Why uproot *Picrorhiza kurrooa*, an endangered medicinal herb?

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***Picrorhiza* (*Picrorhiza kurrooa* Royle ex Benth.) is a small, perennial herb widely used in traditional as well as modern system of medicine. This plant grows in Himalayan region at elevations ranging from 3000 to 5000 m above the sea level and listed as endangered species due to extensive extraction of plant from the nature and lack of organized cultivation. Underground parts (roots and rhizomes) are used for extraction of picrosides, the medicinally important constituents of *Picrorhiza*. We studied the presence of picrosides in leaf tissue. Reverse phase high-performance liquid chromatography was employed for the detection of picrosides in different tissues, viz. root, rhizome and leaf, as well as during different stages of plant development. Picrosides were present in leaf tissues at all the stages of development. Importantly, the leaf biomass was higher compared to that of roots and rhizomes. Results suggested that the leaf, rather than rhizomes and roots may be used as source of picrosides so that the underground parts left after leaf harvest can give rise to new plants in the subsequent year, thus helping in multiplication of plants year after year and conserving *Picrorhiza*.**

Keywords: Conservation, *Picrorhiza kurrooa*, picrosides.

CONSERVATION and sustained use of bioresources are important to keep a balance between nature and welfare of humanity. Apart from ecological significance, bioresources have immense implications to human beings in terms of providing a range of goods and services such as food, medicines, animal feed, fibres and fuels. Overexploitation of bioresources, without ensuring the sustainability, e.g. through cultivation, might lead to extinction. *Picrorhiza* (*Picrorhiza kurrooa* Royle ex Benth.) is one such medicinal plant, which is listed as vulnerable species in *Red Data Book*[1] and subsequently under the 'endangered' species category, according to the International Union for Conservation of Nature criteria[2] due to extensive harvesting and lack of organized cultivation. Convention on International Trade in Endangered Species of Wild Fauna and Flora has listed *Picrorhiza* in its annexure II that restricts its trade across national territories[3].

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Picrorhiza (family Scrophulariaceae) grows in the Himalayan region at elevations ranging between 3000 and 5000 m above the sea level[4]. Traditionally, the mature plants are uprooted, and the rhizomes and roots are traded for preparing medicinal formulations to treat disorders of the liver and upper respiratory tract, dyspepsia, chronic diarrhoea and scorpion sting[5,6]. Several national and international firms such as Life Extension Foundation (http://www.lef.org/Vitamins-Supplements/Item00802/Anti-Alcohol-Antioxidants-with-HepatoProtection-Complex.html), Gero Vita International (http://www.gvi.com/Scripts/prodView.asp?idproduct72), and Nature's Sunshine (http://www.naturessunshine.com/us/products/catalog/product/default.aspx?Stocknum1297) use *Picrorhiza* in drugs as a component to treat various human ailments. Demand for *Picrorhiza* is continuously increasing, e.g. an annual growth rate of 12.9% was recorded for its demand of 220 tonnes in 2001–02 and 317 tonnes during 2004–05 (ref. 7).

Bioactivity/medicinal properties of *Picrorhiza* are attributed to picrosides[8], which are iridoids with iridane skeleton of monoterpene origin. Picroside-I (P-I) and picroside-II (P-II) have been found to affect the activity of P-glycoprotein (P-gp)[9], which is one of the xenobiotic transport proteins implicated in multi-drug resistance in neoplastic tissues. In cancer tissue with high expression of this protein, P-gp functions as a drug export pump that decreases intracellular concentrations of numerous chemotherapeutic agents[10]. P-II has been found to protect hepatocytes against injury and prevent hepatocytes from apoptosis[11]. Also, P-II has been found to protect cells from hydrogen peroxide-induced oxidative stress and is a potential therapeutic for treating nervous disorders[12].

Picrosides are reported to be present in the roots and rhizome of *Picrorhiza*[6,8], however, its unequivocal presence in leaves is not yet established. If picrosides are present in the leaf tissue, the observation will have implications in educating the harvesters and developing appropriate package and practice for harvesting the aerial parts and not uproot the whole plant so as to conserve *Picrorhiza*, an endangered species.

Five hundred rhizomes of *P. kurrooa* were collected from its natural habitat at Rohtang Pass (4000 m altitude, 32°23′N, 77°15′E, India) during December when the plants were in dormant stage and brought to the institute at Palampur (1300 m altitude; 32°06′N, 76°33′E, India). These were transplanted in plastic pots and maintained in the experimental farm of the institute as described by Kawoosa *et al.*[13]. Leaf, rhizome and roots of five separate plants were also collected at Rohtang Pass in July, stored in liquid nitrogen and brought to the institute for picrosides estimations.

Picrorhiza plants grown in pots were randomly selected for analysis of biomass. The plants were gently uprooted, washed with distilled water and blotted dry. Leaf, root and rhizome tissues of individual plants were

Table 1. Biomass and percentage distribution of biomass (on fresh weight and dry weight basis) in different parts of *Picrorhiza*. Data represents the average of eleven independent plants ± SE each, at two different growth stages of plants (60 and 120 days of transplantation (DOT))

		Biomass (in grams)			Biomass (%)		
		Tissue			Tissue		
Growth stage		Root	Rhizome	Leaf	Root	Rhizome	Leaf
60 DOT	Fresh weight	5.77 ± 1.56	3.08 ± 0.60	7.39 ± 2.02	34.23 ± 4.14	22.18 ± 5.07	43.59 ± 5.80
	Dry weight	1.15 ± 0.31	0.89 ± 0.17	1.36 ± 0.34	33.33 ± 5.09	29.10 ± 5.16	37.57 ± 6.15
120 DOT	Fresh weight	7.78 ± 1.16	13.91 ± 0.93	28.48 ± 2.62**	15.56 ± 1.33	28.05 ± 1.48	58.11 ± 1.71**
	Dry weight	1.33 ± 0.19	3.13 ± 0.22	3.73 ± 0.43*	16.28 ± 1.27	37.90 ± 2.28	45.82 ± 1.94*

*Statistical significant difference with corresponding root biomass ($p < 0.05$).
**Statistical significant difference with both corresponding root as well as rhizome biomass ($p < 0.05$).

separated and weighed independently to measure fresh weight. Subsequently, the tissues were dried at 60°C and weighed till the constant weight was achieved to record dry weight. Biomass was estimated at two different stages of growth; one at 60 days of transplantation (DOT) and the other at 120 DOT. Eleven plants were used for biomass analysis at each stage.

Picrosides were extracted essentially as described by Kawoosa *et al.*[13]. Plant tissues (leaf, root and rhizome) were harvested, washed with distilled water, blotted dry, weighed, frozen in liquid nitrogen and stored at –80°C till further use. The frozen samples (100 mg) were ground to fine powder in liquid nitrogen using pestle and mortar followed by the addition of 1 ml of 80% methanol with intermittent grinding for 1 min. Extract was transferred to a centrifuge tube and the pestle and mortar was rinsed with 1 ml of 80% methanol to recover the leftover sample. Extracts were pooled, centrifuged at 15,000 *g* for 15 min and the supernatant was used for picrosides estimation. Samples were filtered through 0.45 µ filter (Millipore, USA) for high-pressure liquid chromatography (HPLC) analysis. The filtered sample extracts were serially diluted 50 times before injecting into the HPLC system. For picrosides extraction, three individual plants were used separately as and when needed.

HPLC analysis was performed on LC 4000 module, 2487 dual λ absorbance detector (both from Waters, UK), and LiChrosorb® RP-18, 250 × 4.0 mm (Hibar) column (Merck & Co., Inc., USA) following the procedures described by Kawoosa *et al.*[13], Sood and Chauhan[14]. The injection volume was 20 µl and the mobile phase (flow rate, 1 ml/min) consisted of a mixture of trifluoroacetic acid (0.05%) and methanol : acetonitrile (1 : 1) in 70 : 30 ratio. Picrosides were identified and quantified following retention time at 270 nm and calculating peak area, respectively of pure P-I and P-II. Stock solutions of standard picrosides (ChromaDex, USA), P-I and P-II (1 mg/ml) were prepared in 80% methanol to inject the desirable amounts (0.2, 0.5, 1.0, 1.5 and 2.0 µg/20 µl) after further dilutions with 80% methanol. In addition, identities of P-I and P-II were confirmed by spiking the extracted samples with standard picrosides, and by comparing the UV spectra of corresponding peaks in samples with those of standard P-I and P-II. Further, mass spectrometry of standard picrosides as well as that of the identified peaks of samples also confirmed the identity of P-I and P-II (Acquity Ultra Performance liquid chromatography (UPLC) system coupled with the photodiode array (PDA) and single quadrupole (SQ) detectors was used; Waters Corporation, Milford, USA).

Leaf tissue (100 mg; leaf at second node position from the top) of 10 individual plants was ground in liquid nitrogen separately and chlorophyll was extracted in 80% acetone. Chlorophyll was estimated according to the method of Porra *et al.*[15] using the following equation: total chlorophyll (µg/ml) = 17.76 ($A_{646.6}$) + 7.34 ($A_{663.6}$), wherein $A_{646.6}$ and $A_{663.6}$ are absorbances at 646.6 and 663.6 nm respectively.

Data was subjected to analysis of variance (ANOVA). For distinguishing the mean differences, which were significantly different, Tukey test was used[16].

Biomass of *Picrorhiza* was estimated on fresh weight as well as on dry weight basis. Leaf, rhizome and root had 37.6%, 29.1% and 33.3% of the biomass on dry weight basis respectively, and 34.2%, 22.2% and 43.6% on fresh weight basis respectively, on 60 DOT (Table 1). At 120 DOT, these values were 45.8%, 37.9% and 16.3% respectively on dry weight basis and 15.6%, 28.0% and 58.1% on fresh weight basis, in the same order. On 60 DOT, the average weight of leaf, rhizome and root was 7.4 g, 3.1 g and 5.8 g which increased to 28.5 g, 13.9 g and 7.8 g respectively on 120 DOT (Table 1).

Differential accumulation of P-I and P-II was detected in different tissues of the *Picrorhiza* (Table 1, Figure 1 *a*). Mass spectrometric analysis confirmed the identity of picrosides P-I and P-II in corresponding peaks (Figure 1 *b*). Picrosides were present in the leaf tissues at all the node positions, with maximum content in the youngest leaf (Table 2). However, leaf tissues were found to contain predominantly P-I. Comparative analysis of picrosides in different tissues revealed significantly higher P-I content in the leaf tissues as compared to the rhizomes and

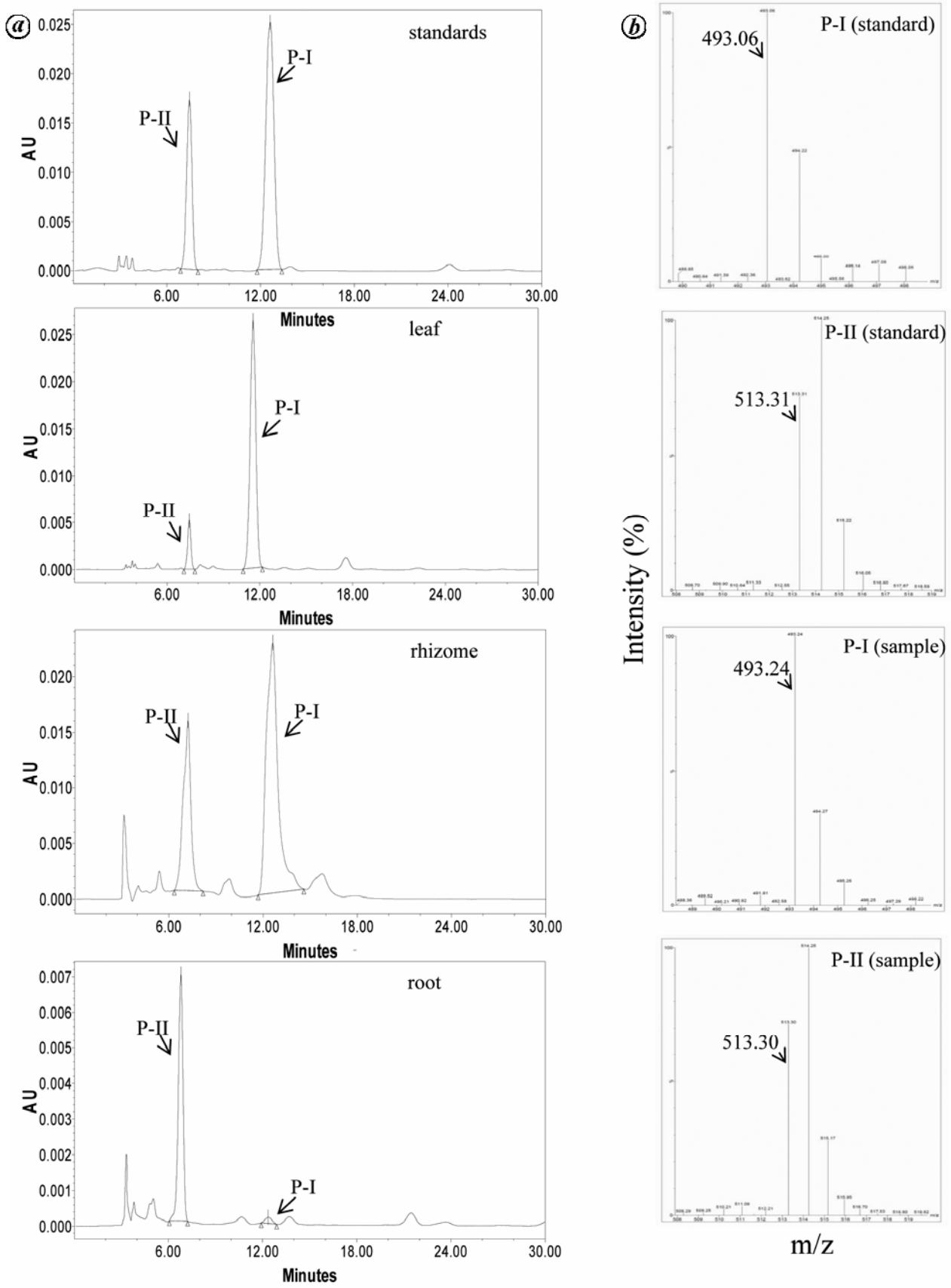

Figure 1. ***a***, HPLC elution profile of picroside-I (P-I) and picroside-II (P-II) standards, extracts of leaf, rhizome and root tissue of *Picrorhiza kurrooa*. ***b***, Mass spectrum (in positive mode of ionization) of P-I (molecular weight, 492.47) and P-II (molecular weight, 412.46).

Table 2. Picrosides content in different tissues/organs of *Picrorhiza*. Leaf positions are numbered starting from the top designated as 1st leaf, the youngest leaf

	Time of the day (h)							
	5:00 (*Light intensity = Not detectable)		9:00 (*Light intensity = 1100 μmol m^{-2} s^{-1})		13:00 (*Light intensity = 1500 μmol m^{-2} s^{-1})		17:00 (*Light intensity = 20 μmol m^{-2} s^{-1})	
	Picroside content (mg/100 mg fresh weight)							
Tissue	P-I	P-II	P-I	P-II	P-I	P-II	P-I	P-II
1st Leaf	$2.40 \pm 0.16^{5,8}$	0.26 ± 0.15^{7}	$2.58 \pm 0.27^{6,7,8}$	0.40 ± 0.23^{7}	$2.31 \pm 0.17^{4,6,7,8}$	1.39 ± 0.70	$2.74 \pm 0.59^{5,6,8}$	1.25 ± 0.66
2nd Leaf	1.52 ± 0.21^{8}	0.14 ± 0.08^{7}	$2.28 \pm 0.35^{7,8}$	0.34 ± 0.27^{7}	$1.71 \pm 0.10^{6,8}$	0.67 ± 0.37	1.43 ± 0.04	0.45 ± 0.39
3rd Leaf	1.43 ± 0.32	0.16 ± 0.09^{7}	1.96 ± 0.17^{8}	0.09 ± 0.03^{7}	$1.86 \pm 0.30^{6,7,8}$	0.70 ± 0.44	1.72 ± 0.49^{8}	0.65 ± 0.32
4th Leaf	1.05 ± 0.18	0.06 ± 0.03^{7}	1.76 ± 0.27^{8}	0.08 ± 0.03^{7}	$1.32 \pm 0.07^{1,8}$	0.18 ± 0.09	1.42 ± 0.36	0.41 ± 0.18
5th Leaf	0.86 ± 0.39^{1}	0.06 ± 0.03^{7}	1.40 ± 0.20^{8}	0.05 ± 0.02^{7}	1.38 ± 0.30^{8}	0.15 ± 0.11^{7}	0.79 ± 0.29^{1}	0.04 ± 0.01^{7}
6th Leaf	1.05 ± 0.49	0.19 ± 0.11^{7}	$1.32 \pm 0.24^{1,8}$	0.03 ± 0.01^{7}	$0.55 \pm 0.12^{1,2,3}$	0.07 ± 0.02^{7}	0.56 ± 0.16^{1}	0.05 ± 0.02^{7}
Rhizome	1.11 ± 0.31	$1.94 \pm 0.42^{1,2,3,4,5,6,8}$	$1.04 \pm 0.06^{1,2}$	$1.84 \pm 0.54^{1,2,3,4,5,6,8}$	0.79 ± 0.26^{1}	$2.04 \pm 0.31^{5,6,8}$	1.11 ± 0.31	$2.04 \pm 0.56^{5,6,8}$
Root	$0.07 \pm 0.04^{1,2}$	0.31 ± 0.14^{7}	$0.05 \pm 0.04^{1,2,3,4,5,6}$	0.27 ± 0.13^{7}	$0.04 \pm 0.04^{1,2,3,4,5}$	0.18 ± 0.13^{7}	$0.01 \pm 0.01^{1,3}$	0.35 ± 0.11^{7}

*Light intensity was measured as photosynthetically active radiation (PAR) using PAR sensor attached to an infrared gas analyser (LI-6400; LI-COR Biosciences, USA). Superscripted numbers 1, 2, 3, 4, 5, 6, 7 and 8 indicate that the corresponding value is statistically different with respect to value of 1st leaf, 2nd leaf, 3rd leaf, 4th leaf, 5th leaf, 6th leaf, rhizome and root respectively, in the same column ($p < 0.05$). No significant difference was observed among corresponding values in a row.
Values are means of three separate biological replicates ± SE.

Table 3. Picrosides content during different stages of development, in leaf, rhizome and root of *Picrorhiza*. Values are means of three separate biological replicates ± SE. In case of leaf tissue, the leaf at position 2 (from the top) was selected due to its presence in the early as well as late stage of plant growth. Young, mature and senescent represent 30, 120 and 180 days of DOT. Numerical values of chlorophyll (chl) are mean ± SE of 10 independent biological samples (2nd leaf) at all three stages (young, mature and senescent)

	Developmental stage					
	Young (chl: 1.08 ± 0.10 mg/g fresh weight)		Mature (chl: 0.98 ± 0.04 mg/g fresh weight)		Senescent (chl: 0.39 ± 0.06 mg/g fresh weight)	
	Picroside content (mg/100 mg fresh weight)					
Tissue	P-I	P-II	P-I	P-II	P-I	P-II
2nd Leaf	0.93 ± 0.090^{3}	$0.07 \pm 0.010^{2,3}$	$2.28 \pm 0.353^{2,3}$	0.34 ± 0.266	$0.95 \pm 0.098^{2,3}$	$0.10 \pm 0.008^{2,3}$
Rhizome	0.69 ± 0.005^{3}	$0.20 \pm 0.004^{1,3}$	$1.04 \pm 0.058^{1,3}$	1.84 ± 0.542^{3}	0.18 ± 0.001^{2}	$0.41 \pm 0.012^{1,3}$
Root	$0.01 \pm 0.002^{1,2}$	$0.39 \pm 0.021^{1,2}$	$0.05 \pm 0.043^{1,2}$	0.27 ± 0.131^{2}	0.01 ± 0.002^{1}	$0.42 \pm 0.011^{1,2}$

Superscripted numbers 1, 2 and 3 indicate that the value is statistically different with respect to value of 2nd leaf, rhizome and root respectively, in the same column ($p < 0.05$).

roots. Roots showed higher amount of P-II as compared to P-I, though the contents of both P-I and P-II were less as compared to that in the leaf. In rhizomes, both picrosides, P-I and P-II were present in comparable amounts.

Further, in order to find out whether or not the picrosides accumulation varies during the day or in other words in response to varying light intensities, picrosides were estimated at four different time points of the day. Picrosides were higher during light period (at 9:00 h and 13:00 h) as compared to the low light/dark period, though statistically the differences were not significant (Table 2). Analysis of picrosides during different growth cycles of the plant showed its presence in all the tissues analysed (leaf root and rhizome) throughout, though the content declined in senescing plants (Table 3). Picrosides were also estimated in the samples collected from natural population growing at Rohtang Pass and the leaf, rhizome and root had $2.32 \pm 0.64\%$, $1.22 \pm 0.25\%$ and $0.10 \pm 0.001\%$ P-I respectively. It was difficult to assign specific age to the populations and compare the data of different locations in the present study, however, our experience showed that naturally growing population always exhibited presence of picrosides in the leaf tissue irrespective of age of the plant (data not shown).

Present data showed the unequivocal presence of picrosides in the leaf tissues, which suggests that aerial portion of the plant should be used for extracting the picrosides and it is not essential to uproot the whole plant. It is

evident from the tissue-based analysis of *Picrorhiza* that the leaves contain higher P-I than that of roots and rhizome (Table 2). For example, in samples harvested at 9:00 h, the leaves were found to contain 63% of the total P-I, whereas rhizomes and roots together contained the remaining 37% of the total P-I (average of all the six leaves analysed is considered). The leaf seems to be an even better source of P-I if both, the biomass and P-I content are taken into consideration. On biomass and P-I content basis, leaf contained 78% of the total P-I of a plant on fresh weight basis and 68% on dry weight basis (Tables 1 and 2). Thus, it clearly indicates that leaves are better source of P-I as compared to rhizomes and roots. This is the first report on the spatio-temporal differential accumulation of picrosides. Such data should be generated for *Picrorhiza* growing in different habitats in light of report by Singh *et al.*[17] wherein the altitudinal variation in picrosides content in rhizomes of *Picrorhiza* has been shown.

Wild herbs and shrubs protect soils from erosion by wind, rainfall and snow. The inappropriate harvest of these herbs particularly of mountain areas endangers land conservation. There is an urgent need to implement appropriate land management which has a large-scale perspective with local relevance[18]. Thus, harvesting of *Picrorhiza* leaves instead of its underground parts (rhizomes and roots) has vital soil conservation aspect also. Because harvesting of rhizomes and roots requires unearthing that in turn is positively associated with more manpower and time requirement and makes the area more prone to soil erosion thus harming the ecosystem. On the other hand, harvesting of leaves do not need earthing up and thus, the undisturbed/leftover rhizomes hold the soil and prevent soil erosion and also, may give rise to plants in subsequent years. This will hold true both for plants growing in natural habitat and plants under cultivation.

Our recommendation for harvesting the aerial portion of *Picrorhiza* instead of the underground parts (rhizomes and roots) should aid checking soil erosion, since the soil remains undisturbed. The undisturbed/leftover rhizomes and roots will give rise to new plants and hence this is a step forward in conserving the plant. Even for cultivation purpose, farmers need not depend on supply of fresh planting material every year for their fields.

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