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Short communication

High-performance thin layer chromatography method for quantitative determination of four major anthraquinone derivatives in *Rheum emodi*☆

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Abstract

A high-performance thin layer chromatographic (HPTLC) method for the rapid and simple quantification of the four major anthraquinone derivatives i.e. physcion, chrysophanol, emodin and chrysophanol glycoside in *Rheum emodi* is described. HPTLC of anthraquinone derivatives was performed on pre-coated RP-18 F_{254S} HPTLC plates. For achieving good separation, the mobile phase of methanol–water–formic acid (80:19:1, v/v/v) was used. The densitometric determination of anthraquinone derivatives was carried out at 445 nm in reflection/absorption mode. The calibration curves were linear in the range of 20–100 ng for physcion, 80–400 ng for chrysophanol and emodin, and 200–1000 ng for chrysophanol glycoside. The method was found to be reproducible and convenient for quantitative analysis of anthraquinone derivatives in the methanolic extract of rhizomes of *R. emodi* collected from three different locations of Western Himalaya, India.
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Keywords: *Rheum emodi*; Anthraquinones; Physcion; Chrysophanol; Emodin; Chrysophanol glycoside; HPTLC

1. Introduction

Rheum emodi Wall. (Syn. *Rheum australe* D. Don, Polygonaceae) [1–2], commonly known as revand-chini, is the Himalayan species of Indian rhubarb distributed in the alpine and sub-alpine zones of the Western Himalaya. This medicinal plant [3–5] is a storehouse of a large number of anthraquinone derivatives such as physcion, chrysophanol, emodin, aloe emodin, rhein, etc. [3–6], which are reportedly known for a large number of biological activities [6–7] including anti-oxidant [8], anti-microbial [9], anti-fungal [10], cytotoxic [11], larvicidal [12], casein kinase II inhibitory [13] and anti-viral activities [14]. Besides, these anthraquinone derivatives have a great potential for dyeing textile [15] and food stuffs [16] as safe and ecofriendly natural dyes. *R. emodi* is available from different locations of western Himalaya. However, there has been no comprehensive studies, made so far, dealing with such variational aspect based on locational and altitudinal differences in anthraquinone derivatives present in *R. emodi*. Although, quantitative analysis of anthraquinone derivatives [17], without locational comparison, have been employed using high-performance liquid chromatography (HPLC) [15,18–20], high speed counter current chromatography (HSCCC) [21–22], micellar electrokinetic chromatography (MEC) [23], capillary zone electrophoresis [24–25], TLC-colorimetric [26] and HPTLC [27]. Among them, HPTLC is a widely accepted technique for its high accuracy, precision, reproducibility of results. In addition, HPTLC is advantageous because of high sample throughput at low operating cost, easy sample preparation, short analysis time and analytical assurance by means of multi-level calibration which has yielded good results in measurement of various analytes [28,29]. However, there is only one reported HPTLC method which discloses quantification of chrysophanol without any locational comparison and also the method remains restricted for a single anthraquinone [27]. In this background, we, herein report quantification of four major anthraquinone derivatives namely physcion (**1**), chrysophanol (**2**), emodin (**3**) and chrysophanol glycoside (**4**) (Fig. 1) in *R. emodi* (rhizomes) and their locational

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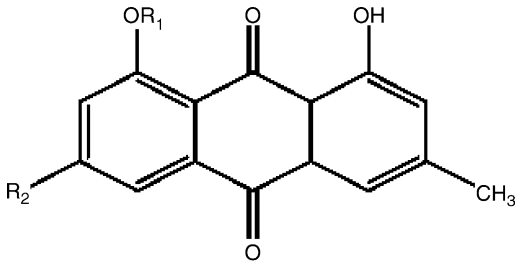

Compound	R_1	R_2
Physcion (1)	H	OCH_3
Chrysophanol (2)	H	H
Emodin (3)	H	OH
Chrysophanol Glycoside (4)	Glc	H

Fig. 1. Structure of Anthraquinone derivatives.

comparison from three different locations (1, 2 and 3) of Western Himalaya by HPTLC. Moreover, we have developed quantification of cytotoxic [11] **4** which, to the best our knowledge, has not been reported so far [17].

2. Experimental

2.1. Materials

The plant material of *R. emodi* was collected from three different locations of western Himalayas. All anthraquinone derivatives (Fig. 1) were isolated and identified in our laboratory. The HPTLC plates RP-18 F_{254S} (20 cm × 10 cm) (E.Merck, Darmastadt, Germany) were used without any pretreatment. All chemicals and solvents used were of analytical and HPLC grade (E.Merck, Ltd, Worli, Mumbai, India).

2.2. Extraction procedure

The dried and powdered rhizomes of *R. emodi* (250 g) were extracted successively with hexane, chloroform and ethyl acetate and concentrated extracts were subjected to column chromatography over silica gel using gradient elution method. Hexane extract upon column purification with ethyl acetate–hexane (5:95, v/v) yielded physcion (**1**) and chrysophanol (**2**) whereas chloroform extract upon column purification with ethyl acetate–hexane (30:70, v/v) yielded compound emodin (**3**). Similarly, ethyl acetate extract upon column purification with methanol–chloroform (20:80, v/v) yielded chrysophanol glycoside (**4**). Structure of all these compounds **1**–**4** were confirmed on the basis of NMR and its comparison with the reported data [11,12,14,30] and were used as standards.

2.3. Standard stock solution and sample preparation

Standard stock solutions containing 0.2 mg/mL of pure compounds **1**–**4** were prepared in a mixture of methanol and chloroform (80:20, v/v) and filtered through 0.45 μm (Millipore) filters for calibration studies.

Samples were prepared from dried and powdered rhizomes (500 mg) of *R. emodi* collected from different locations. The powder was extracted with methanol (3 × 10 mL), filtered and concentrated under vacuum to obtain the crude extract. A known amount of extracts (10 mg) were taken and dissolved in methanol (50 mL) and filtered through 0.45 μm filter for HPTLC analysis.

2.4. HPTLC procedure

A Camag HPTLC system equipped with an automatic TLC sampler (ATS 4), TLC scanner 3 (WINCATS version 1.2.3) and UV cabinet and twin trough glass tank (24.5 cm × 8 cm × 22.5 cm) was used for the analysis. The samples were applied using automated TLC sampler in 6 mm bands at 10 mm from the bottom, both sides and 6 mm space between the two bands.

2.4.1. Calibration and quantification

0.5 mL of compounds **1**–**4** each, taken from standard stock solutions (0.2 mg/mL), was diluted to 20, 5, 5 and 2.5 mL in methanol, respectively. The obtained working solutions were then applied on the HPTLC plate for preparing five point linear calibration curves. Compounds **1**–**3** were applied at 4, 8, 12, 16, 20 μL while **4** was applied at 5, 10, 15, 20, 25 μL.

Twenty five micro liter of each sample solutions (10 mg/50 mL) from different locations were taken and each one of them was applied on the HPTLC plate in triplicate with similar band pattern. The experimental parameters were identical for all above analysis.

2.4.2. Detection of anthraquinone derivatives

The HPTLC plates were developed in a Camag twin-trough glass tank pre-saturated with the developing solvents. The composition of the developing solvent of varying polarities was based up on clear separation of the compounds on the HPTLC plate (Fig. 2). The plates were developed to a height of about 9 cm from the base in methanol–water–formic acid (80:19:1, v/v/v). After development, the plate was removed, dried and spots were visualized under UV light. Quantitative evaluation of the plate was performed in the reflectance/absorbance mode at 445 nm slit width 6 mm × 0.4 mm, scanning speed 20 mm/s and data resolution 100 μm/step.

2.4.3. Recovery

For percent recovery experiments, three different spiking concentrations (10, 25, 50 ng/mL) of the standard stock solutions (0.2 mg/mL) of compounds **1**–**4** were prepared and used accordingly.

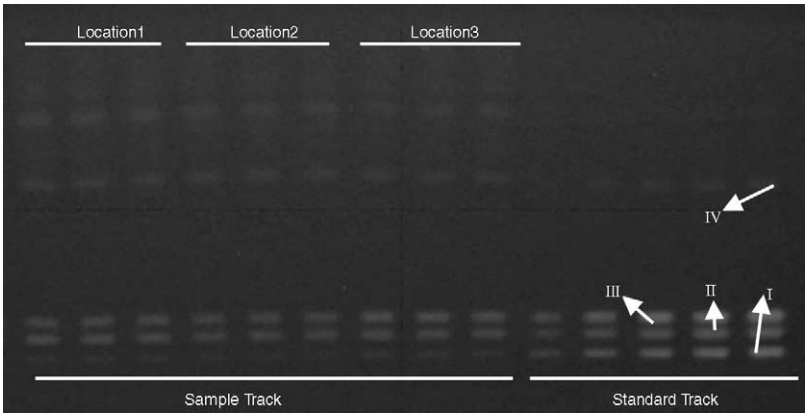

Fig. 2. HPTCL photograph of the anthraquinone separation in three track of each location and standard tracks of five different concentrations for details see Sections 2.2 and 2.3. Peaks I, II, III & IV represents the physcion chrysophanol, emodin and chrysophanol glycoside, respectively.

For percent recovery, three sets were prepared from one of the locations i.e. location 3 (1 mL each, prepared from 10 mg/50 mL). These three sets were then individually spiked with 1 mL of physcion (**1**) and 2 mL of chrysophanol (**2**), emodin (**3**) and chrysophanol glycoside (**4**) from the spiking concentrations. These spiked samples (25 μL) were applied on the HPTLC plate as described in Section 2.4.1.

2.4.4. Limit of detection (LOD) and Limit of quantification (LOQ)

The compounds were identified based on its R_f values and UV–vis spectral overlaying of the standard compounds. Standards from Section 2.3 were serially diluted and applied on HPTLC plate to plot the calibration curve. LOD was determined based on the lowest concentration detected by the instrument from the standard while the LOQ was determined based on the lowest concentration quantified in the sample.

3. Results and discussion

3.1. Method validation

Initially, compounds **1**–**4** were applied on normal phase silica gel HPTLC plate. Among them, compounds **1**–**3** showed good separation whereas **4** moved with the solvent front on TLC plate. To counter the above problem, various combinations of developing solvents with varying polarity were tested, but for no avail. Finally, our attention moved to use of RP-TLC plate which showed good separation of all the four compounds **1**–**4** with R_f values of 0.10, 0.15, 0.20 and 0.65, respectively (Fig. 2) in an optimized developing solvent consisting methanol–water–formic acid (80:19:1, v/v/v). All the compounds showed clear separation and were used as references for the identification and quantification of above four anthraquinones (**1**–**4**) present in the methanolic extract (Fig. 3) of rhizomes of *R. emodi* collected from different locations. Lower limits of detection obtained for compounds **1**–**4** were 15, 75, 65 and 120 ng, respectively with good linearties ranging from 0.997 to 0.999 while the limit of quantification obtained were 20, 80, 80 and 200 ng, respectively (Table 1).

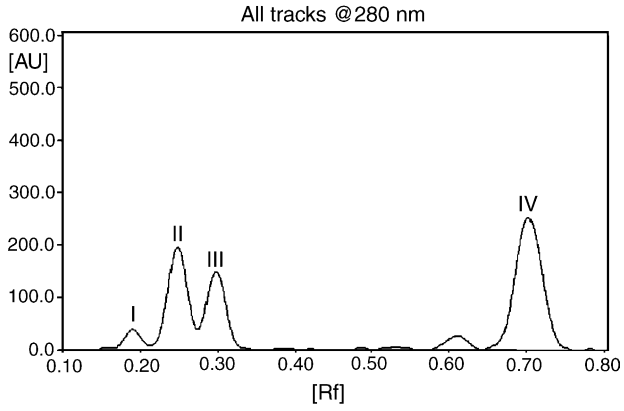

Fig. 3. The HPTCL separation of methanolic extract of *Rheum emodi* and peaks I, II, III and IV represents physcion, chrysophanol, emodin and chrysophanol glycoside, respectively. Detection at 445 nm visible reflectance/absorbance mode.

Table 1
R_f, linear regression, LOD and LOQ for anthraquinone derivatives

Anthraquinone derivative	R_f	Regression equation	r^a	SD (%)	LOD (ng/spot)	LOQ (ng/spot)
Physcion	0.10	$Y = 252.804 + 27.87X$	0.999	1.75	15	20
Chrysophanol	0.15	$Y = 2971.004 + 22.37X$	0.997	2.95	75	80
Emodin	0.20	$Y = 2555.546 + 21.32X$	0.998	2.59	65	80
Chrysophanol glycoside	0.65	$Y = 654.434 + 13.171X$	0.999	2.32	120	200

[a] Correlation coefficient.

Table 2
Recovery studies of standard compounds

Compounds	Amount of compound present in plant material (ng/ml)	Amount of standard added (ng/ml)	Amount of standard found in mixture (ng/ml)	Recovery (%)	CV (%)
Physcion	36.23	10	46.19 ± 0.20	99.67	0.44
		25	61.28 ± 0.09	100.21	0.15
		50	87.02 ± 0.14	101.58	0.16
Chrysophanol	257.62	20	277.34 ± 0.53	98.62	0.19
		50	307.23 ± 0.25	99.23	0.08
		100	359.74 ± 1.64	102.12	0.46
Emodin	131.70	20	151.67 ± 0.72	99.86	0.48
		50	181.51 ± 0.19	98.63	0.10
		100	233.16 ± 0.44	101.46	0.19
Chrysophanol glycoside	791.52	20	811.21 ± 0.60	98.45	0.07
		50	842.10 ± 1.27	101.17	0.15
		100	894.41 ± 0.50	102.89	0.01

Table 3
Amount of anthraquinone derivatives present in *R. emodi* from different locations

Location	Physcion		Chrysophanol		Emodin		Chrysophanol glycoside	
	Average ($n = 3$) (ng/spot)	CV (%)	Average ($n = 3$) (ng/spot)	CV (%)	Average ($n = 3$) (ng/spot)	CV (%)	Average ($n = 3$) (ng/spot)	CV (%)
1	39.94	0.07	340.47	1.30	200.71	0.61	905.65	0.52
2	41.73	0.36	253.30	1.06	119.01	0.63	744.25	0.14
3	36.23	0.32	257.62	1.18	131.70	1.06	791.52	0.64

Good recoveries were obtained by the fortification of the sample at three concentration levels for physcion, chrysophanol, emodin and chrysophanol glycoside. It is evident from the results that the percent recoveries for all the four anthraquinone derivatives (**1**–**4**) after sample processing and applying were in the range of 98.62–102.89%. The high percent recovery yield of compounds **1**–**4** are attributed to minimum processing steps which involved mere dissolution of the sample in methanol as shown in Table 2.

3.2. Quantification of anthraquinone derivatives

The experimental method standardized was employed to determine the anthraquinone derivatives of *R. emodi* collected from different locations of Western Himalayas. The results showed interesting differences in the amounts of these derivatives present in the same species but at different geographical locations (Table 3). In general, the percentage of physcion is lowest followed by emodin, chrysophanol and chrysophanol glycoside. Percentage of physcion was highest in location 2 followed by locations 1 and 3 and values are in the order of 41.73, 39.94, 36.23 ng, respectively. While the percentage of emodin was highest in location 1 followed by locations 3 and 2 (200.71, 131.70 and 119.01 ng, respectively). Similar trend was observed for chrysophanol (340.47, 257.62 and 253.30 ng) and chrysophanol glycoside (905.65, 791.52 and 744.25 ng). It is worth mentioning that we, for the first time, report quantification of four anthraquinones **1**–**4** in *R. emodi* along with their location comparison by HPTLC.

4. Conclusions

The HPTLC method developed here for the quantification of physcion, chrysophanol, emodin and chrysophanol glycoside is simple, rapid, cost effective, eco-friendly (low consumption of solvents) and easily adaptable for screening and quantitative determination as compared to other analytical techniques and methods. Overall, on the basis of HPTLC analysis, quantities of physcion, chrysophanol, emodin and chrysophanol glycoside in different accessions can be ascertained.

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