Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(2): 121-130
Received: 19-01-2016
Accepted: 22-02-2016

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Comparative HPLC analysis of emodin, aloe emodin and rhein in *Rheum emodi* of wild and *in vitro* raised plants

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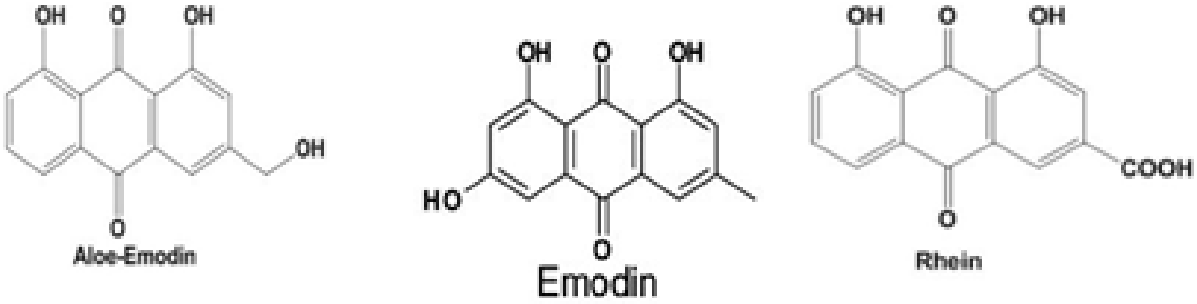
Abstract
The plants are like microbiosynthetic factories having variety of compounds which are their secondary metabolites. These compounds mainly include alkaloids, glycosides, flavonoids, volatile oils, saponins, etc. with tremendous biological activities. The medicinal properties are attributed to the specific bioactive compound or combination of phytochemicals. *Rheum* species contains anthraquinone derivatives, anthrononeses and tannins, etc., in which anthraquinone derivatives including emodin, aloe-emodin, rhein, physcion, chrysophanol and their glucosides are the accepted important active components. The methods commonly used for the determination of the anthraquinone compounds in *Rheum* species are high performance liquid chromatography (HPLC). Aloe-emodin is a natural active compound present in the *Rheum* species. It has also been found that aloe-emodin has numerous biological properties including antiviral, antimicrobial and hepatoprotective activities. Aloe-emodin has been reported to exhibit anticancer activity on neuroectodermal tumors, lung squamous cell carcinoma and hepatoma cells. Another compounds found in *Rheum* species are emodin, rhein, chrysaphanol, physicion etc.

Keywords: *Rheum*, HPLC, Aloe emodin, anthraquinones, phytochemicals, rhein, emodin

Introduction
The old age traditional values attached with the various forest types and the varieties of forest products (i.e., medicinal plants) have gained tremendous importance in the present century [1]. Medicinal plants have been the subject of man's interest since time immemorial and play a key role in human health [2]. Medicinal plants have been identified and used throughout human history. The North Western Himalayan region is endowed with rich wealth of medicinal plants and has large scope for the development of the pharmaceutical and phytochemical industry. In modern medicine, the plants are either directly used as medicine or are isolated or provide bioactive compounds used as drug, used as raw material for semi synthetic drugs or as markers for discovery of new bioactive compounds [3]. A significant variations in phytopharmaceutical products have been reported which are influenced by genetic diversity (both chromosomal and genetic), environmental factors, plant part used, developmental stage of plant, season of collection, cultivation practices, adulteration with this identified plant species intentional mixing, attack of pathogen, etc. [4]. Further, due to multi herbal nature of most of the herbal preparations, it is difficult to identify and quantify the active constituents. Many compounds used today having medicine properties have a complex structure, and synthesizing these bioactive compounds chemically at a low price is not easy [5]. The *Rheum* species are reckoned in endangered list and are under great threat [6] and has been listed as vulnerable by various agencies like IUCN, UNEP and WWF particularly from North western Himalaya. *Rheum* plant has sixty species all over the world. It is very famous for its medicinal value, as its recent studies has proved its one of the anti-cancer plant. It is also an eatable plant and can be taken as a food and its juicy stalks are eaten raw and its leaves are cooked as a vegetable. *Rheum* species are found in all over the world like, India, Pakistan, China, Switzerland, Afghanistan, Balochistan, turkey, Iran, Iraq, Syria and Uzbekistan. In Kashmir valley, it is found in almost all the districts from Ladakh, Guerz to Pahalgam and Gulmarg areas. *R. emodi* is a puragative, stoamchachic, astringent, and tonic and helps in to cure skin diseases [7], antioxidant [8] cytotoxic [9] case and nephroprotective activity [10], used ad textile dyeing [11], anti-microbial, antitumor, anti inlammmatory used in cosmetics and as food colorant [12], live stimulant, purgative, anticholesterolemic, anti-tumor, antiseptic and tonic, anti-fungal, antiparlinson [13]. Chromatography is one of the fast emerging tools by which the quality control and fingerprint of herbs can be maintained. Using this technique, the identification of various chemical markers of the herbal drugs can be easily done and it also helps to identify the same herbs in

combination. Popularity of HPLC analytical method for analysis of herbal drugs due to economic, rapid, simultaneously screening of large number of herbal samples and less time consuming methods. The main active ingredients of the *Rheum* species are a series of anthraquinones, dianthrones, glycosides and tannins. *Rheum* contains anthraquinone derivatives, anthrones and tannins, etc., in which anthraquinone derivatives including emodin, aloe-emodin, rhein, physcion, chrysophanol and their glucosides are the accepted important active components. Rhaponticin, a distyrene derivative, only exists in non-quality (inferior grade) *Rheum*. In quality, *Rheum* and most the content of rhaponticin is not allowed to be detected. Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) is an active constituent of many herbal laxatives. *Rheum spps are* most popular traditional medicinal herbs recorded in Chinese Pharmacopoeia and its species are widely distributed in China. It has been used for the treatment of inflammatory diseases such as peptic ulcers and as a laxative, and others such as skin burns, gallstone, hepatitis, inflammation and osteomyelitis, etc. Rhein, also known as cassic acid, is a substance in the anthraquinone group obtained from *Rheum*. (Pharmacognosy of *Rheum*) Like all such substances, rhein is a cathartic. Rhein is commonly found as a glycoside such as rhein-8-glucoside or glucorhein. (Pharmacognosy of *Rheum*). Rhein was first isolated in [14]. Originally, the *Rheum* plant which contains rhein is used as a laxative. It is believed that rhein along with other anthraquinone glycosides imparted this activity. Rhein has been re-evaluated as an antibacterial agent against *Staphylococcus aureus* [15].



In the current study, the populations of *Rheum emodi* were collected from different locations and were analysed for emodin, aloe-emodin, rhein by HPLC method. Besides, compounds from all the populations were correlated with *in vitro* raised plants of *R. emodi.*

Materials and Methods

Collection of wild germplasm of *Rheum emodi*:

Surveys were done for the collection of *Rheum emodi* from high altitudes of Kashmir Himalaya. *R. emodi* was collected from various altitudes like Yousmarg, Dodhpathri, Thajwas Glacier (Sonmarg), Affarwat (Gulmarg), Dagvan (Tarsar Marsar), Aaroo (Pahalgaam), Laar and Dhara.

Preparation of Plant material

The roots and rhizomes of *Rheum emodi* were kept in brown paper bags dried under room temperature. The *in-vitro* explants *i.e.* leaves, roots and callus [16] of the same species were also dried in room temperature in paper bags. The dried roots and rhizomes of all these species were grinded in pestle and mortar to powder form for material and methanol (HPLC grade) for extraction. The glassware and methanol (HPLC grade) was procured from Merck.

Extraction of plant material:

The powdered roots and rhizomes of each sample (30 g) were charged in a soxhlet apparatus and extracted with 500 ml of HPLC methanol on water bath. The extraction was continued for one week. The extract was concentrated and dried on rotary evaporator under reduced pressure. The resultant semisolid, sticky extract of each sample was stored at 4 ˚C till further analysis. Each extract was subjected to phytochemical screening to detect the different types of constituents present in it.

Qualitative Phytochemical analysis:

a) Alkaloids: A part of each extract was shaken with dilute HCl. The contents were filtered, and the filtrate was used for the following tests for alkaloids. To 2-3 ml filtrate, few drops of Dragendorff's reagent was added (orange brown precipitates show presence of alkaloid).

b) Carbohydrates: Fehling solutions A and B (1ml each) were mixed and boiled for one minute. To this, an equal volume of solution of extract in methanol (TS) was added. The contents were heated on water bath for 5-10 min. (A yellow then brick red precipitates indicate presence of reducing sugars).

c) Proteins and amino acids: The TS (3ml) was mixed with 5 ml Million's reagent (white precipitates which turn brick red or dissolve giving red colour solution on warming indicate presence of proteins and amino acids).

d) Steroids: To a 2 ml of extract in methanol, 2 ml chloroform and 2 ml concentrated H_2SO_4 were added. The mixture was shaken well (chloroform layer becoming red and acid layer becoming greenish yellow fluorescence, indicate presence of steroids)

e) Flavonoids: Extract was mixed with 5 ml of 95% ethanol. Then a few drops of concentrated HCl and 0.5g of magnesium turnings were added (pink colour indicates the presence of flavonoids).

f) Glycoside test: Glacial acetic acid, one drop 5% $FeCl_3$ and concentrated H_2SO_4 were added to a 2ml of extract (reddish brown colour at junction of the two liquid layers and upper layer becoming bluish green, indicates that cardiac glycoside (deoxysugars) are present).

g) Tannins and phenolic compounds: 2 ml of the extract solution was mixed with 5% $FeCl_3$ solution (deep blue-black colour, shows the presence of tanins and phenolic compounds).

h) Fats and Oils: A thin section of drug (rhizomes/roots) on glass slide was placed, and then drop of Sudan III reagent was added. After 2 min, it was washed with 50% alcohol, then it was mounted in glycerine and observed under microscope (red coloured oil globules show presence of fats and oils).

HPLC analysis

Chromatographic system:

The chromatographic analysis was carried out on a Waters HPLC system comprising binary pumps (515), auto injector (2707) and PDA detector (2998), controlled by Empower Pro software. Each standard marker (rhein, emodin, aloe-emodin) and extract (1g extract dissolved in 5 ml methanol) was chromatographed on a C_{18} column (250 mm x 4.6 mm; Sunfire) with gradient elution by using methanol (mobile phase A) and 2% acetic acid (mobile phase B) at a flow rate of 0.5 ml/min. The column was maintained at a temperature of 30 °C. The injection volume was fixed at 10 µl and LC-UV chromatographs were extracted at 254 nm. The gradient program for elution is given in Table 1. Purity of each marker peak in LC-UV chromatogram of each sample was ascertained by PDA analysis.

Anthraquinone Analysis

For quantification of the three markers (aloe-emodin, rhein & emodin), a standard solution containing these three markers (1 µg/ml) in methanol was prepared and analysed (n=6) using the optimized HPLC methanol. Peak area of each marker (mean ±SD) was determined, and it was used to calculate the content of markers in samples of *Rheum* collected from different sources. For sample analyses, each extract obtained after recovery of the solvent was dissolved in 150 ml of methanol. This extract solution was analysed by the HPLC method and contents of markers were calculated as follows:

Content of markers in (µg/30g) = (A_S/A_T) x l x 150

Where, A_S = peak area of marker in standard solution

A_T = peak area of marker in extract solution

1= concentration of marker in standard solution in µg/ml

150 = dilution factor

Statistical analysis

Data were subjected to analysis of variance using SSPP software version 17.0 (SAS Institute Inc., Cary, NC, USA). The amount of anthraquinones among different populations was considered significant according to the magnitude of the F value (P<0.005).

Results

Phytochemical screening

The phytochemical screening of extracts of different samples of *R. emodi* (Table 2) revealed that amino acids were absent in the samples collected from Pahalgaam, Gulmarg and TarsarMarsar. Steroid was absent in TarsarMarsar, Laar and Dhara samples. Glycosides were absent in the samples collected from TarsarMarsar, only. All types of phytoconstituents were found present in different parts of the plant grown in *in-vitro*.

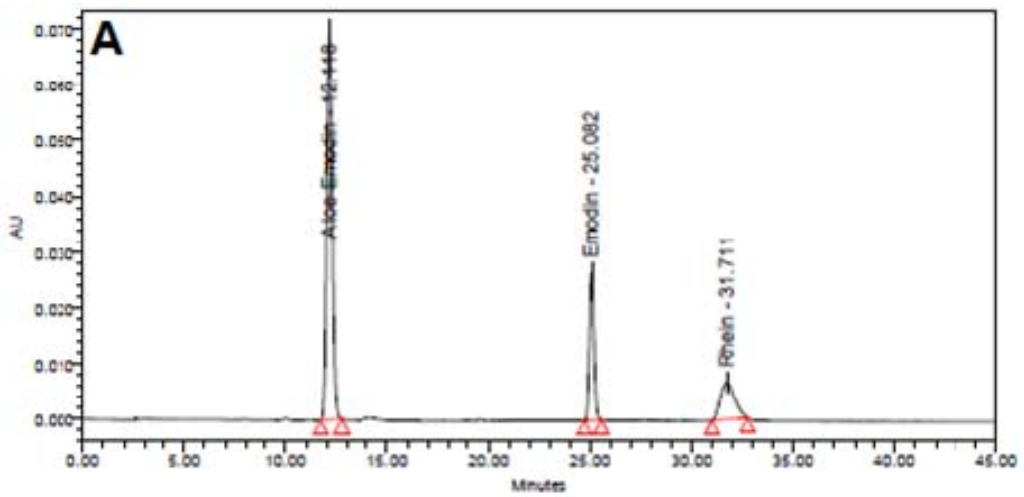
HPLC analysis

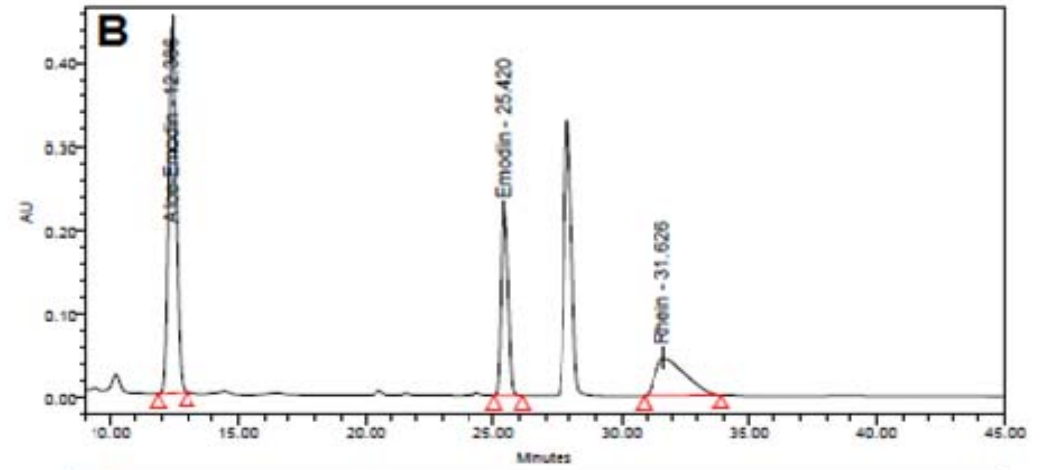
Aloe-emodin, emodin and rhein were eluted as symmetrical peaks at retention time (R_t) of about 12, 25 and 31 min (Fig. 1). The total run time for a single HPLC analysis was 45 min. The area of peaks of aloe-emodin, emodin and rhein (mean ±SD) in standard solutions were found to be 374336 (±15988.7), 927924 (±4086.31), and 1020216 (±12094.66). The LC-UV chromatograms of extracts of different samples of *R. emodi* as well as of *in-vitro* explants (Figure 1).

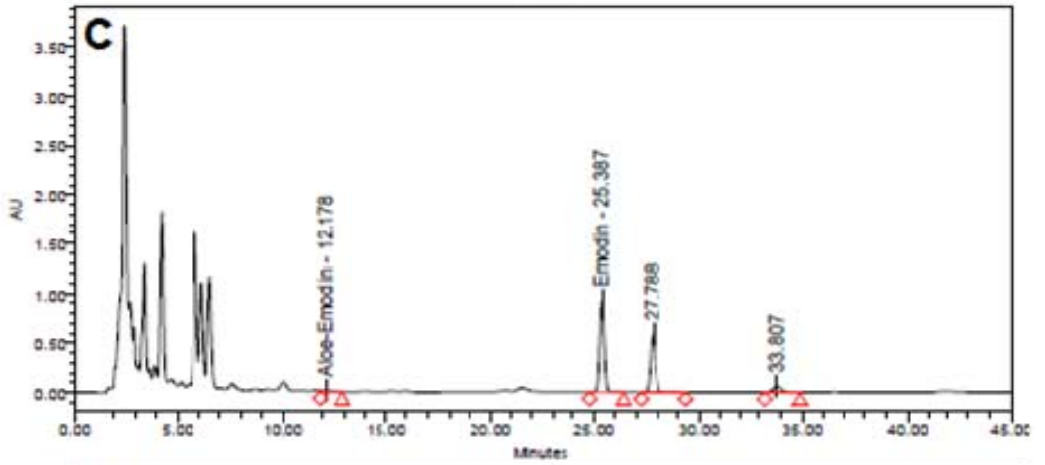
The amount of the three markers calculated in different samples on the HPLC data are given in Table 3. Aloe-emodin and rhein were found to be present in all the collected and *in-vitro* grown sample of *R. emodi*. However, the content of aloe-emodin was found to vary from 4.8 µg/30 g (Yousmarg sample) to 3640.5 µg/30 g (Dhara sample) and of emodin varied from 12.75 µg/30 g (*in-vitro* leaves sample) to 2190 µg/30 g (Dodhpathri sample). In all parts of *in-vitro* grown samples, the content of aloe-emodin was found to the higher than that of emodin (Table 4, Figure 3).

Rhein was not detected at all in samples collected from Yousmarg, Sonamarg and Dodhpathri (Table 3) as well as samples grown *in-vitro* (Table 4, Figure 3). Its content was also found to vary significantly from as low as 6.3 µg/30 g (Laar sample) to as high as 540 µg/30g (Dhara sample).

A comparison of content of these three markers with respect to altitude of the collected sample revealed that there is no co-relation between the amount of markers and the altitude (Figure 2).




B
Aloe-Emodin - 12.366
Emodin - 25.420
Rhein - 31.626
AU
Minutes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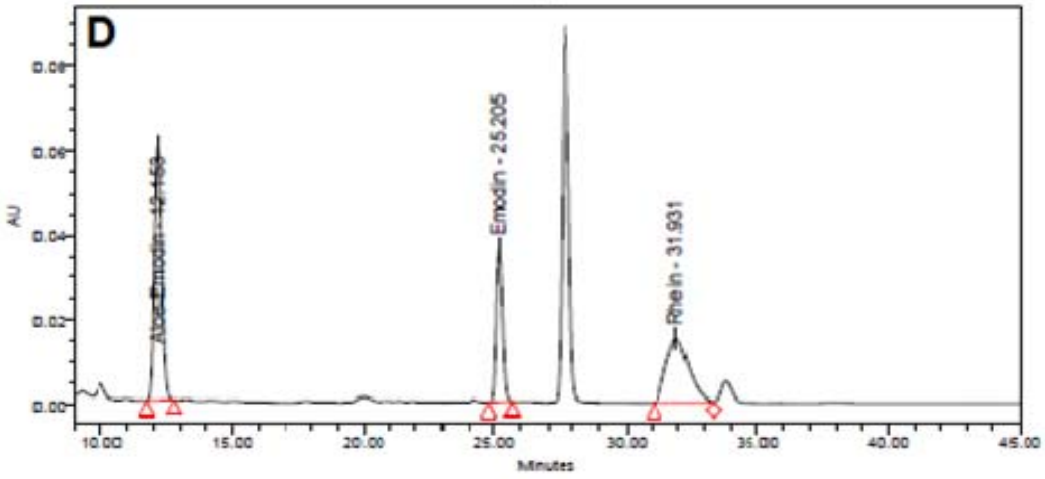
Aloe-Emodin - 12.178
Emodin - 25.387
27.788
33.807
AU
Minutes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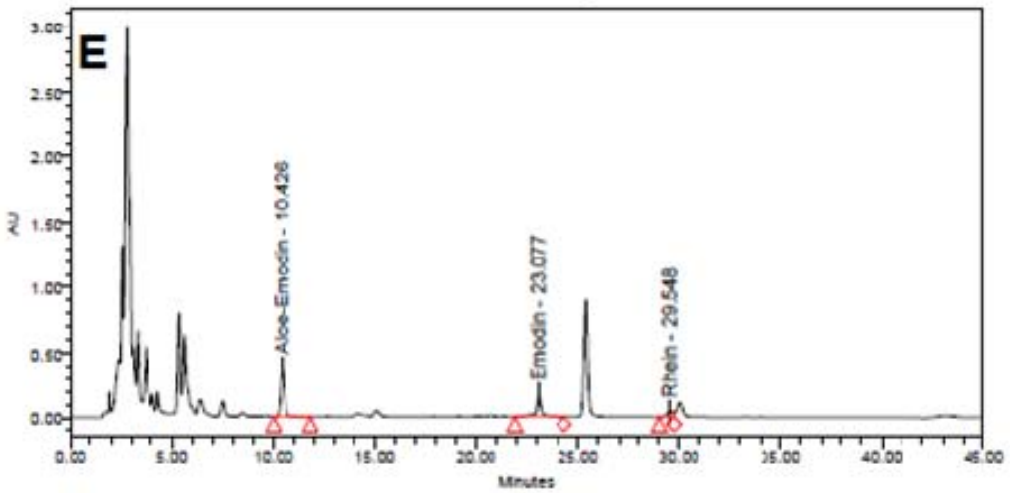
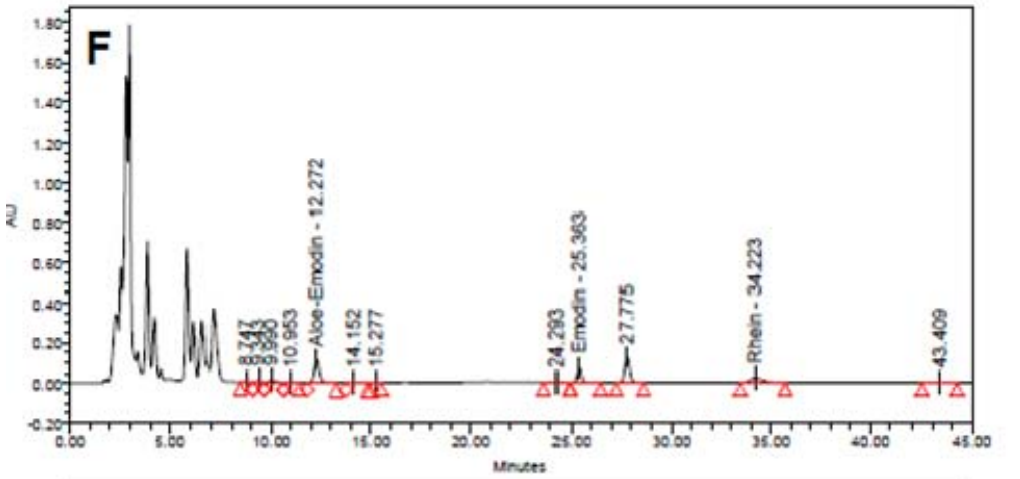
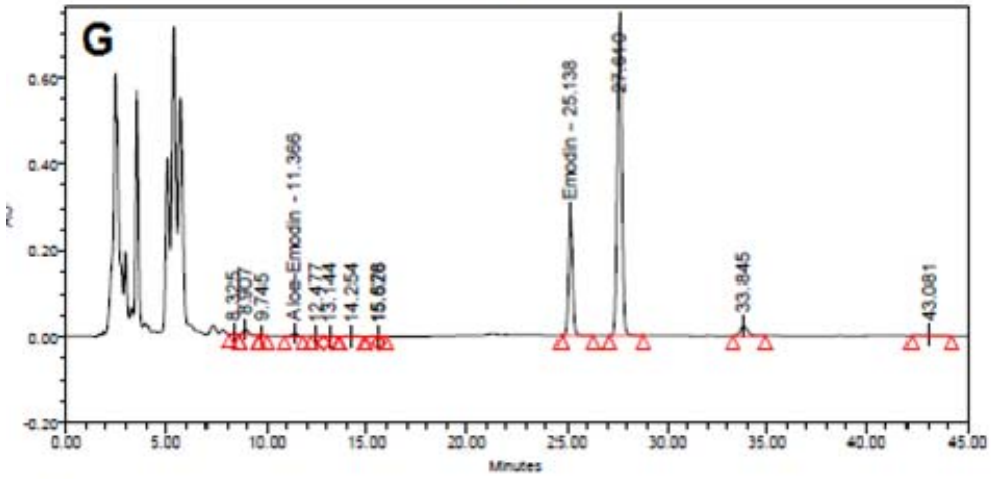
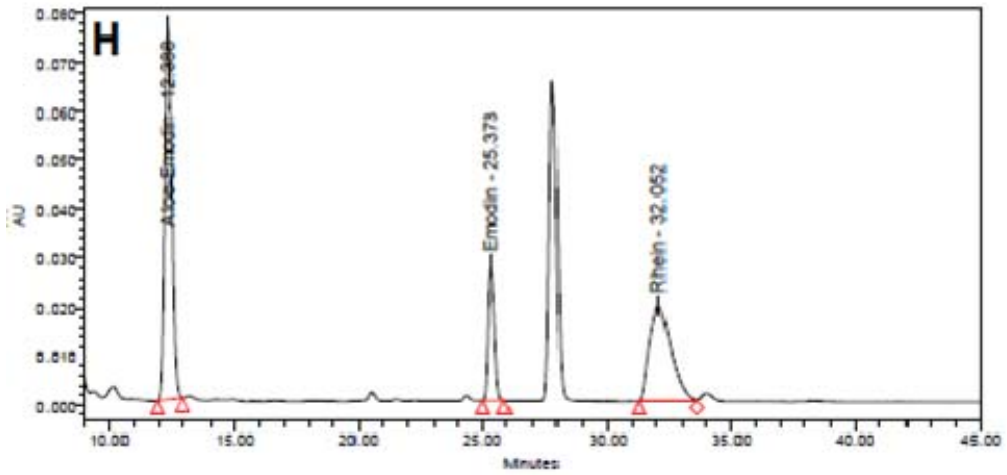
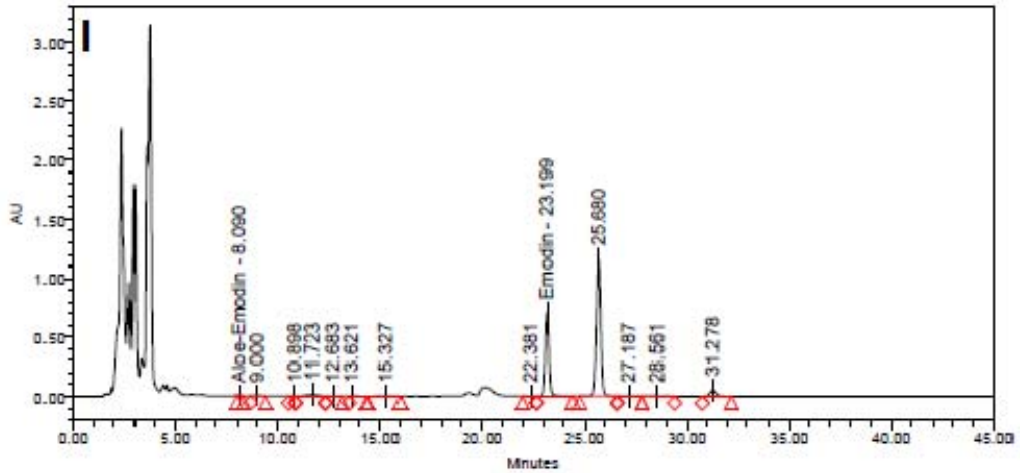
Aloe-Emodin - 12.153
Emodin - 25.205
Rhein - 31.931
AU
Minutes


E
Aloe-Emodin - 10.426
Emodin - 23.077
Rhein - 29.548
AU
Minutes


F
AU
Aloe-Emodin - 12.272
Emodin - 25.363
Rhein - 34.223
Minutes


G
AU
Aloe-Emodin - 11.366
Emodin - 25.138
33.845
43.081
Minutes


H
AU
Emodin - 25.373
Rhein - 32.052
Minutes


I
AU
Aloe-Emodin - 8.090
Emodin - 23.199
25.680
Minutes

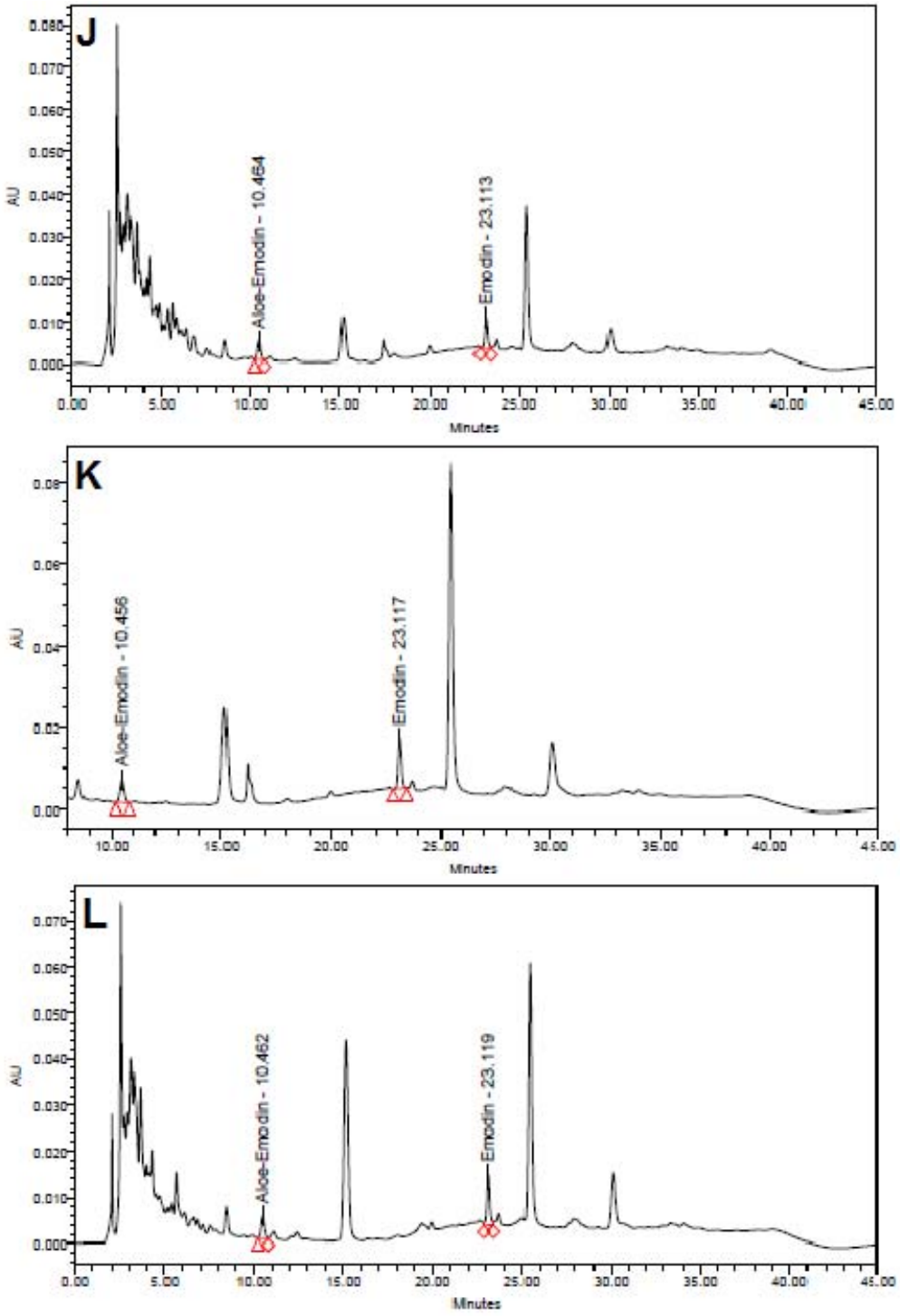


Fig. 1 (A-L): LC-UV chromatographs of standard solutions of aloe-emodin, emodin , rhein (A) and of *R. emodi* sample collected from Dhara (B), Dodhpathri (C) , Gulmarg (D), Laar (E), Pahalgaam (F), Sonamarg (G), TarsarMarsar (H), Yousmarg (I), *In-vitro* leaves (J), *In-vitro* roots (K) and *In-vitro* callus (L).

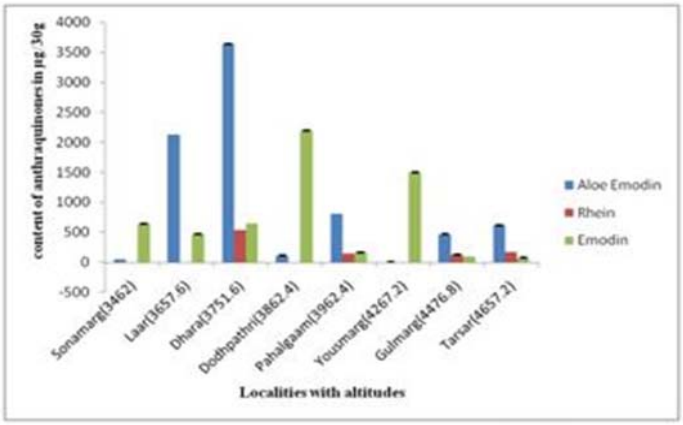


Fig 2: Content of anthraquinone markers in ***R. emodi.***

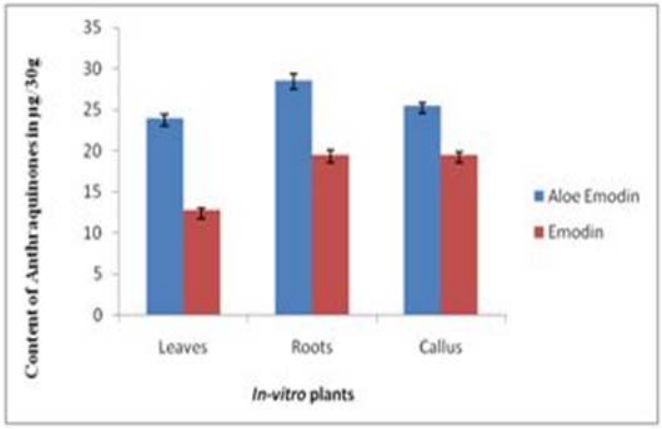


Fig. 3: Anthraquinones derived from *in-vitro* explants of *R. emodi*

Table 1: Gradient elution programme

Time (min)	Flow (ml/min)	% A (Methanol)	% B (2% Acetic acid)
-	1.00	70%	30%
13.00	1.00	70%	30%
18.00	1.00	85%	15%
40.00	1.00	85%	15%
45.00	1.00	70%	30%

Table 2: Tests from methanolic extracts of roots of *R. emodi.*

Samples	Alkaloids	Carbohydrates	Proteins	Amino acids	Steroids	Flavonoids	Glycosides	Tannins and phenols	Fats and oils
Yousmarg	+	+	+	+	+	+	+	+	+
Sonamarg	+	+	+	+	+	+	+	+	+
Pahalgaam	+	+	+	-	+	+	+	+	+
Dodhpathri	+	+	+	+	+	+	+	+	+
Gulmarg	+	+	+	_	+	+	+	+	+
TarsarMarsar	+	+	+	_	_	+	_	+	+
Dhara	+	+	+	+	_	+	+	+	+
Laar	+	+	+	+	_	+	+	+	+
Roots (*in-vitro*)	+	+	+	+	+	+	+	+	+
Leaves (*in-vitro*)	+	+	+	+	+	+	+	+	+
Callus(*in-vitro*)	+	+	+	+	+	+	+	+	+

(+ = Present, - = Absent).

Table 3: Content of markers in different samples of *R. emodi*.

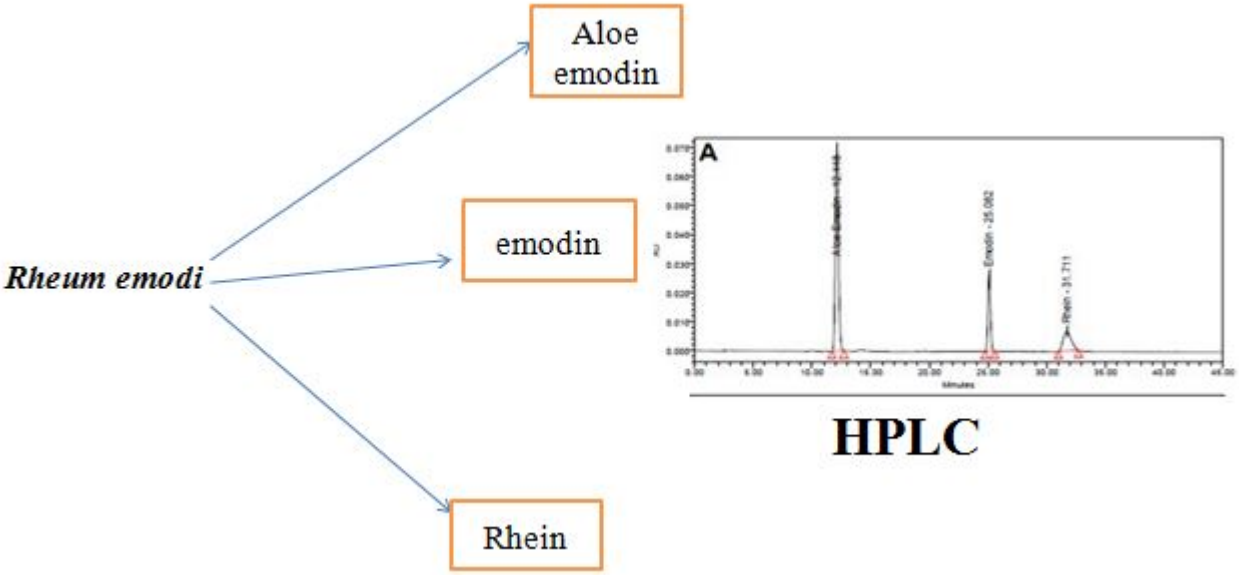
Collection Area	Altitude (m)	Content (µg/30 g of plant material)		
		Aloe emodin	Emodin	Rhein
Yousmarg	4267.2	4.8±0.1	1500.0±9.6	ND
Sonamarg	3462.2	46.5±1.2	640.5±7.8	ND
Pahalgaam	3962.4	808.5±6.3	163.5±3.2	142.5±1.6
Dodhpathri	3862.4	115.5±3.7	2190±13.4	ND
Gulmarg	4476.8	468.0±4.9	97.5±2.8	129.0±1.7
TarsarMarsar	4657.2	619.5±7.2	78.0±1.3	168.0±2.4
Dhara	3751.6	3640.5±12.9	646.5±7.1	540.0±9.5
Laar	3657.6	2130.0±10.6	471.0±5.4	6.3±0.2

ND= not detected

Table 4 Content of markers in *in-vitro* plant material of *R. emodi.*

***In-vitro* explants**	**Content (µg/30 g of plant material)**		
	Aloe-emodin	**Emodin**	**Rhein**
Leaves	24±0.4	12.75±0.3	ND
Roots	28.5±0.9	19.5±0.6	ND
Callus	25.5±0.4	19.5±0.4	ND

ND=not detected



Discussion

Rheum is a well-known medicinal plant having anti-cancer and anti-oxidant activities. Anthraquinone is the major class of phytochemicals, which is responsible for its pharmacological activities. These constituents are mainly present in roots and rhizomes. The main members of anthraquinone class include aloe-emodin, emodin, chrysophanol, physcion and rhein, which are proved as anticancer agents [17]. Anthocyanins and flavonols are also found in *Rheum* [18, 19]. In the present study, three anthraquinones were analysed i.e. aloe-emodin, rhein and emodin. Aloe-emodin (1, 8-Dihydroxy-3-(hydroxymethyl)-9, 10-anthraquinone) has been reported to exhibit anticancer activity on neuroectodermal tumors, lung squamous cell carcinoma and hepatoma cells [20].

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) is an active constituent of many herbal laxatives. It has been used for the treatment of inflammatory diseases such as peptic ulcers and as a laxative, and others such as skin burns, gallstone, hepatitis, inflammation and osteomyelitis, etc. [21]. Rhein (1,8-dihydroxy-3-carboxyl anthraquinone) is also known as cassic acid, is a substance in the anthraquinone group obtained from rhubarb.

Preliminary phytochemical screening is the first step towards standardization of a medicinal plant. This can lead to a qualitative as well as quantitative estimation of bioactive principles. Amino acids were absent in the sample of *R. emodi* collected from Pahalgaam, Gulmarg and Tarsar Marsar. Steroids were absent in Tarsar Marsar, Laar and Dhara samples. Glycosides were absent only in Tarsar Marsar. Alkaloids, proteins, flavonoids, carbohydrates, tannins and phenolic compounds, fats and oils were present in all the collected samples. All these constituents were also present in *in-vitro* explants. The results obtained from these studies are in full agreement with the previous reports in literature [22, 23]. It was reported [24] that pharmacognostical and phytochemical evaluation of *R. emodi*. In their study, detection of different constituents was also done using HPLC method.

In present studies, the glycosides were screened in roots of *Rheum* species and also from *in-vitro* explants (roots, callus and leaves).

In *R.emodi*, it was observed in present studies, that the content of aloe-emodin was about 3640 µm/30g and rhein was about 540 µm/30g in Dhara sample whereas emodin was about 2190 µm/30g in Dodhpathri sample, whereas, [25] also reported anthraquinone derivatives in the roots of *R. ribes* and *R. emodi* by HPLC method in which they analysed content of aloe-emodin 0.39% (*R. ribes*), 0.48% (*R. emodi*) and content of emodin 0.014% (*R. ribes*) and 0.32% (*R. emodi*). [26] also gave report on detection of emodin, chrysophanol and physcion from leaves of *Rheum undulatum* and detected the emodin approximately 50% of the total anthranoid content, followed by physcion (30%) and chrysophanol (20%) and the petiole of *R. undulatum*. an anthranoid content from 0.001 to 0.004% (dry weight) . In both these reports the content of anthraquinones were less as compared to the content analysed in our present studies. The content of aloe-emodin (4.8±0.1) and content of emodin (78.0±1.3) was reported from roots of *R.emodi*, whereas [27] also reported the extraction of anthraquinones from roots of *R.emodi* through HPLC method in which they analysed content of aloe-emodin 0.46+/-0.05 and content of emodin 15+/-0.05, which is very less than the content analysed in our present study. [28] also reported 0.33%, 0.70% w/w of emodin by HPLC method in *R. emodi* rhizome which was also less than the content analysed in the present study. The content of aloe-emodin was 28 µm/30g and emodin was 19.5 µm/30g in *in-vitro* root explants whereas content observed in leaf explants aloe-emodin (24 µm/30g) and emodin 12 µm/30g which was less than the content present in *in-vitro* roots, this observation resembles to some extent with the report of [29] in *R. emodi* in which the anthraquinones and their glycosides were more in rhizome as compared to leaves.

It was observed in the present study, that the amount of these compounds show great variation, to check the variation we must go for authenticated plants and must explore extensively area to know the best chemotype plant. The value of contents must be varying due to genetic diversity and/ or ecological factors, so we must mark out the best genotype growing in specific type of environment which is having the maximum amount of these active principles, only then we can have the standardized drug with specific amount of the active principles. Also, the amount of these active principles is known to vary with the age of plants, so we must study the amount of these active principles in the cultivated plants at different stages of age. The tissue cultured plants show less amount of active principles as compared to wild grown plants which might be due to the young age of tissue cultured plants as they were hardly 4-5 months old whereas the wild plants were very old, as the amount of active principles increase with

increase in age of plants, so the age differences between tissue cultured and wild grown plants might showed the variation in content of active principles.

Correlation in amount of secondary metabolite with altitude has been reported in *R. emodi* [30, 31, 32]. It was reported [33] that the concentration of active constituents and calorific value of *R. emodi* and *R. nobile* in Sikkim Himalaya. In their study, high calorific values was recorded in *R. nobile* in comparison to *R. emodi*, and active constituents of both the plant species were found to decrease in low-altitudes conserved plants compared to the wild plants. But in the present study, it was found that no co-relation between altitude and content of markers was found. This lack of correlation may be attributed to the other factors such as age of the plant and magnitude of exposure of the plant to sunlight also affect concentration of markers in the plants.

Conclusion

In some samples of *R. emodi*, amino acids, steroids and glycosides were absent. The highest amount of aloe- emodin (3640.5/ 30g), emodin (1500.0/ 30g) and rhein (540.0/ 30g) was observed in Dhara, Yousmarg and Dhara samples respectively. Rhein was absent in some of the samples of *R. emodi* and in all the samples of *in-vitro* explants of *R. emodi*.

These variations and differences which were observed for all these compounds could have been because of shifting the plants from their natural conditions or from wild to cultivation conditions. Other reasons of variations, differences and absences of some compounds must be due to different collection seasons (which range from May to October) and the age of plants.

Conflict of Interest

The authors hereby declare that they have no conflict of interest.

Author's contributions

All authors equally participated in designing experiments analysis and interpretation of data. All authors read and approved the final manuscript.

Acknowledgement

This study was supported by DST, GoI, New Delhi funded women entrepreneurship project, the assistance of which is highly acknowledged.

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