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Determination and locational variations in the quantity of hydroxyanthraquinones and their glycosides in rhizomes of *Rheum emodi* using high-performance liquid chromatography☆

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Abstract

Locational variations in the quantity of five hydroxyanthraquinone derivatives (emodin glycoside (**1**), chrysophanol glycoside (**2**), emodin (**3**), chrysophanol (**4**) and physcion (**5**)) in the rhizomes of *Rheum emodi* are described. A simple and reliable method was developed for quantitation of compounds (**1**–**5**) in the methanolic extract of rhizomes of *R. emodi* using reverse-phase high-performance liquid chromatography (HPLC) with photo-diode array detector (PDA). The separation was carried out using a Purospher®-Star RP-18e column (4.6 mm i.d. × 250 mm, 5 μm) under the following conditions: acetonitrile:methanol (95:5, v/v) (solvent A) and water:acetic acid (99.9:0.1, v/v) (solvent B) as mobile phase with a linear gradient elution at a flow rate of 0.8 mL/min. The detection wavelength was set at 290 nm. Regression equation revealed a linear relationship ($r^2 > 0.9901$) between the mass of hydroxyanthraquinone derivatives injected and the peak areas. The detection limits ($S/N = 3$) ranged from 0.56 to 3.50 ng/mL and the recoveries ranged from 95.7 to 103.5% for five hydroxyanthraquinone derivatives. Compound **2** was found in maximum quantity (up to 2.23%) in the rhizomes from all the three locations (L_1, L_2 and L_3) while compound **5** was found in the least quantity (up to 0.19%).

Keywords: HPLC; *Rheum emodi*; Emodin glycoside; Chrysophanol glycoside; Emodin; Chrysophanol; Physcion

1. Introduction

Anthraquinone pigments, including their glycosides are a wide group of plant secondary metabolites, present in tissue of plants belonging to the different families such as Polygonaceae, Rhamnaceae, Fabaceae, Asphodelaceae, Rubiaceae and others [1]. *Rheum emodi*, one of the oldest and best known Indian herbal medicine of Polygonaceae family, is used or recommended as a purgative, stomachic, astringent and tonic as well as in certain skin diseases [2,3]. This herbal medicine is also administered in biliousness, piles, lumbago, chronic bronchitis and asthma. Rhizomes of *R. emodi* contain a large number of hydroxyanthraquinone derivatives such as physcion, chrysophanol, emodin, aloe-emodin, rhein and their glycosides [4–9], which are reportedly known for various biological activities including anti-oxidant [10–12], cytotoxic [13], casein kinase II inhibitory [14], anti-viral [15] and nephroprotective activities [16]. In addition, these hydroxyanthraquinone derivatives are also used in preserving food stuffs [10] and in textile dyeing as safe and ecofriendly natural dyes [17,18].

Various methods for anthraquinone derivatives identification and quantification in plant extracts have been reported such as high-speed counter-current chromatography (HSCCC) [19,20], micellar electrokinetic chromatography (MEC) [21], capillary zone electrophoresis [22,23], high-performance thin layer chromatography (HPTLC) [24–26]

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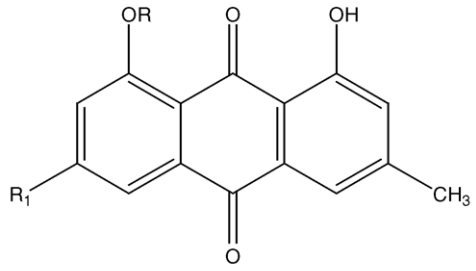

Compound	R	R_1
Emodin glycoside (**1**)	Glc	OH
Chrysophanol glycoside (**2**)	Glc	H
Emodin (**3**)	H	OH
Chrysophanol (**4**)	H	H
Physcion (**5**)	H	OCH_3

Fig. 1. Chemical structure of hydroxyanthraquinone derivatives (**1**–**5**).

and most commonly used high-performance liquid chromatography (HPLC) [12,20,27–29]. However, there has been no comprehensive study, made so far, dealing with such variational aspect based on locational differences in hydroxyanthraquinone derivatives present in rhizomes of *R. emodi* by HPLC. Hence, in the present communication, we report for the first time, a simple analytical method using HPLC for quantitative determination of five hydroxyanthraquinone derivatives, namely emodin glycoside (**1**), chrysophanol glycoside (**2**), emodin (**3**), chrysophanol (**4**) and physcion (**5**) (Fig. 1) and their locational variations in rhizomes of *R. emodi* collected from three different locations (L_1, L_2 and L_3) of western Himalaya. The study would be useful in commercial utilization of *R. emodi*.

2. Experimental

2.1. Materials and chemicals

The rhizomes of *R. emodi* were collected from three different locations (L_1, L_2 and L_3) of western Himalaya of Himachal Pradesh, India. The rhizomes were dried under the gentle stream of air in the laboratory (temperature $25 \pm 2\,^{\circ}C$ and relative humidity $65 \pm 5\%$) and powdered in an electric grinder. The powdered rhizomes were used for the extraction of hydroxyanthraquinone derivatives. All solvents used were of analytical and HPLC grade (Merck, Mumbai, India). Ultra-pure distilled water with resistivity greater than 18 MΩ was used. For samples and solvents filtration, 0.45 μm membrane filters (Millipore, Germany) were used, and solvents were degassed prior to use.

2.2. Instruments and conditions

The HPLC (Waters model 600E system, Waters, Milford, MA, USA) equipped with Purospher®-Star RP-18e column (4.6 mm i.d. × 250 mm, 5 μm, Merck, Darmstadt, Germany), a photo-diode array detector (PDA) (Waters 2996), an inline-degasser AF (Waters), a 20 μL loop manual injector and Waters empower software, was used for analysis of hydroxyanthraquinone derivatives. Acetonitrile:methanol (95:5, v/v) (solvent A) and water:acetic acid (99.9:0.1, v/v, pH 3.5) (solvent B) were used as mobile phase with a linear gradient elution as follows: 0–15 min, 20% A; 15–25 min, 50% A; 25–30 min, 70% A; 30–40 min, 100% A; at a flow rate of 0.8 mL/min. The detection wavelength was set at 290 nm. The column temperature was 30 °C, and the injection volume of samples was 20 μL.

An ultrasonicator (Sonics, Vibra cell, ultrasound waves for 30 min with a pulse rate of 5 s) was used for the extraction of hydroxyanthraquinone derivatives (**1**–**5**) from the rhizomes of *R. emodi*. UV–vis spectra were recorded on Analytikjena Specord 200 spectrophotometer.

2.3. Extraction and isolation of hydroxyanthraquinone derivatives (*1*–*5*)

The powdered rhizomes (225 g) were extracted with three different solvents (3 × 500 mL each with hexane, chloroform and ethyl acetate) under ultrasonication (3 × 10 min). The extracts were filtered and solvents were removed under reduced pressure. Concentrated extracts obtained from different solvents 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 chrysophanol (**4**) and physcion (**5**), whereas chloroform extract upon column purification with ethyl acetate–hexane (30:70, v/v) yielded emodin (**3**). Similarly, ethyl acetate extract upon column purification with methanol–chloroform (20:80, v/v) and (30:70, v/v) yielded chrysophanol glycoside (**2**) and emodin glycoside (**1**), respectively [26]. The purity and structure of compounds (**1**–**5**) were confirmed by matching their reported physio-chemical (mp and co-TLC) and spectral data (NMR and UV–vis) [13,15,20,30–32] (Fig. 2). These hydroxyanthraquinone derivatives (**1**–**5**) were used as standards for HPLC analysis.

2.4. Method validation

2.4.1. Calibration curve

The stock solution (1 mg/mL) of each standard hydroxyanthraquinone derivatives (**1**–**5**) was freshly prepared in a mixture of methanol:acetonitrile (80:20, v/v) and desired concentrations were obtained by serial dilution for standard curve preparation (Table 1). The calibration graphs were plotted after linear regression of the peak area versus concentration and both detection limits (LODs) and quantitation

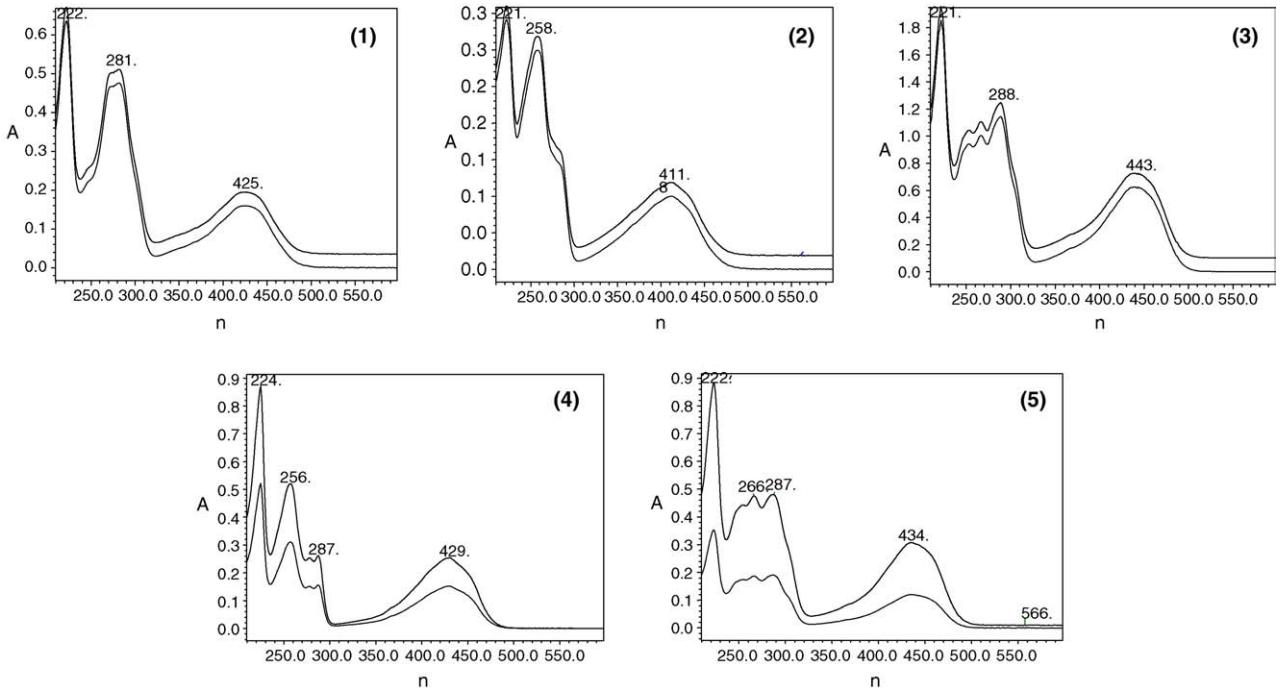

Fig. 2. UV–vis spectra of hydroxyanthraquinone derivatives (**1**–**5**) recorded with the HPLC-PDA (black line) and a spectrophotometer (dotted line).

limits (LOQs) were measured following the standard methods [33].

2.4.2. Repeatability

The precision of the chromatographic determination for the proposed method, expressed as a relative standard deviation (RSD), was calculated by five replicate injections of each compound (intra-day and inter-day). The standard solutions used for repeatability experiments were the same as used in the calibration curve experiment.

2.4.3. Recovery

For percent recovery, three sets of methanolic extract of rhizomes of *R. emodi* were prepared from one of the locations, i.e. location L_1 (1 mL each, 1 mg/mL). Three different concentrations of standard compounds **1** (20, 40 and 60 μg/mL); **2** (60, 80 and 100 μg/mL); **3** (10, 20 and, 40 μg/mL); **4** (20, 40 and 80 μg/mL); and **5** (25, 50 and 100 μg/mL) were prepared. These three sets were then individually spiked with 1 mL of each hydroxyanthraquinone derivatives (**1–5**) from all the three spiking concentrations. The samples, to which standards were added, were pretreated and analysed using the developed HPLC method. Twenty microlitres of each concentration was injected into the HPLC for measuring the percentage recovery.

2.5. Sample preparation

The powdered rhizomes (1.0 g) of *R. emodi* from three different locations (L_1, L_2 and L_3) were extracted separately with 20 mL of methanol using an ultrasonicator (ultra-sound waves used for 3×10 min). Extracts were evaporated to dryness under reduced pressure. Each obtained extract was dissolved separately in methanol to get 1 mg/mL concentration and was subjected to HPLC analysis for quantitative determination of hydroxyanthraquinone derivatives (**1**–**5**).

3. Results and discussion

3.1. Method development for separation of hydroxyanthraquinone derivatives (*1*–*5*)

HPLC quantitative determination for a large number of hydroxyanthraquinone derivatives from *Rheum* spp. is well

Table 1
Parameters of the linearity, detection limit and quantitation limit for hydroxyanthraquinone derivatives (**1**–**5**)

Compound	Linearity range (μg/mL)	Linear equation	r^2	LOD (ng/mL)	LOQ (μg/mL)
Emodin glycoside	20–100	$y = 260049x - 1557023$	0.9993	0.78	0.11
Chrysophanol glycoside	60–200	$y = 5101x - 69864$	0.9979	0.89	0.16
Emodin	1–100	$y = 65718x - 263159$	0.9991	0.56	0.24
Chrysophanol	10–100	$y = 35034x - 325578$	0.9901	0.68	0.26
Physcion	25–400	$y = 16100x - 269319$	0.9959	3.50	0.35

r^2 = correlation coefficient.

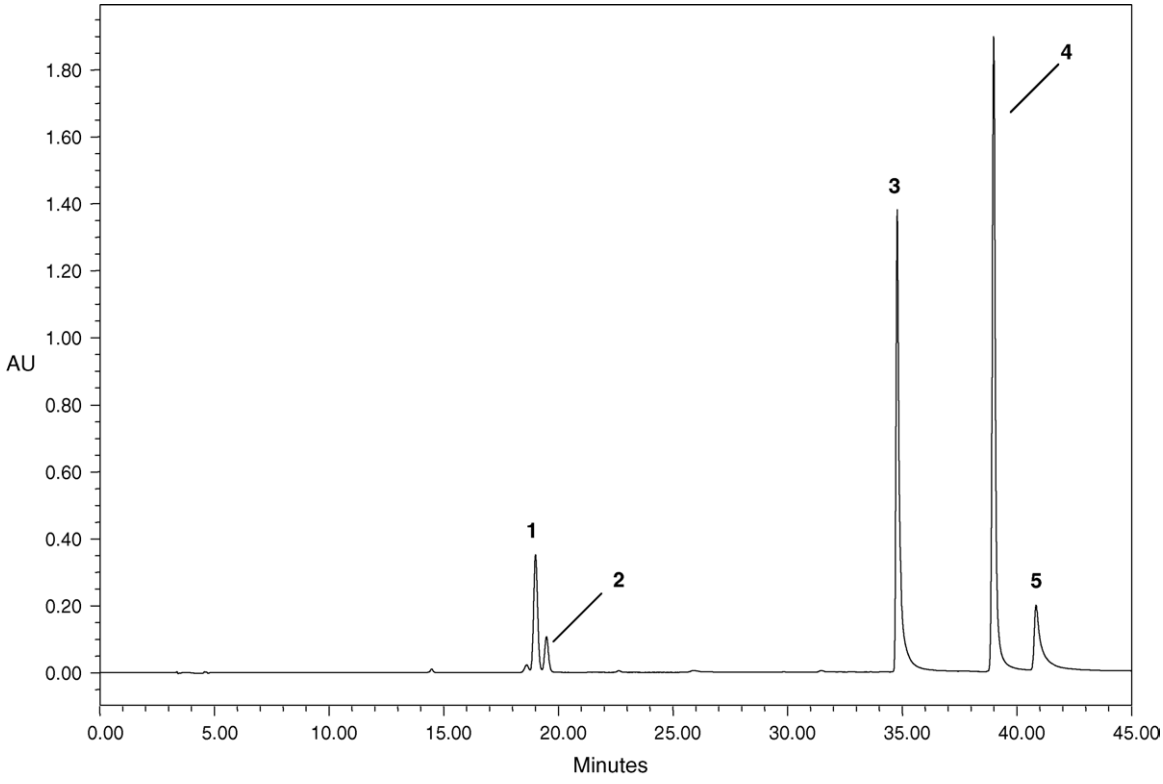

Fig. 3. HPLC chromatogram of standard hydroxyanthraquinone derivatives (for HPLC conditions, see Section 2.2). Peaks: **1** = emodin glycoside; **2** = chrysophanol glycoside; **3** = emodin; **4** = chrysophanol; **5** = physcion.

known [12,20,27–29]; however, to the best of our knowledge, an analysis of such glycosidic hydroxyanthraquinone derivatives is not reported in literature, which may be primarily because of three main reasons. First, compounds **1** and **2** are not commercially available due to the difficulty of their isolation from natural sources. Secondly, glycosylation [34] of hydroxyanthraquinone derivatives involves a multi-step reaction, which is again a tedious process. Lastly, the use of acidic eluent required to avoid peak tailing during resolution of hydroxyanthraquinone derivatives might hydrolyze the glycosidic linkage of compounds **1** and **2**. Therefore, the present study was aimed at developing a simple and reliable HPLC method for better separation of mixture of hydroxyanthraquinone derivatives (**1**–**5**). Initially, solvent system A (acetonitrile) and solvent system B (water:acetic acid, 99.9:0.1, v/v) in mobile phase (with gradient elution of solvent system A from 20 to 100% in 45 min with solvent system B) showed no resolution between compounds **1** and **2**. Further, addition of methanol in solvent system A (i.e. acetonitrile:methanol, 95:5, v/v) with solvent system B (water:acetic acid, 99.9:0.1, v/v) in mobile phase with similar condition showed better resolution between compounds **1** and **2** with a difference of 0.5 min in retention time. Increasing the ratio of solvent system A (>20%) in mobile phase resulted in a quick elution of all hydroxyanthraquinone derivatives (**1**–**5**), thus, badly affecting the resolution of mixture of hydroxyanthraquinone derivatives (**1**–**5**). In solvent system B, the presence of acetic acid in the range 0.08–0.13% was crucial for better peak shapes of hydroxyanthraquinone derivatives (**1**–**5**) and improved detection limit of compound **5** as shown in Fig. 3. Thus, combination of solvent system A (acetonitrile:methanol, 95:5, v/v) and solvent system B (water:acetic acid, 99.9:0.1, v/v) provided a better choice of solvents for separation of hydroxyanthraquinone derivatives (**1**–**5**) with retention times of 18.8, 19.3, 34.5, 38.8 and 40.7 min, respectively (Fig. 3). The same conditions were followed for the separation of hydroxyanthraquinone derivatives (**1**–**5**) present in the crude methanolic extract of rhizomes of *R. emodi* and the obtained results were satisfactory (Fig. 4).

3.2. Parameters for method validation

The linearity, limit of detection (LOD) and limit of quantitation (LOQ) for hydroxyanthraquinone derivatives (**1**–**5**) were investigated and the results are presented in Table 1. The linear equation between the concentration of the standards injected and the peak area can be expressed as $y = mx + c$, where y is the concentration and x is the peak area of the standard, and m and c are constants. A good linearity was achieved in the range of 0.9901–0.9993 for all hydroxyanthraquinone derivatives (**1**–**5**) (Table 1). Detection limit is the lowest amount of the analyte in a sample that can be detected, but not necessarily quantitated. The lowest limit is usually evaluated as the signal to noise ratio that is equivalent to three times the standard deviation of the noise ($S/N = 3$). The LOD and LOQ were estimated in accordance with base

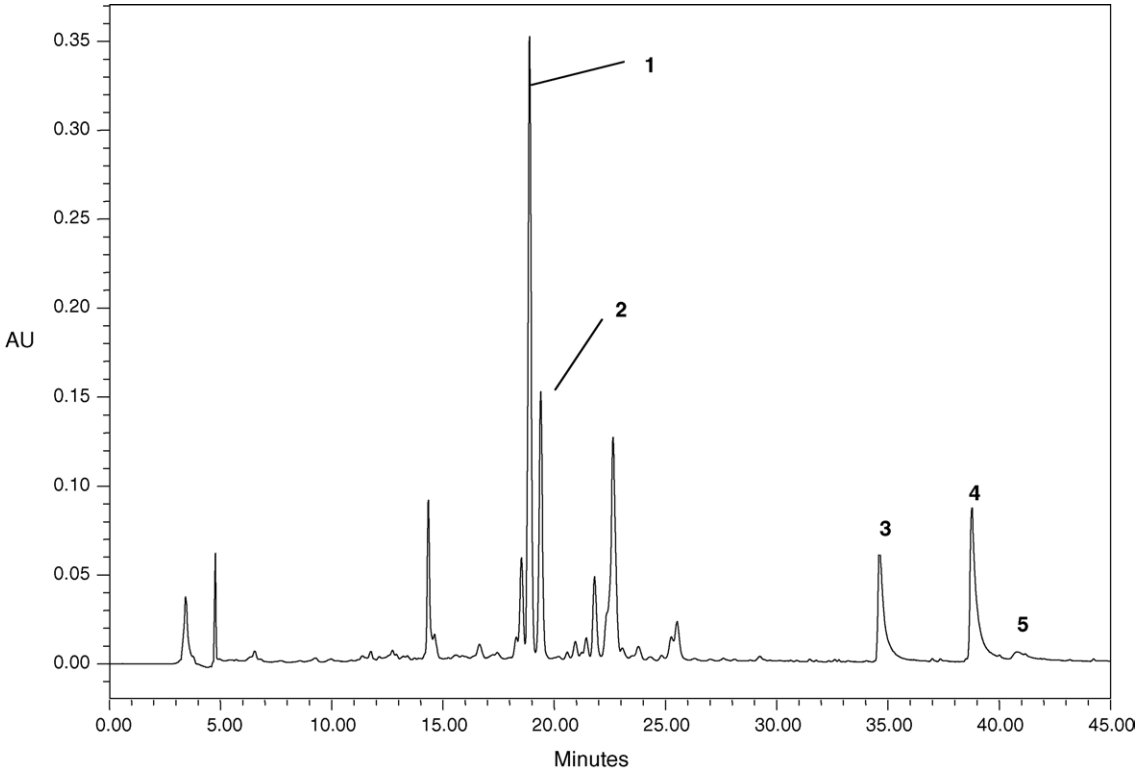

Fig. 4. HPLC chromatogram of methanolic extract of rhizomes of *R. emodi* (for HPLC conditions, see Section 2.2).

line noise, which was evaluated by recording the detector response over a period of as much as the 10 times of the peak width. The LOD for hydroxyanthraquinone derivatives (**1**–**5**) were in the range of 0.56–3.50 ng/mL. The LOQ, which is defined as the lowest concentration that can be determined with acceptable accuracy and precision, can be established at a signal to noise ratio of 10. The LOQ for hydroxyanthraquinone derivatives (**1**–**5**) was experimentally verified by six injections and was found in the range of 0.11–0.35 μg/mL (Table 1).

The instrument precision was measured by performing the intra-day and inter-day experiments by five replicate injections of each hydroxyanthraquinone derivative (**1**–**5**) in three

Table 2
Repeatability of intra-day and inter-day analysis

Compound	Concentration (μL/mL)	RSD (%)	
		Intra-day (n = 5)	Inter-day (n = 5)
Emodin glycoside	80	0.23	0.98
	40	0.31	1.15
	20	0.76	2.10
Chrysophanol glycoside	40	4.91	2.84
	20	3.21	2.25
	10	4.98	4.24
Emodin	40	0.45	1.10
	20	0.57	3.50
	10	3.81	3.30
Chrysophanol	80	0.95	1.28
	40	2.96	1.74
	10	4.21	3.71
Physcion	200	2.41	3.25
	100	2.08	2.95
	60	3.59	4.65

RSD = relative standard deviation; n = No. of injections.

Table 3
Recovery data by standard addition

Compound	Concentration (μg/mL)	Added (μg/mL)	Obtained (μg/mL)	Recovery (%)
Emodin glycoside	2.50	20	22.34	99.2
		40	42.59	100.2
		60	62.42	99.8
Chrysophanol glycoside	10.24	60	70.60	103.5
		80	90.48	102.3
		100	110.16	99.2
Emodin	4.25	10	13.92	97.6
		20	24.44	100.7
		40	44.31	100.1
Chrysophanol	7.50	20	26.92	97.8
		40	47.18	99.3
		80	88.86	101.5
Physcion	0.93	25	24.83	95.7
		50	51.68	101.4
		100	98.94	98.0

Table 4
Locational comparison of the hydroxyanthraquinone derivatives (**1–5**) content present in rhizomes of *R. emodi*

Location	Emodin glycoside (%)	Chrysophanol glycoside (%)	Emodin (%)	Chrysophanol (%)	Physcion (%)
L_1	0.51	2.23	0.90	1.44	0.19
L_2	0.44	1.56	0.49	0.99	0.15
L_3	0.58	1.98	0.64	0.88	0.16

different working concentrations. The intra-day and inter-day RSDs of chromatographic determination were observed in the range of 0.23–4.98% and 0.98–4.65%, respectively (Table 2). The results showed good precision of the method.

Quantitative results are expressed in terms of recovery percentage. The recoveries accomplished for all the hydroxyanthraquinone derivative (**1**–**5**) were in the range of 95.7–103.5% (Table 3).

*3.3. Quantitation of hydroxyanthraquinone derivatives (**1–5**)*

Hydroxyanthraquinone derivatives (**1**–**5**) in the methanolic extract of the rhizomes of *R. emodi* were quantitated using developed HPLC method. The calculated percentage of hydroxyanthraquinone derivatives (**1**–**5**) in the rhizomes collected from three locations (L_1, L_2 and L_3) were in the range of 0.44–0.58%, 1.56–2.23%, 0.49–0.90%, 0.88–1.44% and 0.15–0.19%, respectively. In general, the percentage of chrysophanol glycoside (**2**) is highest followed by chrysophanol (**4**), emodin (**3**), emodin glycoside (**1**) and physcion (**5**) in all the locations as clearly evident from Table 4.

Quantitatively and qualitatively variations in secondary metabolites in the plant of the same species grown in different geographical locations [35–37] is well understood. The above study clearly reveals the locational variation in the chemical composition of the plants collected from the different locations with respect to the above mentioned hydroxyanthraquinone derivatives (**1**–**5**). Further, quantitation and identification of remaining secondary metabolites present in rhizomes of *R. emodi* by HPLC is under progress.

4. Conclusion

A simple and reliable HPLC method for the simultaneous determination and locational variation of five hydroxyanthraquinone derivatives (**1**–**5**) in rhizomes of *R. emodi* was developed. The method enabled good resolution for hydroxyanthraquinone glycosides **1** and **2** having close polarities and chromatographic behavior. The developed method has been satisfactorily verified for its accuracy, precision and selectivity. In addition, the HPLC study of locational variation allowed us to conclude that the percentage variation of hydroxyanthraquinone derivatives (**1**–**5**) in all the three locations (L_1, L_2 and L_3) remain in the order **2** > **4** > **3** > **1** > **5**. The method can be used for quality control of herbal formulations containing hydroxyanthraquinone derivatives and it can be used for pharmacokinetic studies of related extracts and drugs.

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