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Original Article

Determination of Dried Root Extract of *Rheum emodi* and its Commercial Products Using Rhein and Chrysophanol as Standard Markers by HPTLC Densitometric Analysis

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ABSTRACT

Background: Standardization and quality control of medicinal plants and their commercial products are one of the critical needs for ensuring their quality, safety and efficacy which assists in validation as well as regulation of herbal medicines and their herbal products. *Rheum emodi*, Himalayan rhubarb or Revand chini is a perennial, traditional Indian medicinal herb from family Polygonaceae, used as laxative, tonic, diuretic and to treat fever, cough, indigestion, menstrual disorder. Emodin, aloe-emodin, rhein and chrysophanol are the anthraquinones found in high concentration in this plant. **Objectives:** The present study aimed at qualitative and quantitative estimation of rhein and chrysophanol in the methanolic extract of *Rheum emodi* roots and its commercial products by HPTLC densitometric analysis which has not been reported in the literature so far. **Materials and Methods:** The separation and quantification of rhein and chrysophanol was performed on pre-coated Silica gel 60 F_{254} HPTLC plates using Toluene: Ethyl Acetate: Glacial Acetic Acid (6:3.5:0.5, v/v/v) as mobile phase. The quantification of rhein and chrysophanol was carried out at 254nm and 366nm using Camag TLC scanner III using Wincats1.2.3 software. **Results:** The developed method was linear, accurate, precise, robust and specific and was found to be more sensitive to chrysophanol than the reported method of Singh *et al*. The proposed method was linear from 100 to 2000 ng. The limit of detection and quantification were found to be 18.915±0.754, 15.849±0.839 ng/spot and 57.318±1.162, 48.028±1.007 ng/spot for rhein and chrysophanol respectively. The content of rhein and chrysophanol was found to be 108.002±0.323 and 67.143±0.0884 µg/mg in *Rheum emodi* root extract, 26.829±0.138, 1.652±0.0031 µg/mg in marketed formulation 1 and 123.691±0.758, 30.476±0.0884 µg/mg in marketed formulation 2. **Conclusion:** The developed method can have great application for standardization of herbal formulations and commercial products containing *R. emodi* plant extract in the herbal industry.

Keywords: *Rheum emodi*, Herbal formulation, Standardization, Anthraquinones, Rhein, Chrysophanol, Densitometry.

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INTRODUCTION

Indian traditional system of medicine has a long history of using medicinal plants for the treatment for various disorders in order to maintain health and wellness for human beings.[1] Due to the plethora of major and minor phytochemicals present in medicinal plants, they exhibit diverse synergistic, agonistic and antagonistic biological effects, some are beneficial, some help in counteracting each other's side effects while few also lead to undesirable side effects.[2] Despite the tremendous amount of research carried out on medicinal plants and which has been documented well in the literature, quality-based standardization of herbal medicines and their derived products by using a systematic approach and well-designed methodologies is still considered one of the most important critical needs to ensure quality, safety and efficacy of herbal raw materials and herbal formulations.[3] Availability of reproducible standards of plants, their authentication and characterization as well as access to suitable and pure biomarkers, are essential for achieving freedom from adulteration and effective quality control of herbal products.[4,5]

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Standardization of herbal medicines emphasizes on chemical profiling of phytoconstituents by their quality and quantity-based assessment which is important from the regulatory point of view.[6-8] Various chromatographic and spectral analytical techniques have been used for the analysis of medicinal plants and generate scientific evidence which helps in the regulation of herbal medicine or products.[9-13]

Rhubarb (*Rheum emodi*, family Polygonaceae) is a perennial herb. It is commonly used in ailments of kidney, for relieving edema by diuretic action, hepatostimulant, to produce catharsis, to lower plasma cholesterol levels and for its anticancer and antimicrobial actions. There are reports that this plant is referred to as "the wondrous drug" due to its broad activity profile that includes anti-inflammatory anti-platelet, anti-diabetic, antiulcer and nephroprotective activities. It is known for its remarkable content of several anthraquinones such as rhein, chrysophanol, physcion, emodin and aloe-emodin which have been reported to possess additionally anti-Parkinson's, antifungal, immunostimulant, antiviral and antioxidant activities.[14] Some newer bioconstituents have been isolated from the same plant in recent years like revandchinone-1, revandchinone-2, revandchinone-3, revandchinone-4, sulfemodin 8-O-β-d-glucoside, 6-methyl-rhein and 6-methyl aloe-emodin.[15]

Being a potential medicinal herb, quality control and standardization of RC as herbal medicine and herbal product are important goals to monitor and maintain their quality and purity.[16]

Singh *et al.* (2005) have developed an HPTLC method for quantification of 4-anthraquinones i, e. emodin, physcion, chrysophanol and chrysophanol glycoside in *Rheum emodi* plant using methanol, water and formic acid as a mobile phase at 445 nm in reflection/absorption mode. The method was found to be linear in the range of 20-100 ng for physcion, 80-400 ng for chrysophanol and emodin and 200-1000 ng for chrysophanol glycoside. [17]

Arvindekar *et al.* (2013) worked on the estimation of anthraquinones and their glycoside from Indian rhubarb (*Rheum emodi*) viz. emodin, physcion and chrysophanol by an HPLC method using fluorescence detection.[18]

Ahmad *et al.* (2014) selected emodin and chrysophanic acid as chemical markers Safoof-e-Pathar Phori (SPP), a traditional polyherbal formulation used for its anti-urolithiatic activity and quantified using Simultaneous HPTLC and RP-HPLC methods *in Rheum emodi* and in SPP.[19]

Alqarni *et al.* (2022) studied to design and validate a sensitive and sustainable reverse-phase high-performance thin-layer chromatography method for the simultaneous estimation of rhein and aloe-emodin in a traditional extract and ultrasound-based extract of commercial Rhubarb and Rhubarb plant extracts in comparison to the conventional normal-phase HPTLC method.[20]

Waseem *et al.* (2013) have added new quality control parameters for standardization of *Rheum emodi* Wall. like assays for phenolics and flavonoid constituents, fingerprinting using HPLC, HPTLC and co-chromatography techniques using e22modin and chrysophanic acid as standard markers. Their report claims to be useful in assisting to identify, authenticate and check the purity of the plant material to ensure its safety and efficacy.[21]

To establish the novelty of the proposed work, a comparison of the reported literature was carried out. The HPTLC method developed by Singh *et al.* (2005) is for emodin, physcion, chrysophanol and chrysophanol glycoside in *Rheum emodi* plant using methanol, water and formic acid as a mobile phase at 445 nm in reflection/absorption mode while the proposed method was developed specifically for rhein and chrysophanol using Toluene: Ethyl Acetate: Glacial Acetic Acid (6:3.5:0.5, v/v/v) as developing system, at 2 different wavelengths i.e. 254 nm and 366 nm. Singh *et al.* did not carry out analysis of any marketed formulation with their developed method while we have applied the present developed method to two marketed formulations that highlights a direct commercial application of the method and demonstrates how it can be a useful tool for standardization of extracts of *R emodi* and its marketed products. This underlines the innovativeness of the proposed work. The linearity range reported by Singh *et al.* for chrysophanol was 80-400 ng while in the present work it was found to have a wide range of linearity between 100-2000 ng/spot for both rhein and chrysophanol. LOD and LOQ reported by Singh *et al.* for chrysophanol is 75±2.95 ng/spot and 80±2.95 ng/spot respectively while the proposed method was found to be more sensitive because LOD for chrysophanol was found to be 15.849±0.839 ng/spot and 8.553±0.537 ng/spot at 254 nm and 366 nm respectively and LOQ was found to be 48.028±1.007 ng/spot and 25.918±0.992 ng/spot at 254 nm and 366 nm respectively.

The other references cited above clearly indicate that so far neither HPLC or HPTLC methods have been reported in the literature for simultaneous estimation of rhein and chrysophanol in the dried roots of *R emodi* which highlights the novelty of the present work that deals with the densitometric analysis of dried root extract of *R. emodi* and two marketed formulations containing extract of *R. emodi*, by a validated HPTLC method using two anthraquinone markers rhein (Figure 1a) and chrysophanol (Figure 1b). The developed HPTLC method may find application in authentication and identification of plant material and analysis of adulteration, ensuring bio-efficacy of the *R. emodi* and its herbal products.

MATERIALS AND METHODS

Plant materials and commercial products

The roots of *R. emodi* were purchased from the local market in Lucknow, Uttar Pradesh, India. Authentication was carried out by Prof. R.B. Ram, Dean, School of Agricultural Sciences and Technology (SAST), Baba Sahab Bhim Rao Ambedkar University, Lucknow through letter no: 01/Dean /SAST/BBAU/LKO, dated: 05/01/2021 and a voucher specimen was deposited for further reference, by correlating their morphological and microscopical characters with those given in the literature. The roots were dried in the shade, finely powdered and stored in an airtight container at room temperature (30±2°C). Commercial product no. 1 and 2 containing the dried roots of *R. emodi* were purchased from Win-Naturals, Bharatganj, Loadhhari, Raebareli, Uttar Pradesh, India and Hamid Unani and Ayurvedic, Lucknow, Uttar Pradesh, India respectively.

Reagents, chemicals and instrumentation

Pre-coated TLC plates, Silica gel 60 F_{254} thickness 0.25 mm were purchased from Merck. Rhein (R7269) and chrysophanol (01542) were procured from Sigma Aldrich Co. via voucher no: SSC/TI/20-21/05 and SSC/TI/20-21/05 respectively. HPTLC CAMMAG scanner 3.0 and UV chamber were used for analysis at the Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India. All other analytical grade chemicals and solvents used were available commercially.

Preparation of extract

Dried roots of *R. emodi* (10 g) and commercial products 1 and 2 (10 g) each, were powdered separately using an electric grinder. The powders were extracted separately using 100 mL methanol under reflux at 50°C for 2 hr. The extracts were filtered using muslin cloth, concentrated using Rotavac and stored in a dry and cool place for further use.

Determination of percentage yield of extract

The percentage yield of extract was calculated using the following equation:

Percentage yield= [W2-W1] / W X100

Where W= 10 g (weight of powdered root of *R. emodi* or formulations), $W_{1=}$weight of empty China dish in (g) and $W_{2=}$weight of the China dish and dried extract (g).

Extraction of *R. emodi* roots and its commercial products was achieved using methanol.

Qualitative analysis of *R. emodi* plant extract

Qualitative analysis of a freshly prepared extract of *R. emodi* for the presence of chemical constituents was performed using standard procedures.[22,23]

After completion of extraction, determination of percentage yields of extracts and qualitative analysis of *R. emodi* root extract, HPTLC densitometric analysis was performed to establish the chemical profiling of each sample and quantify the marker compounds.

Method Development

Optimization of solvent system

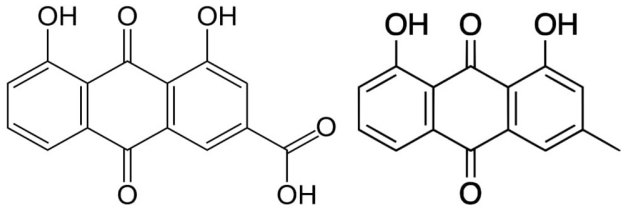
Various solvent systems like Toluene: Ethyl Acetate: Formic Acid (4.5:4.5:0.5), Toluene: Ethyl Acetate: Formic Acid (7:3:0.5), Toluene: Ethyl Acetate: Glacial A.A(5:4:1), Toluene: Ethyl Acetate: Glacial A.A (7:3:0.5), Toluene: Ethyl Acetate: Glacial A.A (6:3.5:0.5) were employed for optimization of developing system.

Preparation of sample for HPTLC analysis

The extract of roots of *R. emodi* and commercial products (30 mg) were solubilized in 1 ml of HPLC grade methanol, vortexed, centrifuged (10 min) and then the supernatant was separated into a fresh vial and used for analysis.

Preparation of standard solution

Each standard (rhein and chrysophanol, 1 mg each) was solubilized in 1 mL HPLC grade methanol, vortexed, centrifuged (10 min) and the supernatant was separated into a fresh vial labeled as standard. Thereafter, half of the volume from each vial of the standard solution was transferred to another vial to obtain a mixed standard of 0.5 mg/mL concentration.



a. Rhein **b. Chrysophanol**

Figure 1: a. Rhein b. Chrysophanol

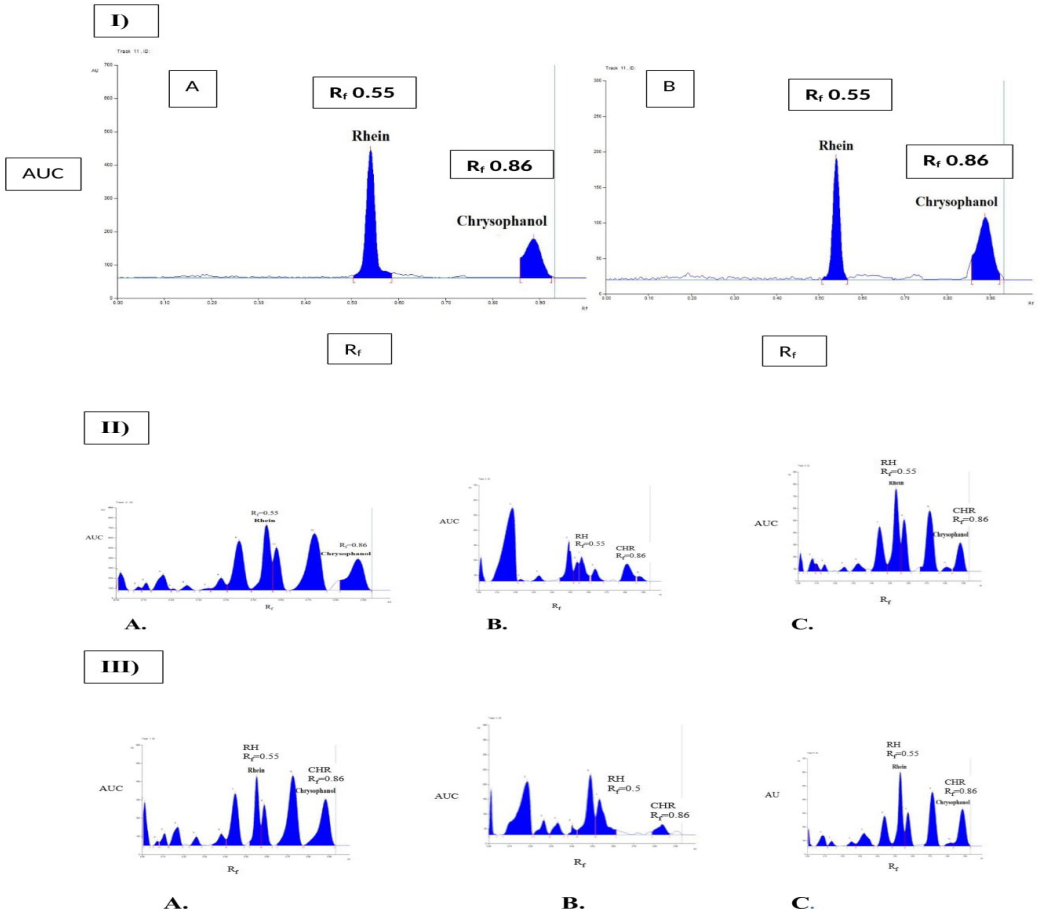


Figure 2: I) HPTLC chromatograms of standard markers rhein and chrysophanol at R_f 0.55 and 0.86 respectively at 254 nm (A) and 366 nm (B), II) HPTLC chromatogram of (A) *Rheum emodi* root extract, (B) Marketed formulation 1, (C) Marketed formulation 2 showing Rhein (RH) and Chrysophanol (CHR) at R_f 0.55 and 0.86 respectively at 254 nm, III)) HPTLC chromatogram of (A) *Rheum emodi* root extract, (B) Marketed formulation 1, (C) Marketed formulation 2 showing Rhein (RH) and Chrysophanol (CHR) at R_f 0.55 and 0.86 respectively at 366 nm.

HPTLC profiling and quantitative estimation of rhein and chrysophanol

From the stock solution of samples and standards, 4 µL from each sample and 0.2-4 µL (0.2, 0.5, 1.0, 2.0 and 4.0 µL) from mixed standard (rhein and chrysiphanol in 50:50 proportion) were introduced with a band length of 4 mm on a pre-washed and activated pre-coated HPTLC plates of Silica gel 60 F_{254} (20x10 cm; Merck, Germany) with the help of nitrogen gas under pressure that provided flow speed of 150 nL/s. The concentrations of the applied samples corresponded to 100, 250, 500, 1000 and 2000 ng/spot for plotting the calibration curve. The TLC development chamber which was maintained at room temperature (25°C), was saturated with developing system comprising of toluene, ethyl acetate, glacial acetic acid (6:3.5:0.5; v/v/v). The plates (in duplicate) were allowed to develop till 80 mm distance and then dried. The visualization of spots was conducted under visible (white), short UV (254 nm) and long UV (366 nm) lights. The quantification of rhein and chrysophanol was carried out at 254 nm and 366 nm using Camag TLC scanner III using Wincats1.2.3 software.[7]

Method validation

Standard guidelines of the International Conference on Harmonization (ICH)[24] were used to validate the developed densitometric method for parameters such as linearity, specificity, the Limit of Detection (LOD), Limit of Quantitation (LOQ), precision, accuracy and robustness.

Linearity

Different concentrations of the mixed standard sample (100, 250, 500, 1000 and 2000 ng/spot) were applied on the HPTLC plate to obtain a linear standard calibration curve. Each application was made in duplicate. The Standard Deviation (SD), regression coefficient (r^2), slope and intercept were estimated from the calibration curve to validate the linearity of the developed method.

Specificity

Standard and test solutions were applied to check the specificity of the developed method. Later their R_f values were confirmed to see whether they were different from the other related compounds.

Table 1: Qualitative analysis of *R. emodi* dried root extract.

Sl. No.	Chemical Constituents	Presence/ Absence
1	Alkaloids	+
2	Steroids	+
3	Anthraquinones	+
4	Phenolics	++
5	Flavonoids	+
6	Tannins	-
7	Cardiac glycosides	-
8	Saponins	+

Table 2: Chromatographic conditions for method development

Parameters	Chromatographic conditions
Saturation time	20 min
Detection wavelength	254, 366 nm
Distance between spots	4 mm
Drying time	10 min
Drying temperature	45°C
Development time	15 min
Slit dimension	6×0.3 mm
Temperature for densitometric analysis	25°C±2°C

LOD and LOQ

The LOD is the lowermost limit of the marker compound detected in an analyte while LOQ is the lowermost limit of the marker compound quantified in an analyte. The analytes can be detected and quantified with adequate accuracy, precision and variability through the standard deviation of the response and the slope of the calibration curve.[25] Equation 1 and 2 were used to compute the LOD and LOQ values respectively.

$$LOD = 3.3\sigma / S \quad \text{----------(1)}$$

$$LOQ = 10\ \sigma / S \quad \text{----------(2)}$$

Where σ denotes standard deviation of the response and S corresponds to calibration curve slope.

Precision

The inter-day and intra-day precision were estimated in the form of percentage Relative Standard Deviation (%RSD), or the coefficient of variation for the developed method. The preciseness of the developed method was considered as per the referenced protocol.[25] The intra-day and inter- day precision of the developed method were estimated at five different concentrations (100, 250, 500, 1000 and 2000 ng/spot) followed by estimation of mean peak area, standard deviation, drug content and %RSD. The following formula was used to calculate the %RSD.

% RSD=Standard deviation/ Mean x100

Accuracy

The accuracy of the developed method was determined for the estimation of percentage recovery of drug at a known concentration by spiking to the sample with 0%, 50%, 100% and 150%. Comparisons were made between the theoretical and experimental values of percentage drug content recovery and the outcomes were considered as the parameter to measure the accuracy of the developed method.[25]

Robustness

A few parameters such as mobile-phase composition and chamber saturation time were altered deliberately to assess whether the method was robust or not. The changes tried in mobile phase ratio were, Toluene: Ethyl acetate: Glacial acetic acid (6: 3: 1; v/ v/v), Toluene: Ethyl acetate: Glacial acetic acid (6:3.5:0.5; v/v/v) and Toluene: Ethyl acetate: Glacial acetic acid (8: 2: 1; v/ v/v). The changes implemented in chamber saturation time were 20 min, 25 min and 30 min.[25]

RESULTS AND DISCUSSION

Extraction of *R. emodi* dried roots and commercial products were performed using methanol. The percentage yield was found to be 10.172±0.449%, 16.036±0.264 and 14.418±0.593 for the *R. emodi*, commercial product 1and 2 respectively. The extractive value of *R. emodi* was found to be within the prescribed limit of Ayurvedic Pharmacopoeia of India (should not be less than 10%).

Qualitative analysis of *R. emodi* extract (Table 1) established presence of various bioactive constituents like alkaloids, steroids, anthraquinones, phenolics, flavonoids and saponin. Tannins and cardiac glycosides were found to be absent.

HPTLC analysis for simultaneous estimation of rhein and chrysophanol

HPTLC method was developed for simultaneous quantification of rhein and chrysophanol present in dried roots of *R. emodi* and its marketed products. Chromatographic conditions used for method development are mentioned in Table 2.

For optimization of mobile phase, mixtures of Toluene: Ethyl Acetate: Formic Acid and Toluene: Ethyl Acetate: Glacial Acetic Acid in different proportions was employed. Toluene: Ethyl Acetate: Formic Acid (4.5:4.5:0.5), Toluene: Ethyl Acetate: Glacial A.A. (5:4:1) and Toluene: Ethyl Acetate: Glacial A.A. (7:3:0.5) gave poor resolution while Toluene: Ethyl Acetate: Formic Acid (7:3:0.5) demonstrated poor resolution with tailing. After many trials and errors, Toluene: Ethyl Acetate: Glacial Acetic Acid (6:3.5:0.5, v/v/v) was chosen as the optimized developing system as it resolved rhein and chrysophanol at R_f 0.55 and 0.86 respectively, demonstrating well-defined and sharp peaks, at

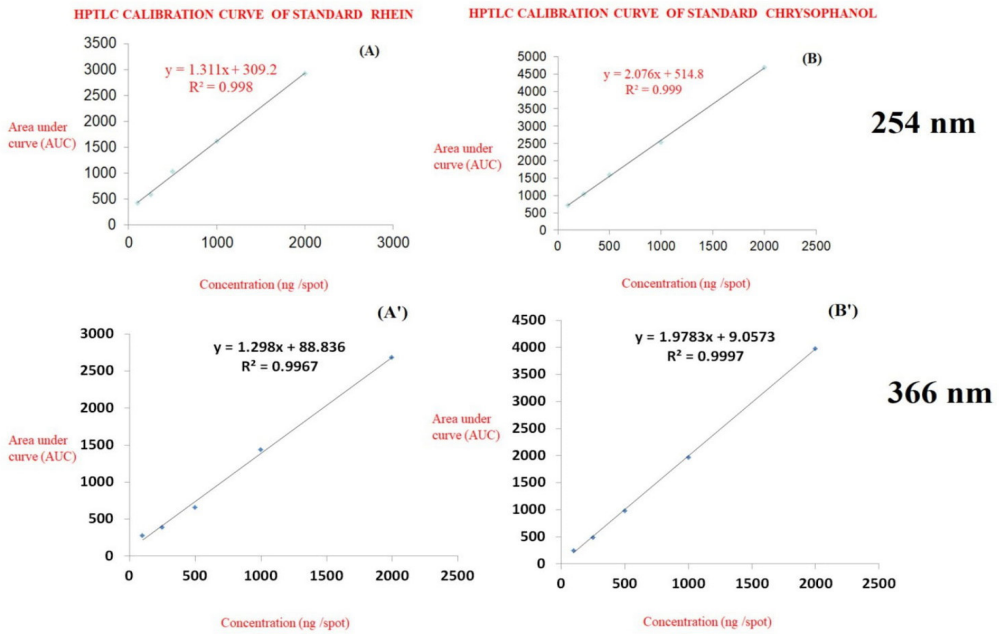


Figure 3: Calibration curves of rhein (A; A') and chrysophanol (B; B') at 254 nm and 366 nm, respectively.

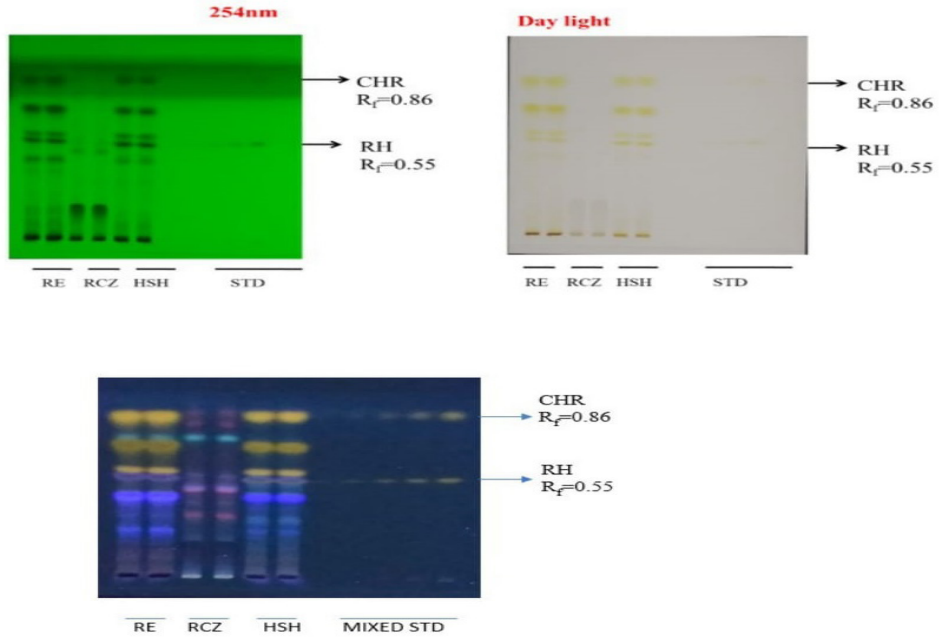


Figure 4: Developed HPTLC Plates at Day-Light, 254 nm and 366 nm. RE: *Rheum emodi* root extract, RCZ: Marketed formulation 1, HSH: Marketed formulation 2, MIXED STD: (50% Rhein+50% Chrysophanol); RH: Rhein; CHR: Chrysophanol.

estimation wavelengths of 254 nm and 366 nm (Figure 2 IA and IB respectively).

Method validation

The validation of the developed method was performed as per the ICH guidelines Q2(R1): 2005.[23] The parameters such as linearity, specificity, LOD, LOQ, accuracy, precision and robustness were determined successively.

Linearity

The linearity of the developed method was checked by plotting peak area versus concentration of applied sample from 100-2000 ng (100, 250, 500, 1000 and 2000 ng/spot) at 254 nm and 366 nm (Figure 3). The resulted data (Table 3) revealed that the developed method was linear which exhibited good linearity (regression equation: 1.3114x+309.22 and 2.076x+514.84 for rhein and

Table 3: Linearity parameters of developed method.

Parameters	Biomarkers			
	Rhein		Chrysophanol	
R_f value	0.55		0.86	
Linearity range (ng/spot)	100-2000 ng/spot		100-2000 ng/spot	
Scanning wavelength	254 nm	366 nm	254 nm	366 nm
Regression equation	y=1.3114x+309.22	y=1.298x+88.836	y=2.076x+514.84	y=1.978x+9.0573
Regression coefficient±SD	0.9983±0.0003	0.9967±0.0002	0.9995±0.0011	0.9997±0.0005
Slop±SD	1.3114±0.0016	1.298±0.0015	2.076±0.0153	1.978±0.0019

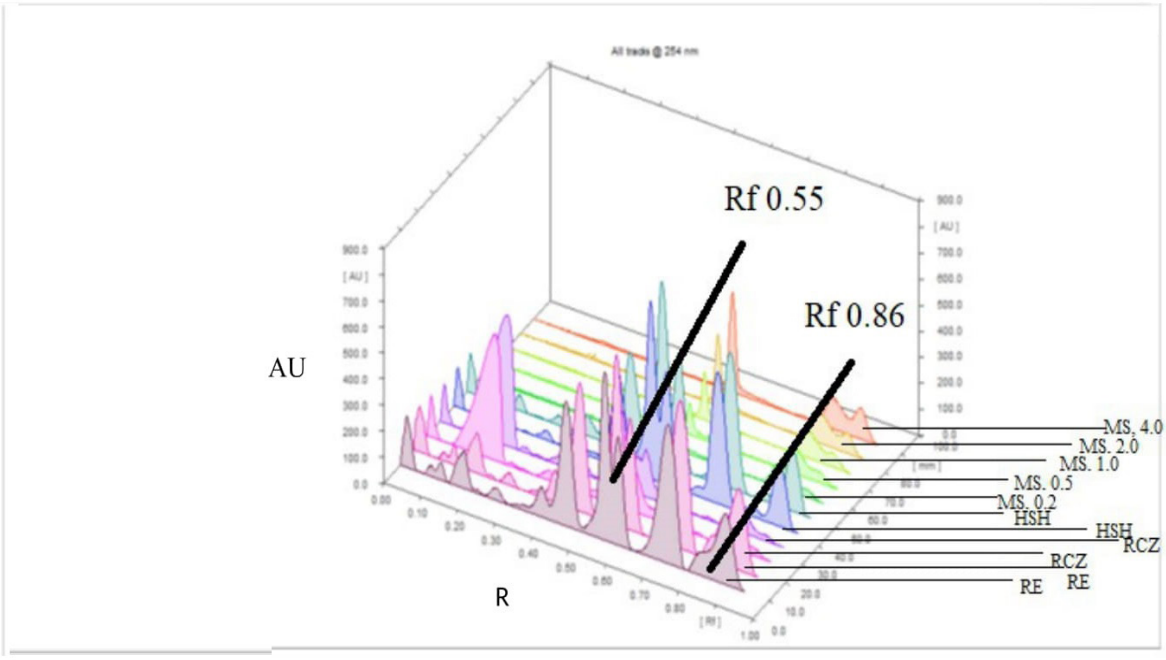


Figure 5: Overlay 3D chromatogram of standard rhein and chrysophanol at R_f 0.55 and 0.86 observed in *R. emodi* root Extract (RE), Marketed formulation 1 (RCZ) and Marketed formulation 2 (HSH) at 254 nm. Tracks 1 and 2 for RE, tracks 3 and 4 for RCZ, tracks 5 and 6 for HSH and tracks 7-11 are of mixed standard rhein and chrysophanol at concentrations of 100, 250, 500,1000 and 2000 ng/spot.

chrysophanol, at 254 nm and 1.298x+88.83 and 1.978x+9.0573 for rhein and chrysophanol, at 366 nm respectively.

Specificity

Specificity is the capability of a method to identify the analyte in presence of other components without their interference. When the R_f values of standards were confirmed, they were found to be different than other closely related compounds, showing that the method was specific. No peak tailing or altered retention factor or peak areas were observed for standards.

LOD and LOQ

The LOD for marker compounds (rhein and chrysophanol) at 254 nm and 366 nm was found as 18.915±0.754, 15.849±0.839 ng/spot and 16.343±0.825, 8.553±0.537 ng/spot respectively. LOQ for marker compounds (rhein and chrysophanol) at 254 nm and 366 nm was found as 57.318±1.162, 48.028±1.007 ng/spot and 49.524±0.983, 25.918±0.992 ng/spot respectively.

Precision

The intra-day and inter-day precision of the developed method was estimated at all the concentrations at the wavelengths 254 nm and 366 nm followed by estimation of mean peak area, standard deviation, found drug content and %RSD. The experimental observations revealed no significant changes in the mean peak area and concentration of the analyte at different applications per spot. The %RSD values suggest the reproducibility of the developed method. The precision data is summarized in Tables 4 and 5. The intra-day and inter-day precision of rhein and chrysophanol was estimated in terms of %RSD which was observed to be lower than 2% as prescribed by ICH guidelines.

Table 4: Intra-day and Inter-day precision of developed and validated method at 254 nm.

	Theoretical content (ng/spot)	Mean peak area ±SD	Content found (ng/spot)	Content found (%)	% RSD	Mean peak area ±SD	Content found (ng/spot)	Content found (%)	% RSD
	Intra day					Inter day			
Rhein	100	432.85±7.566	90.290±1.248	90.290	1.747	437.02 ±5.904	97.486±1.047	97.486	1.351
	250	612.505±8.636	231.268±1.475	92.507	1.40	624.48±8.251	240.615±2.5615	96.246	1.311
	500	1046±18.950	552.198±1.898	110.439	1.811	1065.37±14.248	576.777±2.477	115.355	1.337
	1000	1641.7±24.890	1003.725±5.178	100.372	1.516	1650.5±12.445	1023.096±2.479	102.309	0.754
	2000	2924.05±6.150	1992.732±2.001	99.636	0.210	2911.95±17.783	1985.320±1.728	99.266	0.610
Chrysophanol	100	728.73±9.941	99.932±0.958	99.932	1.377	736.745±11.334	106.890±1.478	106.890	1.538
	250	1037.21±13.341	251.169±1.128	100.467	1.308	1075.875±18.844	270.248±4.248	108.099	1.751
	500	1596.60±13.010	518.144±1.883	103.628	0.819	1591.700±19.940	518.718±2.458	103.743	1.252
	1000	2549.95±25.950	974.280±2.479	97.428	1.025	2559.125±12.975	984.723±5.478	98.472	0.507
	2000	4665.5±30.829	2015.681±5.498	100.784	0.657	4658.100±20.364	1995.789±5.783	99.789	0.437

Table 5: Intra-day and inter-day precision of developed and validated method at 366 nm.

	Theoretical content (ng/spot)	Mean peak area ±SD	Content found	Content found (%)	% RSD	Mean peak area ±SD	Content found	Content found (%)	% RSD
	Intra day					Inter day			
Rhein	100	217.732±3.257	99.30±1.411	99.30	1.49	220.232±3.61	101.229±1.532	101.229	1.63
	250	396.852±4.160	237.300±1.271	94.92	1.04	381.955±5.283	227.228±1.634	97.057	1.38
	500	696.732±8.560	468.332±2.52	93.66	1.22	671.385±9.946	451.593±1.889	102.728	1.48
	1000	1436.42±16.326	1044.64±3.478	104.46	1.13	1463.635±19.35	1065.740±2.648	102.019	1.35
	2000	2680.44±29.330	2009.24±2.993	100.45	1.09	2694.58±21.023	2019.961±3.558	100.545	0.78
Chrysophanol	100	245.839±5.106	120.197±1.634	106.197	1.95	283.52±5.550	139.327±1.996	109.327	1.95
	250	488.96±9.342	243.609±2.638	97.443	1.58	472.92±7.496	235.467±2.035	94.186	1.58
	500	979.73±16.139	492.732±2.996	98.546	1.29	955.59±12.388	480.477±2.367	96.095	1.29
	1000	1966.74±21.983	993.753±3.648	99.375	0.96	1969.155±19.056	994.977±3.468	99.497	0.96
	2000	3980.48±22.486	2015.95±3.992	100.79	0.68	3946.425±27.159	1998.667±3.664	99.933	0.68

Accuracy

Samples of rhein and chrysophanol were applied at known concentrations of 500 ng and then spiked further by 50%, 100% and 150% w/w amount of respective analyte in triplicate and the accuracy was then calculated as percentage of analyte recovered from the assay. The recovery study was carried out at 254 nm and 366 nm for both the markers. The resulted data revealed 99.24%, 98.87%, 99.23%, 99.43% recovery for rhein and 99.66%, 99.29%. 98.37%, 98.60% for chrysophanol, respectively at 254 nm while 98.55%, 98.30%, 97.63%, 99.00% for rhein and 97.54%, 97.48%, 98.25%, 98.52% for chrysophanol, at 366 nm respectively. This confirmed the accuracy of the developed method. The accuracy data of the analyzed marker compounds is summarized in Table 6.

Robustness

Mobile-phase composition and chamber saturation time were deliberately altered to demonstrate the robustness of the developed and no significant variation with a %RSD was observed i.e. it was found to be lower than 2%, indicating the robustness of the method. The robustness data is compiled in Table 7.

Analysis of *R. emodi* extract and marketed formulations for quantification of rhein and chrysophanol

The aim of this study was to develop and validate a HPTLC method for simultaneously estimating the concentration of 2 marker compounds namely rhein and chrysophanol in *R. emodi* plant extract and two marketed formulations of dried roots of *R. emodi*. The solvent system of Toluene: Ethyl Acetate: Glacial Acetic Acid (6:3.5:0.5, v/v/v) was able to provide excellent separation and resolution of rhein and chrysophanol peaks

Table 6: Accuracy of developed and validated method at 254 and 366 nm.

Drug	Percentage of standard spiked to the sample	Theoretical content (ng/spot)	Amount of drug recovered (ng/spot)		% of drug recovered (ng/spot)	
			254 nm	366 nm	254 nm	366 nm
Rhein	0	500	496.21	492.78	99.24	98.55
	50	750	741.53	737.26	98.87	98.30
	100	1000	992.34	979.63	99.23	97.63
	150	1250	1242.92	1237.56	99.43	99.00
Chrysophanol	0	500	498.34	487.73	99.66	97.54
	50	750	744.68	731.11	99.29	97.48
	100	1000	983.72	982.54	98.37	98.25
	150	1250	1237.94	1232.53	98.60	98.52

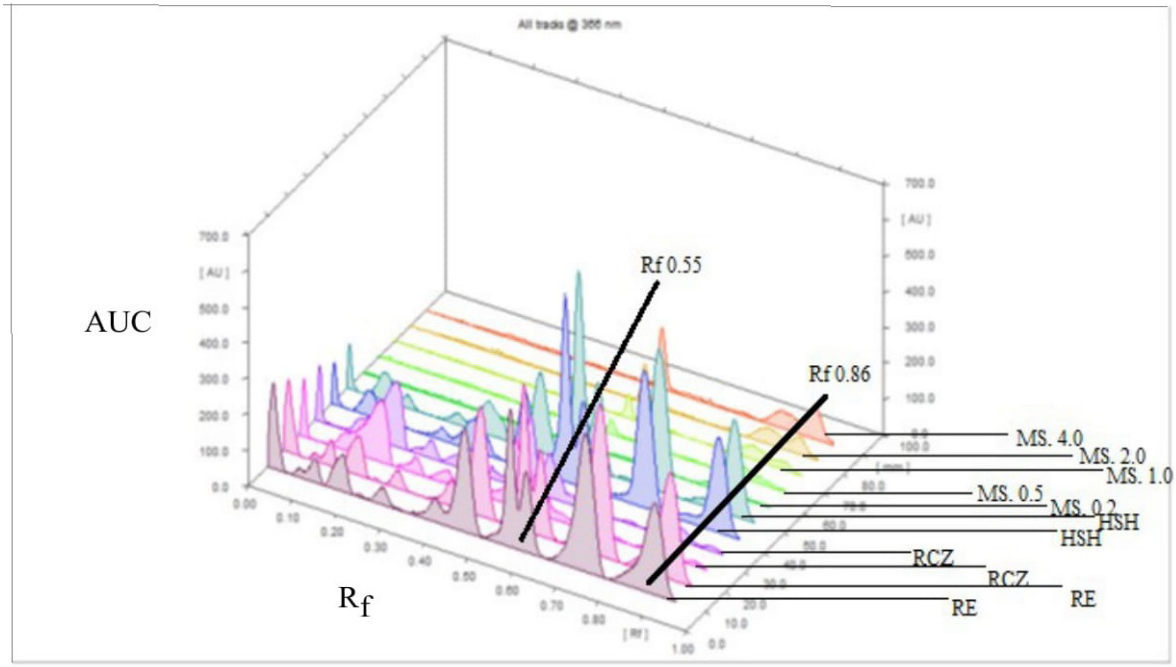


Figure 6: Overlay 3D chromatogram of standard rhein and chrysophanol at R_f 0.55 and 0.86 observed in *R. emodi* root Extract (RE), Marketed formulation 1 (RCZ) and Marketed formulation 2 (HSH) at 366 nm. Tracks 1 and 2 for RE, tracks 3 and 4 for RCZ, tracks 5 and 6 for HSH and tracks 7-11 are of mixed standard rhein and chrysophanol at concentrations of 100, 250, 500,1000 and 2000 ng/spot.

without any disturbance from the other compounds present in extract or marketed formulations (Figures 2 II, 2 III and 4).

The quantification of rhein and chrysophanol was determined using the calibration equations mentioned in Table 1. The drug content of each marker was expressed in µg/mg, w/w of the sample extract or marketed formulation. Overlay of 3D chromatograms of root extract, two formulations and mixed standards of rhein and chrysophanol are depicted in Figures 5 and 6 given under supplementary materials. The content of each marker compound has been described in Table 8.

Table 7: Robustness study of the developed HPTLC method.

Change in mobile phase ratio; Toluene, Ethyl acetate, Glacial acetic acid (6:3.5:0.5; v/v/v)				
Drug	**Mobile phase ratio**	**R_f**	**Area±SD (ng/Spot)**	**% RSD**
Rhein	6: 3: 1; v/ v/v	0.53	2931.545±12.582	0.429
	6:3.5:0.5; v/v/v	0.55	2924.05±6.151	0.210
	8: 2: 1; v/ v/v	0.48	2912.842±9.845	0.337
Chrysophanol	6: 3: 1; v/ v/v	0.84	4657.845±39.786	0.854
	6:3.5:0.5; v/v/v	0.86	4665.569±30.829	0.660
	8: 2: 1; v/ v/v	0.79	4678.561±39.845	0.851
Change in the chamber saturation time				
	Time in min	R_f	Area±SD (ng/Spot)	% RSD
Rhein	20	0.54	2925.952±9.746	0.333
	25	0.54	2931.854±9.845	0.335
	30	0.55	2924.057±6.151	0.210
Chrysophanol	20	0.85	4638.512±39.786	0.857
	25	0.85	4663.842±39.845	0.660
	30	0.86	4665.569±30.829	0.854

Table 8: Drug content of marker compounds in *R. emodi* extract and its commercial products.

Sample	Content of drug in sample (µg/mg)			
	Rhein		Chrysophanol	
	254 nm	366 nm	254 nm	366 nm
Rheum emodi plant extract	108.002±0.323	99.703±0.301	67.143±0.0884	63.006±0.081
Marketed formulation 1	26.829±0.138	24.241±0.119	1.652±0.0031	1.405±0.004
Marketed formulation 2	123.691±0.758	117.660±0.823	30.476±0.0884	27.605±0.0875

CONCLUSION

The phytochemical screening of *R. emodi* plant extract and its commercial products indicated the presence of several major and minor metabolites along with rhein and chrysophanol as the two prominent anthraquinones. There are many commercial products containing *R. emodi* extracts in the market that are used as laxatives. However, very few validated analytical methods are available which can help in quantifying these anthraquinones with the purpose of standardization of these herbal preparations. Present study demonstrated that though both the commercial preparations contain *R. emodi* extract, the content of rhein and chrysophanol in commercial product no. 2 is more (123.691±0.758, 30.476±0.0884 µg/mg respectively) than commercial product no. 1 (26.829±0.138, 1.652±0.0031 µg/mg of rhein and chrysophanol respectively. The developed method is simple, reproducible, sensitive, specific, accurate, precise and robust that can have great application for the standardization of herbal formulations and commercial products containing *R. emodi* plant extract.

AUTHOR'S CONTRIBUTION

Rizwan Ahmad: acquisition of the data, analysis and interpretation of the data, drafting of the article, statistical expertise, collection and assembly of data. Zeeshan Fatima: critical revision of the article for important intellectual content, final approval of the article, statistical expertise, administrative, technical, or logistic support. Sadath Ali: conception and design, critical revision of the article for important intellectual content, final approval of the article, statistical expertise, administrative, technical, or logistic support. Suneela Dhaneshwar: conception and design, acquisition of the data, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, administrative, technical, or logistic support, collection and assembly of data. Sayeed Ahmad: conception and design, acquisition of the data, analysis and interpretation of the data, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, administrative, technical, or logistic support, collection and assembly of data.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

R. emodi: *Rheum emodi;* **ICH:** International Conference on Harmonization; **r^2:** Regression coefficient; **R_f:** Retardation factor; **HPTLC:** High performance thin layer chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **Σ:** Standard deviation; **RSD:** Relative standard deviation; **µg/mg:** Microgram/milligram; **µL:** Microliter; **Ng:** Nanogram; **S:** Slope; **Y=mx+c:** Linearity equation.

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