ORIGINAL ARTICLE

Estimation of Rhein from *Cassia fistula* Linn. using validated HPTLC method

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Background: Rhein is the major anthraquinone found in *Cassia fistula* Linn, which is traditionally used as an important component of plant based medicine. **Aims and Objective:** To develop and validate a High Performance Thin Layer Chromatographic method for the estimation of Rhein from *C. fistula* Linn. **Materials and Methods:** Chromatographic separation was achieved on silica gel 60 F_{254} plates with n-hexane: Ethyl acetate: Formic acid (5:5:0.2, v/v/v) as a mobile phase. Detection of Rhein was carried out by derivatizing the plate with 10% ethanolic KOH reagent followed by heating at 110°C for 10 min. CAMAG TLC scanner 4 equipped with winCATS software was used for densitometric scanning at 550 nm. The accuracy of the method was checked by conducting various validation parameters according to ICH (International Conference on Harmonization) guidelines. **Results:** The method was found applicable to evaluate the content of Rhein in different plant parts of *C. fistula* and the impact of different geographical regions on the Rhein content in fruit pulp of *C. fistula* was also studied. Estimation of Rhein from a herbal formulation containing *C. fistula* fruit pulp was also carried out using the validated HPTLC method. The developed method was found sensitive and accurate for the estimation of Rhein from various plant matrices. **Conclusion:** The developed method was found useful for the quantitation of bioactive marker Rhein and can be used as a routine quality control tool for the assessment of botanicals.

Key words: *Cassia fistula*, fruit pulp, geographical variation, HPTLC, Rhein

INTRODUCTION

Cassia fistula Linn., (Fabaceae) commonly known as Amaltas; is a deciduous tree and grows throughout the greater parts of India.[1] It has become extensively diffused in various countries including Mauritius, Sri Lanka, Amazon, Malaysia, South Africa, Mexico, China, West Indies, East Africa and Brazil as an ornamental tree and known for its beautiful bunches of yellow flowers.[2] Various parts of *C. fistula* such as stem bark, leaves, flowers, fruit pulp and seeds are extensively used as medicine for a wide range of ailments. Both the leaves and pods of *C. fistula* are widely used in traditional medicine as strong purgatives and laxatives.[3] Flowers, fruits and seeds of *C. fistula* are used to treat skin diseases, fever, abdominal pain and leprosy.[4] Black viscid pulp of *C. fistula* fruit has a good commercial value for its medicinal properties. It is used in diabetes, rheumatism, gout; colic pains and has purgative, hepatoprotective and estrogenic properties.[1,5,6] Several herbal industries have been using the *C. fistula* fruit pulp in various Ayurvedic, Unani and herbal formulations as a strong laxative agent.

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Rhein (1, 8-dihydroxyanthraquinone-3-carboxylic acid) is a major anthraquinone found in *Rheum* species, *Senna* leaves; and also in several species of *Cassia*. Rhein is used for alleviating pain, fever and has anti-inflammatory, laxative,[7] anti-tumour,[8] anti-bacterial,[9] and anti-angiogenic[10,11] properties. Estimation of Rhein has been carried out using the various analytical techniques. A reversed-phase high-performance liquid chromatographic method is described for the determination of Rhein in *Senna alata* leaves[12] and *Cassia alata* leaves.[13] Recently, a validated TLC technique has been reported for quantitation of Rhein from the aqueous extract of *C. fistula* fruit pulp.[14]

Literature survey reveals that neither Rhein has been quantitated from various morphological parts of *C. fistula* nor the impact of regional variation on the content of Rhein in *C. fistula* fruit pulp has been studied so far using the HPTLC technique. Thus, the present investigation describes the development of HPTLC technique for the estimation of Rhein from different samples of *C. fistula*.

MATERIALS AND METHODS

Plant Material

Plant material was collected from Mumbai, India

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authenticated by Agharkar Institute (Auth 12-12) and a voucher specimen was deposited for further reference. Fruit pulp samples were collected from Malaysia (Southeast Asia) and regions of India such as Udaipur (Rajasthan), Ahmedabad and Rajkot (Gujarat), Mumbai (Maharashtra) and Dehradun (Uttarakhand) in order to study the impact of regional variation on Rhein content. Various morphological plant parts such as stem bark, leaves, fruit pulp, flowers and seeds were also collected in order to study the morphological variation. Samples were powdered, sieved through BSS (British Standard Specification) sieve (85 mesh) and stored in air-tight containers. Laxived formulation containing fruit pulp of *C. fistula* was purchased from the local market.

Chemicals

Analytical grade solvents like n-hexane, ethyl acetate and formic acid were procured from Merck Specialities Pvt. Ltd., Mumbai. Standard Rhein (≥98% purity) [Figure 1] was procured from Sigma Aldrich, Germany.

HPTLC Conditions

Chromatographic separation was achieved on TLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with the aluminium sheet support. Samples were spotted using the CAMAG Linomat 5 sample spotter (CAMAG Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Plates were developed in a glass twin trough chamber (CAMAG) pre-saturated with mobile phase n-hexane: Ethyl acetate: Formic acid (5:5:0.2, v/v/v). Scanning device used was CAMAG TLC Scanner 4 equipped with winCATS software. The experimental condition was maintained at 25 ± 2°C. CAMAG -Reprostar 3 was used for photo-documentation.

Preparation of Standard Stock Solutions and Extraction Conditions for Plant Samples

A stock solution of Rhein (1000.0 µg/mL) was prepared in methanol. Seven calibrant samples ranging from 5.0 µg/mL to 60.0 µg/mL and three quality control samples of Rhein namely low, mid and high (6.5, 20.0, 50.0 µg/mL respectively) were prepared in methanol using the stock solution. Accurately weighed (1.0 g) of powdered sample was extracted with a methanol (10.0 mL). The sample was vortexed for 1-2 min, kept standing overnight at room temperature and filtered through Whatmann filter paper No. 1 (E. Merck, India). The filtrate was subjected to HPTLC analysis. Similar extraction procedure was followed for other plant samples and laxived formulation.

Method Validation

The developed HPTLC method for estimation of Rhein was validated as per ICH guidelines[15] for the parameters such as sensitivity, linearity, precision, recovery, specificity and ruggedness.

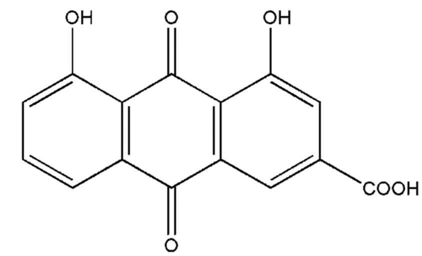

Figure 1: Structure of Rhein

Estimation of Rhein from *C. fistula* fruit pulp

Relative response for the characteristic band of Rhein in *C. fistula* fruit pulp samples and formulations were obtained and the content of Rhein in each sample was determined using the regression equation obtained from the regression analysis of the calibration curve.

Statistical Analysis

Microsoft Excel-2007 was used for the statistical evaluation of results.

RESULTS AND DISCUSSION

HPTLC methods are commonly applied for the identification, assay or content uniformity of herbal raw materials and their formulations.[16] *C. fistula* is one of the most commonly used herbs in traditional systems of medicine. Its therapeutic benefits are largely based on folkloric rather than scientific evidences. Rhein is the major anthraquinone found in this plant.[14]

Considering the biological importance of Rhein a rapid, simple and accurate HPTLC densitometric method was developed for its quantitation from *C. fistula*. Of the various solvent systems tried, mixture containing n-hexane: Ethyl acetate: Formic acid (5:5:0.2, v/v/v) gave the best resolution of Rhein (R_f = 0.37) from the other components in the methanolic extract of *C. fistula*. The identity of the band of Rhein in plant matrix was confirmed by comparing the R_f and colour of the band with that of the standard Rhein. The method was validated as per ICH guidelines and found a linear over the range of 5.0-60.0 µg/mL of Rhein. The method was found to be precise with % RSD (relative standard deviation) of 0.03% and 0.06% during the intra-day and inter-day precision studies respectively [Table 1]. The method was also found sensitive with a limit of detection and limit of quantification values 0.5 µg/mL and 5.0 µg/mL respectively, and the results were incompliance with other published reports.[12-14] The average recovery for quality control samples of Rhein was found to be 101.23% [Table 1]. The method was also found rugged for the parameters such as change in analysts, change in mobile phase composition and change in spotting volume.

Using the regression equation, the exact content of Rhein was determined from the different samples of *C. fistula* [Tables 2 and 3]. Among the different morphological parts of *C. fistula*, leaves showed the maximum Rhein content [Figure 2]. Hence, leaves can be a good source of Rhein. Method was also applied to determine the Rhein content in *C. fistula* fruit pulp collected from different geographical regions [Figure 3]. Sample collected from Mumbai showed the maximum Rhein content while sample from Dehradun had minimum. Method was also found applicable to evaluate the Rhein content from a herbal formulation laxived containing *Cassia fistula* fruit pulp [Figure 4]. The Rhein content in laxived formulation was found to be 1.80 ± 0.13 mg/g. 3-D overlay of the HPTLC

Table 1: Results of method validation parameters for Rhein

Parameters	Results
LOD (μg/mL)	0.5
LOQ (μg/mL)	5
Linear working range (μg/mL)	5-60
Regression equation	y=5.7218x+307.62
Coefficient of determination (r^2)	0.990
Intra-day precision (% RSD, *n*=7)	0.03
Inter-day precision (% RSD, *n*=7)	0.06
Instrumental precision (% RSD, *n*=7)	0.82
Repeatability (% RSD, *n*=5)	0.30
Ruggedness	Rugged
Recovery (%)	101.23±4.56

LOD – Limit of detection; LOQ – Limit of quantification; RSD – Relative standard deviation

Table 2: Rhein content in the fruit pulp of *Cassia fistula* collected from different geographical regions

Place of collection	Content in mg/g (Mean±SD, *n*=7)
Malaysia	1.49±0.11
Udaipur	1.05±0.08
Baroda	1.24±0.13
Rajkot	1.18±0.10
Mumbai	1.92±0.14
Dehradun	0.17±0.01

Table 3: Rhein content in different morphological parts of *Cassia fistula*

Parts of *Cassia fistula*	Content in mg/g (mean±SD, *n*=7)
Leaves	2.21±0.18
Fruit pulp	1.90±0.17
Flowers	0.89±0.12
Seeds	0.19±0.02

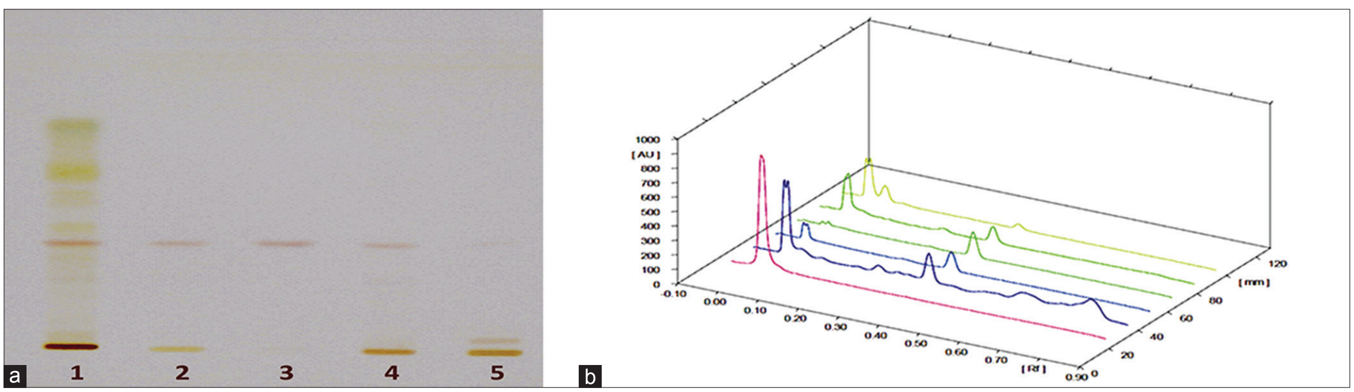

Figure 2: HPTLC plate photo (a) and overlay (b) of different morphological parts of *Cassia fistula* (methanolic extract) with Rhein at 550 nm. Track details: (1) leaves; (2) fruit pulp; (3) Rhein 40 μg/mL; (4) flowers; (5) seeds

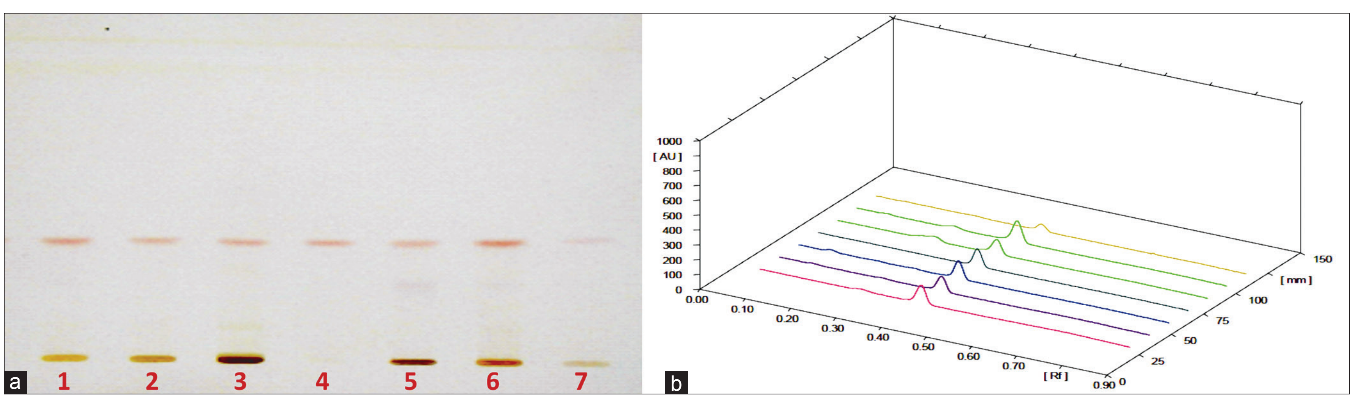

Figure 3: HPTLC plate photo (a) and overlay (b) of *Cassia fistula* fruit pulp collected from different geographical regions (methanolic extract) with Rhein at 550 nm. Track details: (1) Malaysia; (2) Udaipur; (3) Baroda; (4) Rhein 40 μg/mL; (5) Rajkot; (6) Mumbai; (7) Dehradun

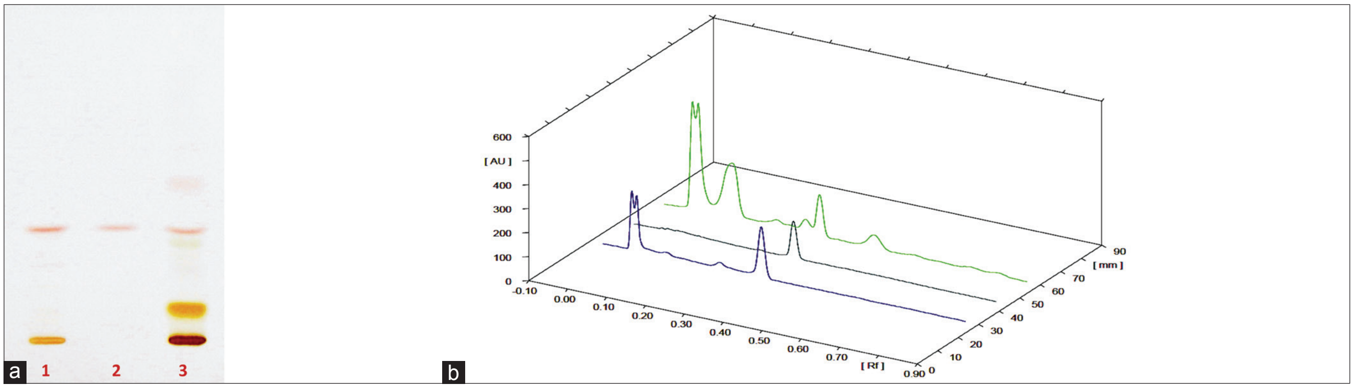

Figure 4: HPTLC plate photo (a) and overlay (b) of *Cassia fistula* fruit pulp and its formulation (methanolic extract) with Rhein at 550 nm. Track details, (1) *Cassia fistula* fruit pulp; (2) Rhein 40 μg/mL; (3) Laxived formulation

chromatograms is also shown in Figures 2-4. Thus, the HPTLC method developed was found to be suitable for the quantification of Rhein in the herbal raw materials of varied matrices. The developed method can be used as a powerful quality control tool for a botanical identification of plants and to detect adulterants in plant raw materials.

CONCLUSIONS

Results of the current study could be used by industries for the characterization of *C. fistula* samples and its formulations in order to check their uniformity. Using such validated methods, *C. fistula* with precise quality can be encouraged in herbal industries.

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