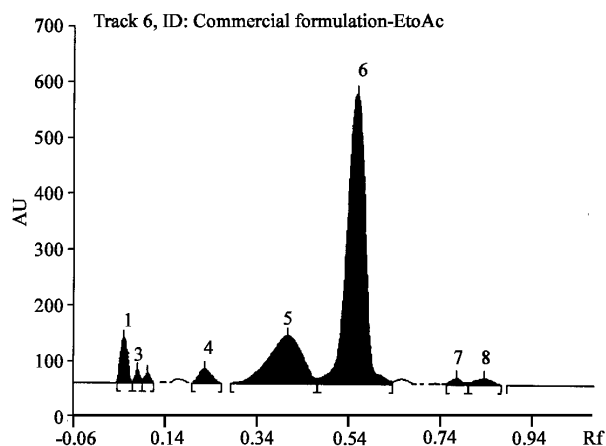
Fig. 8: Chromatogram of methanol ext. of TPC (CF)

Fig. 9: Chromatogram of ethyl acetate ext. of TPC (CF)

Table 4: Results of method validation

Analytical method	Accuracy (% recovery)	Precision (SD)	Linearity	Coefficient of variation (CV) (%)
HPTLC of EA	99.20	0.1145	125-500 ng	5.20
HPTLC of GA	98.13	0.2482	1.25-5.0 μg	3.24

Specificity

It was observed that other constituents present in the formulations did not interfere either with the peak of ellagic acid or gallic acid. Therefore the method was specific. The spectrum of standard ellagic acid and standard gallic acid spots and ellagic acid and gallic acid spots present in the samples were found to be similar or overlap.

Limit of Detection

The minimum detectable limit was found to be 125 ng spot^{-1} for ellagic acid and 450 ng spot^{-1} for gallic acid.

CONCLUSIONS

The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of ellagic acid and gallic acid in different formulation extracts. The recovery values of EA and GA were found to be 99.20 and 99.36%, respectively, which shows the reliability and suitability of the method. The ellagic acid and gallic are the main marker compounds of this formulation. Hence, the assay results of these compounds can be kept as standard for comparison and evaluation of other commercial samples available in the market. The method was found to be useful in detecting the genuiness of the formulation. In the present study, though both formulation can be used for therapeutic activity, the quality of commercial formulation is not up to the level of authentic formulation.

ACKNOWLEDGMENT

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