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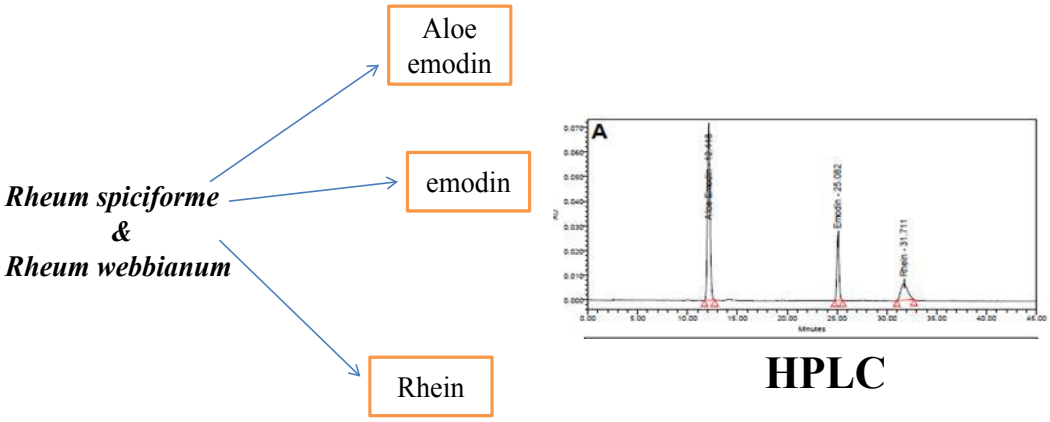
Phytochemical Analysis of Wild and *In vitro* Raised Plants of *Rheum* Species Using HPLC

Tabin S[1]*, Gupta RC[1], Kamili AN[2] and Bansal G[3]

[1]*Department of Botany, Punjabi University Patiala, Punjab, India*
[2]*Centre of Research for Development, University of Kashmir, Srinagar, India*
[3]*Department of Pharmaceutical Sciences and Drug Research, Punjabi University Patiala, Punjab, India*

Abstract

The plants are micro-biosynthetic factories for variety of compounds which are their secondary metabolites. These mainly include alkaloids, glycosides, flavonoids, volatile oils, saponins, etc. The medicinal properties are attributed to the specific bioactive compound or combination of phytochemicals. In present study, Phytochemical analysis of two *Rheum* species namely, *Rheum spiciforme* and *Rheum webbianum* was carried out using different plant parts from wild populations and also from tissue culture raised plants derived from these wild populations. Anthraquinone derivatives including emodin, aloe-emodin and rhein was quantified in analysed plant parts using HPLC method and a comparative analysis was done. The analyzed samples showed presence of various alkaloids, carbohydrates, proteins and tannins. Furthermore, *Rheum* spp. are shown to contain emodin, aloe-emodin and rhein as main active principles. Among the various populations of *R. spiciforme* maximum yield of aloe-emodin and Rhein was detected in Chakwali population while maximum amount of emodin was found in Dahi nala. Similarly, in *R. webbianum* maximum yield of aloe-emodin and rhein was detected in Panzila top population while maximum amount of emodin was found in Tangsti population. The different regenerants from tissue cultured plants showed very low yield of these active compounds as compared to wild populations in both the species. The reported contents of different phytochemicals can be useful in determination of best chemotypes which will be significant for the future use of these chemotypes in pharmaceutical industries (Graphical Abstract).



Keywords: *Rheum*; HPLC; Aloe-emodin; Anthraquinones; Phytochemicals; Anticancer; Antioxidant compounds

Introduction

Rheum species, commonly called rhubarb, are included in endangered plants list and are under great threat. It has been listed as vulnerable by various agencies like IUCN, UNEP and WWF particularly from Kashmir Himalaya [1]. *Rheum* has sixty species all over the world and mostly famous for its medicinal value, as recent studies have proved rhubarb as one of the anticancer plant. *Rheum* includes perennial, stout herbs, mainly distributed in the temperate and sub- tropical regions of world chiefly in Asian countries viz. India, Nepal, Bhutan, China, Pakistan, Korea, Turkey, Russia and Tibet. Many compounds used in today's medicine have a complex structure, and synthesizing these bioactive compounds chemically at a low price is not easy [2]. The age old 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 [3]. In china, *Rheum* plant is worshipped as it cures so many diseases and it is called as Dahuang in China. The *Rheum* plant is an eatable plant. It can be taken as a food ad it can be also cooked. Its juicy stalks are eaten raw and its leaves are cooked as a vegetable. In India it is found in Western Himalaya, and Northern Himalaya. In

***Corresponding author:** Shagoon Tabin, Department of Botany, Punjabi University Patiala, Punjab-147002, India, Tel: 919419024683; E-mail: shagoonkhan@gmail.com

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Kashmir it is found in all in over the Kashmir Himalaya which includes regions of Ladakh, Guerz, Anantnag and Baramulla districts. *R. webbianum* is used in treatment of indigestion, abdominal disorders, boils, wounds and flactuaence [4]. It is also helpful in managing cancers [5]. *R. emodi* is puragative, stomachache, astringent, tonic and effective in curing skin diseases [6,7] it is an antioxidant [8] and [9] cytotoxic in nature [10], Kinase II inhibitory [11], anti-viral [12] and nephroprotective [13]. Moreover, it is used as textile dyeing [14], anti-microbial, anti-tumor, anti-inflammatory [15] used in cosmetics ad as food colorant [16], live stimulant, purgative, anticholesterololeamic, antitumor, antiseptic, tonic, and, antiparlinson [17]. Hypatoprotective principles that can prevent and treat liver damage [18], blocks the binding of SARS-CoV S proein to ACE2 and infectivity of S protein pseudotyped retrovirus to vero E6 cells [19], Antidiabetic, similar to insulin [20], Nephroroprotective properties [21] anticancer, anti-oxidant [22,23]. *R. spiciforme* is also an adulterant and also used also in the treatment of boils, wounds, rheumatic pain. Roots are frequently used for the treatment of bone fractures, backache and joints pain [24]. Chromatography is one of the fast emerging tools by which the quality control and fingerprint of herbs can be assessed accurately. Using this technique, the identification of various chemical markers of the herbal drugs can be easily done and it also helps to identify the specific herb in combination of other herbs. Popularity of HPLC for analysis of herbal drugs is due to its economic, rapid and simultaneous screening of large number of herbal samples in less time. The main active ingredients of the *Rheum* species are a series of anthraquinones, dianthrones, glycosides and tannins. The anthraquinone derivatives including emodin, aloe-emodin, rhein, physcion, chrysophanol and their glucosides are the accepted important active principles. Rhaponticin, a distyrene derivative, only exists in non-quality (inferior-grade) rhubarb. In quality rhubarb and most exported rhubarbs, the content of rhaponticin is not detected. Like all such substances, rhein is a cathartic. Rhein is commonly found as a glycoside such as rhein-8-glucoside or glucorhein. Rhein was first isolated in Yu et al. [25]. Originally the rhubarb plant which contains rhein was used as a laxative. It was believed that rhein along with other anthraquinone glycosides imparted this activity. Rhein has been reevaluated as an antibacterial agent against *Staphylococcus aureus* [26]. Present study was aimed to find out the anthraquinones from the two *Rheum spp.* for which three standards were used i.e., aloe emodin, emodin and rhein.

Materials and Methods

The roots and rhizomes of *R. webbianum* and *R. spiciforme* were kept in brown paper bags dried under room temperature. The *in vitro* explants i.e., leaves, roots and callus [27] of these 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 making methanol (HPLC grade) extracts. The glassware and methanol (HPLC grade) was procured from commercial suppliers. Triple distilled water was used in the laboratory for different steps.

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. We used Dragendorff's test for alkaloids, Fehling solutions test for carbohydrates, Millions test for proteins and amino acids, Salkowski reaction for steroids, Shinoda test for Flavonoids, Keller-Killiani test for glycosides, $FeCl_3$ test for tannins and phenolic compounds and Sudan Reagent test for Fats and oils.

HPLC analysis

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 (1 g extract dissolved in 5 ml methanol) was chromatographed on a C_{18} column (250 mm × 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.

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:

$$\text{Content of markers in } (\mu g/30\ g) = (A_S/A_T) \times l \times 150$$

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 150 µg/ml = dilution factor.

Results

Rheum webbianum

The phytochemical screening of extracts of *R. webbianum* showed that the amino acids were absent in the samples of Panzila Top, Khardungla and Tangsti, whereas glycosides were absent in the samples of Khardungla and Tangsti. It was also observed that all types of phytoconstituents were present in each of the *in vitro* explants also (Table 1).

HPLC analysis

The contents of aloe-emodin, emodin and rhein in various samples of *R. webbianum* were found to be in the range of 67.5-4500, 255-2385 and 0.4-1143 µg/30 g of the plant material, respectively (Table 2). The LC-UV chromatograms of extracts of different samples of *R. webbianum* along with *in vitro* explants are shown in Figure 1. The roots showed the presence of anthraquinones i.e., aloe-emodin, emodin and rhein in all the observed samples. The maximum content of aloe-emodin and rhein was found in Panzila Top sample, whereas maximum content of emodin was found in Tangsti sample (Table 2 and Figure 2). Of these, Panzila Top, populations (2n=22), whereas all other populations are tetraploid (2n=44). The amount of aloe emodin and rhein is found to be very high in diploid cytotype, compared with all the populations of tetraploid cytotypes. However, the amount of emodin is more than two populations and less than other two populations. The leaves, roots and callus of *in vitro* explants were also analysed for anthraquinones content (Table 2 and Figure 2**).** The maximum content of the markers of aloe-emodin and rhein was found in *in vitro* callus, while the maximum content of emodin was found in *in vitro* leaves (Table 2, Figure 2).

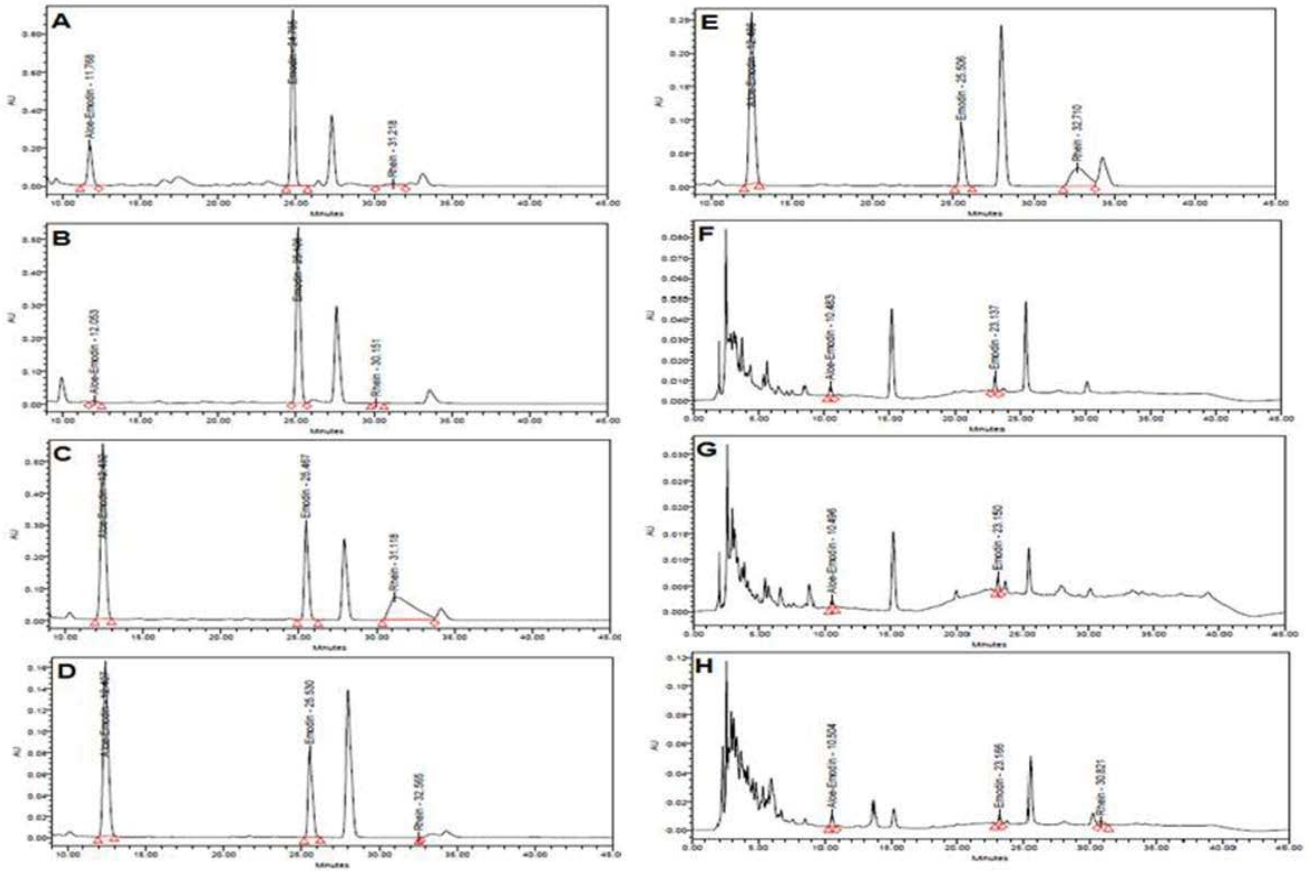

Figure 1: (A-H): LC-UV chromatographs of *R. webbianum* samples collected from Tangsti (A), Khardungla (B), Pazila top (C), Parakhchik (D), Tangole (E), *In vitro* leaves (F), *In vitro* roots (G), *In vitro* callus (H).

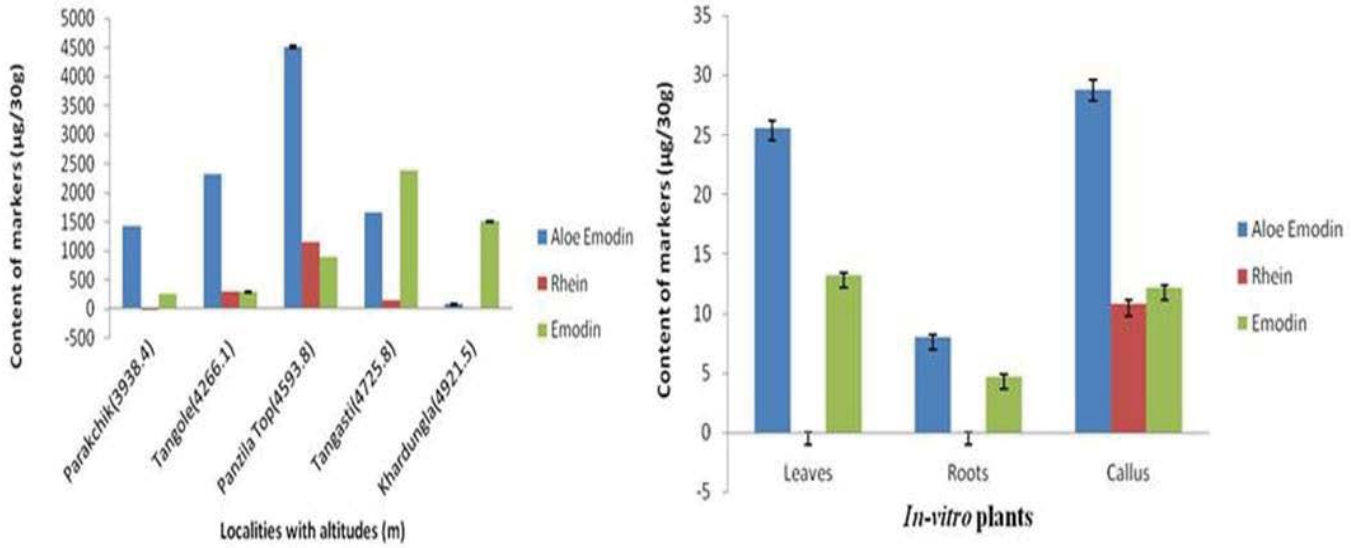

Figure 2: Content of anthraquinone markers in *R. webbianum* samples of wild and *in vitro* raised plants.

Samples	Alkaloids	Carbohydrates	proteins	Amino acids	Steroids	Flavonoids	Glycosides	Tannins and phenols	Fats and oils
Panzila top (4x)	+	+	+	–	+	+	+	+	+
Tangole (4x)	+	+	+	+	+	+	+	+	+
Parakhchik (2x)	+	+	+	+	+	+	+	+	+
Tangtsi (4x)	+	+	+	–	+	+	–	+	+
Khardungla (4x)	+	+	+	–	+	+	–	+	+
Roots (*in-vitro*)	+	+	+	+	+	+	+	+	+
Leaves (*in-vitro*)	+	+	+	+	+	+	+	+	+
Callus(*in-vitro*)	+	+	+	+	+	+	+	+	+

(+): Present; (-): Absent

Table 1: Estimation of different compounds from methanolic extracts of *R. webbianum.*

Samples	Altitude (m)	Content (µg/30 g of samples)		
		Aloe-emodin	Emodin	Rhein
Khardungla (4x)	4921.5	67.5 ± 0.8	1500.0 ± 9.3	5.7 ± 0.1
Tangsti (4x)	4725.8	1650 ± 10.4	2385 ± 18.6	130.5 ± 1.7
Panzila top (2x)	4593.8	4500 ± 29.2	885 ± 9.5	1143.0 ± 12.7
Tangole (4x)	4266.1	2130 ± 16.7	279.0 ± 3.1	282.0 ± 3.7
Parakachik (4x)	3938.4	1410.0 ± 12.3	255.0 ± 3.6	0.4 ± 0.01
Leaves (*in vitro* raised)	-	15.3 ± 0.7	13.2 ± 0.2	ND
Roots (*in vitro* raised)	-	7.95 ± 0.3	4.65 ± 0.2	ND
Callus (*in vitro* raised)	-	48 ± 0.8	12.15 ± 0.2	10.8 ± 0.3

ND: Not Detected

Table 2: Content of different active constituents in various samples of wild *R. webbianum* populations and in regenerants of *in vitro* raised plants.

Rheum spiciforme

Phytochemical screening: The extracts along with the *in vitro* explants of *R. spiciforme* were screened for phytochemical tests, and it was observed that all the compounds i.e., amino acids, steroids, glycosides, etc., were present in all the samples (Table 3).

HPLC analysis: The sharp and symmetrical peaks were observed for all the three marker anthraquinones i.e., aloe-emodin, emodin and rhein in all the samples of *R. spiciforme* (Figure 3 and Table 3). The maximum content of aloe-emodin (3409.5 µg/30 g) and rhein (531.4 µg/30 g) was found in Chakwali, whereas, maximum content of emodin (915.0 µg/30 g) was found in Dahi Nala. The different parts of *in vitro* explant i.e., leaves, roots and callus were also analysed for markers. The maximum content of aloe-emodin and emodin was found in *in vitro* callus whereas rhein was absent in all the three explants (Table 4 and Figure 4).

Discussion

Plants are rich source of effective and safe medicines due to presence of different bioactive molecules such as alkaloids, flavonoids, glycosides, tannins, phenolic compounds, etc. [28,29]. *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, chrysaphanol, physcion and rhein, which are proved as anticancer agents [30]. Anthocyanins and flavonols are also found in *Rheum* [31,32]. 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 [33]. 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. [34]. Rhein (1,8-dihydroxy-3-carboxyl anthraquinone) is also known as cassic acid, is a substance in the anthraquinone group obtained from rhubarb. Rhein is a cathartic and is commonly found as a glycoside such as rhein-8-glucoside or glucorhein. Rhein was first isolated [25]. Originally the rhubarb plant which contains rhein was used as a laxative. It was believed that rhein along with other anthraquinone glycosides imparted this activity. Rhein has been reevaluated as an antibacterial agent against *Staphylococcus aureus* in 2008 [26].

In the present study, the two species of *Rheum* i.e., *R. webbianum* and *R. spiciforme* were screened for the presence of different phytoconstituents. These were collected from the different parts of Kashmir (India) at different altitudes and were also grown *in vitro*. There were total 17 samples, including the *in vitro* explants of each species from which the anthraquinones were derived. In case of *R. webbianum*, amino acids were absent in the sample collected from Panzila Top, Khardungla and Tangsti. Glycosides were absent in Khardungla and Tangsti samples. All these compounds were also present in *in vitro* explants. Raashid [35] has also reported the similar phytochemical behavior for *R. webbianum*. All these constituents i.e.,

Altitudes	Alkaloids	Carbohydrates	proteins	Amino acids	Steroids	Flavonoids	Glycosides	Tannins and phenols	Fats & oils
Dawar Hills	+	+	+	+	+	+	+	+	+
Satni Mountain	+	+	+	+	+	+	+	+	+
Tragbal	+	+	+	+	+	+	+	+	+
Dahinala	+	+	+	+	+	+	+	+	+
Chakwali	+	+	+	+	+	+	+	+	+
Habakhatoon Mountain	+	+	+	+	+	+	+	+	+
Roots (*in-vitro*)	+	+	+	+	+	+	+	+	+
Leaves (*in-vitro*)	+	+	+	+	+	+	+	+	+
Callus (*in-vitro*)	+	+	+	+	+	+	+	+	+

(+): Present, (-): Absent

Table 3: Estimation of different compounds from methanolic extracts of *R. spiciforme*.

Collection Area	Altitude (m)	Content (µg/30 g of samples)		
		Aloe emodin	Emodin	Rhein
Dawar Hills	4419.6	996 ± 12.6	189.0 ± 2.7	279.1 ± 4.1
Chakwali	4684.8	3409.5 ± 28.4	538.5 ± 7.6	531.4 ± 8.3
Dahi Nala	3810.0	3102.0 ± 32.6	915.0 ± 16.6	384.2 ± 5.6
Satni Mountain	3962.4	2124.0 ± 19.4	376.5 ± 5.2	301.5 ± 5.0
Habbakhatoon Mountain	4756.8	2856.0 ± 31.7	622.5 ± 8.9	421.5 ± 6.4
Tragbal	3352.8	2887.5 ± 40.2	505.5 ± 7.6	478.5 ± 7.2
Leaves (*in vitro* raised)	-	7.95 ± 0.5	3 ± 0.5	ND
Roots (*in vitro* raised)	-	31.5 ± 0.3	15 ± 0.1	ND
Callus (*in vitro* raised)	-	31.35 ± 0.3	22.5 ± 0.3	ND

ND: Not Detected

Table 4: Content of different active constituents in various samples of wild *R. spiciforme* populations and in regenerants of *in vitro* raised plants.

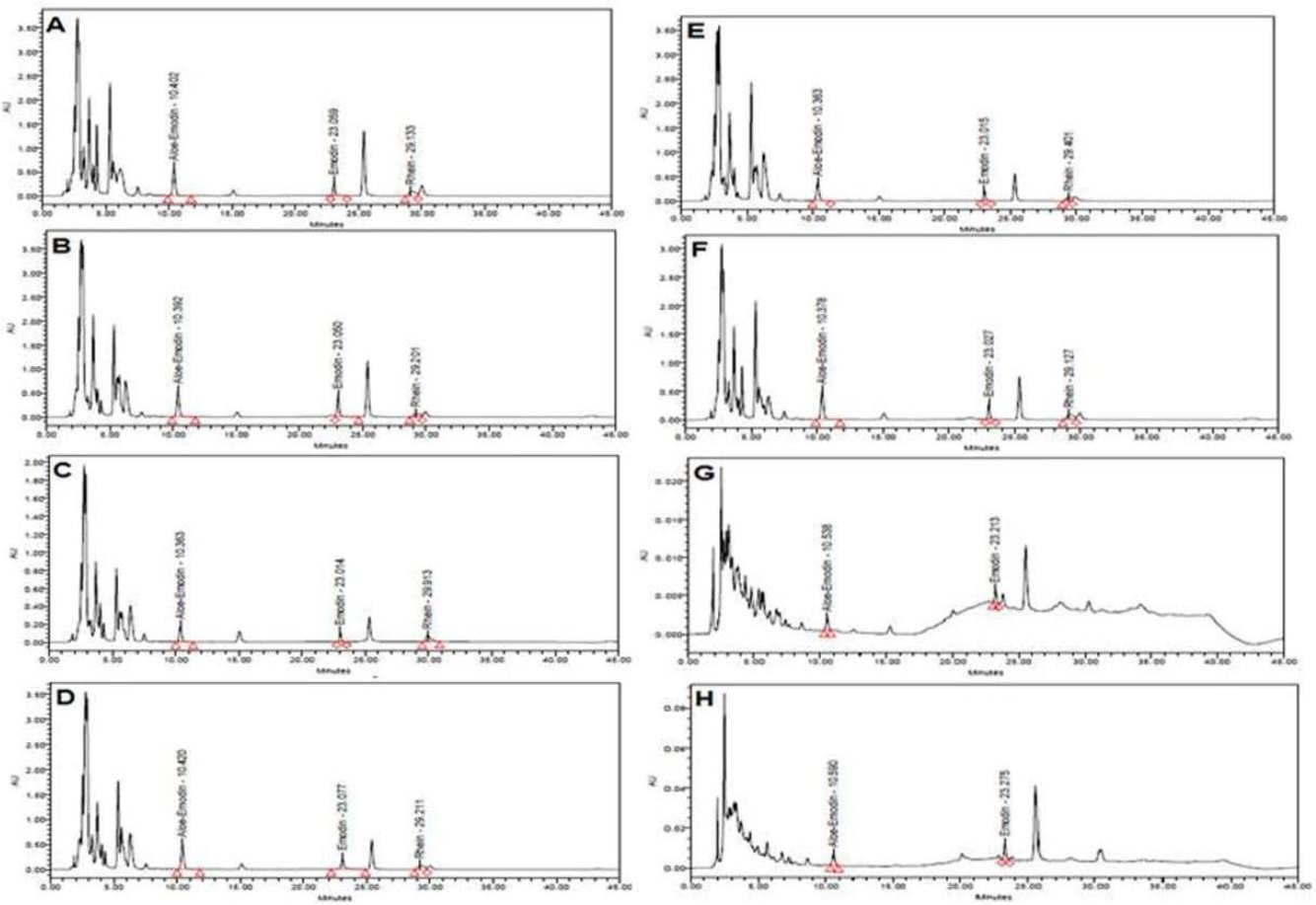

Figure 3: (A-I): LC-UV chromatographs of *R. spiciforme* samples collected from (A) Chakwali, (B) Dahinala, (C) Dawar hills, (D) Habba Khatoon mountain, (E) Satni mountain, (F) Tragbal, (G) *In vitro* leaves, (H) *In vitro* roots, (I) *In vitro* callus.

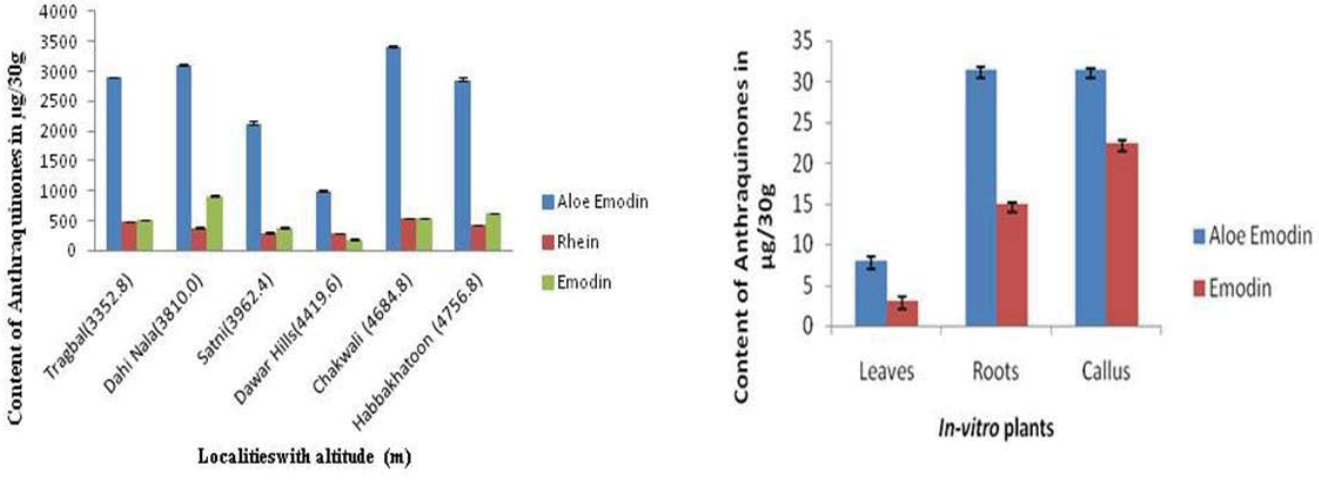

Figure 4: Content of anthraquinone markers in *R. spiciforme* samples of wild and *in vitro* raised plants.

alkaloids, proteins, flavonoids, carbohydrates, amino acids, steroids, glycosides, tannins and phenols, fats and oils were also present in all the samples of *R. spiciforme*, as well as in the *in vitro* raised explants. These results indicate that there is no uniform effect of altitude on the presence of phytochemicals in any of the two species. In present studies, the glycosides were screened in roots of *Rheum* species and also from *in vitro* explants (roots, callus and leaves). All samples of *R. webbianum* were also analyzed for the content of these three markers. All the three markers were present in all samples of *R. webbianum*. The maximum content of aloe-emodin (4500 µm/30 g) and rhein (1143 µm/30 g) was found in sample collected from Panzila Top whereas maximum content of emodin (2385 µm/30 g) was found in Tangsti sample. Rhein was also present in all these samples. In the case of *in vitro* explants, the maximum content of aloe-emodin (48 µm/30 g) was found in *in vitro* callus, the maximum content of emodin (13 µm/30 g) was found in *in vitro* leaves, rhein was absent in *in vitro* roots and leaves, whereas rhein (10 µm/30 g) was only present in *in vitro* callus. The amount of these anthraquinones and their derivatives has also been studied in many other species of *Rheum* such as *R. tanguticum* [36]. *R. officinale* [37], *R. palmatum* [38] and *R. ribes* [39] etc. (Supplementary Table S1).

The nine samples of *R. spiciforme* were also analyzed through HPLC for the quantification of the markers. All of these markers were present in each sample of *R. spiciforme*. The maximum content of aloe-emodin (3409 µm/30 g) was found in Chakwali, whereas, maximum content of emodin (915 µm/30 g) was found in Dahi Nala sample. The maximum content of rhein (531 µm/30 g) was also found in Chakwali. It was also observed that rhein was present in all the samples of *R. spiciforme,* whereas it was absent in some of the samples of *R. emodi*. In case of *in vitro* explants, it was noted that the maximum content of aloe-emodin (31.35 µm/30 g) and emodin (22 µm/30 g) was found in *in vitro* callus whereas rhein was absent in all these three explants.

It was observed that maximum aloe-emodin, emodin and rhein were found in *R. webbianum* as compared to *R. spiciforme*. The maximum content of aloe-emodin and rhein of the *in vitro* explants was also found in *R. webbianum,* whereas rhein was absent in explants (*in vitro)* of *R. spiciforme*. Only maximum content of emodin was found in *in vitro* explants of *R. spiciforme*.

Present study is the first report on *R. spiciforme* from India for phytochemical screening as no previous work was done in India and it was also observed that the *R. webbianum* contains highest content of these three anthraquinones and it was also observed that in *R. webbianum has* the amount of active compounds present in tetraploid plants was less than diploid plant. Similarly, the amount of active principle is reported to be more in the diploid than polyploids by some other workers [40-42]. Generally the amount of these active principles are found to be more in polyploids as compared to diploids [43,44].

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* [7,45-46]. Prasad and Purohit [7] reported 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 affects concentration of markers in the plants.

Conclusion

Phytochemical screening showed the presence of amino acids, alkaloids, flavonoids, steroids, fats and oils, tannins and phenols, carbohydrates and glycosides in different samples of *Rheum* species. In some samples of *R. emodi*, amino acids, steroids and glycosides were absent. In R. *webbianum*, constituents like alkaloids, proteins, steroids, etc., were also present. Only amino acids and glycosides were absent in two of the samples, whereas all the compounds were present in *in vitro* explants of *R. webbianum*. In *R. spiciforme*, all constituents i.e., alkaloids, proteins, flavonoids, carbohydrates, amino acids, steroids, glycosides, tannins and phenols, fats and oils were also present in all the samples as well as in samples of *in vitro* explants. These species of *Rheum* were also screened to quantify the anthraquinone markers (aloe-emodin, emodin and rhein). The methanolic extracts were analysed to determine the content of aloe-emodin, emodin and rhein by HPLC method. All the three anthraquinones i.e., aloe-emodin, emodin and rhein were present in *R. webbianum*, but in case of *in vitro* explants of *R. webbianum*, rhein was present only in callus. The highest amount of aloe emodin (4500/30 g), emodin (1500.0/30 g) and rhein (1143.0/30 g) was observed in Panzila Top, Khardungla and Panzila Top samples respectively. In *R. spiciforme* highest content of aloe-emodin (3409.5/30 g) and rhein (531.4/30 g) was found in Chakwali samples, while highest content of emodin (915.0/30 g) was present in the samples of Dahi Nala. Rhein was absent in all *in vitro* explants of this species. Among all the three species, the maximum contents of aloe-emodin, emodin and rhein were found in samples of *R. webbianum* and amongst the *in vitro* explants, the maximum content of aloe-emodin and rhein was found in *R. webbianum* and that of emodin was observed in *R. spiciforme.* The observation of present study showed that *R. webbianum* is most abundant and rich in contents of active compounds. The results of present study can be efficiently utilized in future research programmes for the selection of elite material and for devising the utilization strategies for best chemotypes.

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.

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