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RESEARCH ARTICLE

Differential biosynthesis and accumulation of picrosides in an endangered medicinal herb *Picrorhiza kurroa*

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Abstract *Picrorhiza kurroa* Royle ex Benth (Family: Scrophulariaceae) is a medicinal herb, mainly found in the North-Western Himalayas. Extensive harvesting for pharmaceutical purposes, lack of organized cultivation and unorganized methods of uprooting the plants because of unawareness has brought an endangered status to this important herb in nature. The medicinal property of this plant is attributed to monoterpenoid picrosides. The influence of developmental status of different growth stages on picrosides content is poorly understood in *Picrorhiza kurroa*. Picroside-I (P-I) content increased from 0.05 % to 0.76 % in different growth stages of shoots. Significant increase in the contents of P-I (0.15–0.50 %) and Picroside-II (P-II) (0.1–0.45 %) was observed in rhizomes of different developmental stages. Highest amounts of P-I (8.7 %) and P-II (5.3 %) was detected in uppermost part of mature dried rhizomes compared to bottom part with 2.9 % and 2.2 % of P-I and P-II, respectively. *P. kurroa* grown at high altitude (Sairopa, 4,500 amsl) showed 1.75-folds increase in P-I in leaves whereas exponential increase in the P-I content was detected (0.05–1.7 %) in the leaves of different developmental stages (L1-L5) of *P. kurroa* grown at lower altitude (Jagatsukh, 1,900 m). Variable amounts of P-I and P-II in different growth and developmental stages of *P. kurroa* imply importance of selection of plant material (rhizomes and roots). The study undertaken explored the status of metabolites accumulation and biosynthesis in the field grown plants of *P. kurroa* where not only environmental parameters but different morphogenetic stages of its developmental cycles, different age groups and different parts of plantlets were extensively analysed and estimated for medicinally important picrosides.

Keywords HPLC · Developmental stages · *Picrorhiza kurroa* · Picrosides · Rhizomes

Abbreviations

P-I	Picroside-I
P-II	Picroside-II
HPLC	High performance liquid chromatography

Introduction

Picrorhiza kurroa Royle ex Benth (Family: Scrophulariaceae) is a medicinal herb, mainly found in the North-Western Himalayan regions of India at altitude of 3,000 to 4,500 m (Chettri et al. 2005). *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting (Sood and Chauhan 2010). The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatoprotective (Chander et al. 1992), antioxidant (particularly in liver) (Ansari et al. 1998), antiallergic and antiasthamatic (Dorch et al. 1991), anticancerous activity particularly in liver (Joy et al. 2000), and immunomodulatory, (Gupta et al. 2006). The herbal drug formulations are mainly prepared from the rhizomes and roots of *P. kurroa*, which are rich source of hepatoprotective components, P-I and P-II and other metabolites like Picroside-III, Picroside-IV, Apocynin, Androsin, Catechol, Kutkoside,

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etc. (Weinges et al. 1972; Stuppner and Wagner 1989). Recently rhizome extracts of *P. kurroa* have also shown to possess anti-malarial activity (Singh and Banyal 2011).

A hepatoprotective drug formulation, Picroliv has been prepared from the roots and rhizome extracts of *P. kurroa* for which 3–4 years old plants were used as a raw material, (Dwivedi et al. 1997; Katoch et al. 2011). Picroliv, which was launched as a herbal drug formulation, is prepared from a standardized iridoid fraction containing 60 % of P-I and Kutkoside in a 1: 1.5 ratio (Dwivedi et al. 1992; Ansari et al. 1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections (Puri et al. 1992; Gupta et al. 2006). In addition to picroliv there are several commercially available drug formulations consisting of P-I and P-II, e.g. Katuki, Zandu Pharma works Ltd. (Mumbai, India) having P-I (1.29 %) and P-II (1.16 %), Arogya, Zandu Pharma works Ltd. (Vapi, GJ, India) consisting of P-I (1.01 %) and P-II (0.55 %), Livocare, Dindayl Aushdi Pvt Ltd. (Gawalior, MP, India) having P-I (4.17 %) and P-II (3.25 %) and Livplus, BACFO Pharmaceuticals Ltd. consisting P-I (0.07 %) and P-II (0.01 %) (Bhandari et al. 2008). P-I and P-II are the major ingredients of Picroliv (Verma et al. 2009), thus suggesting that a proper concentration of these compounds is highly desirable in an herbal drug formulation.

The *P. kurroa* plants have been recklessly collected from its natural habitat, thereby, putting it under the category of endangered plant species (Nayar and Sastri 1990). The *P. kurroa* propagates vegetatively through stolons, which initially emerge as a young bud, grow to a mature stolon and then eventually into a rhizome with independent shoots and roots (Raina et al. 2010) (Fig. 1). The *P. kurroa* plants are uprooted from their natural habitat for obtaining mature rhizomes, however, along with all other young stolons, the rhizomes also get uprooted, thereby, disrupting the natural propagation of *P. kurroa* (Fig. 1). The increasing national and international demand for *P. kurroa* raw material coupled with limited cultivation has made it a critically endangered plant species (Nayar and Sastri 1990; Rai et al. 2000). The plant produces relatively small amounts of picrosides and that too in the rhizomes, which necessitate efforts to increase the yield of picrosides.

The biosynthesis of P-I and P-II occurs differentially in shoots and roots, respectively, whereas both accumulate in rhizomes (Sood and Chauhan 2010). The P-I and P-II contents in rhizomes can be influenced by several factors such as environment (altitude, light intensity, soil conditions, etc), strains, rhizome age and developmental stage of shoots, roots and rhizomes. Understanding the dynamics of P-I and P-II contents in different growth and developmental stages of *P. kurroa* is of paramount importance not only for regulating the quality of herbal drug formulations prepared from *P. kurroa* but also optimizing agro-based technologies for the commercial cultivation and production of uniform plant material. Recent studies have suggested that the leaves, rather than the underground parts such as rhizome and roots, might be used as a plant material for the preparation of herbal drug formulations from *P. kurroa*

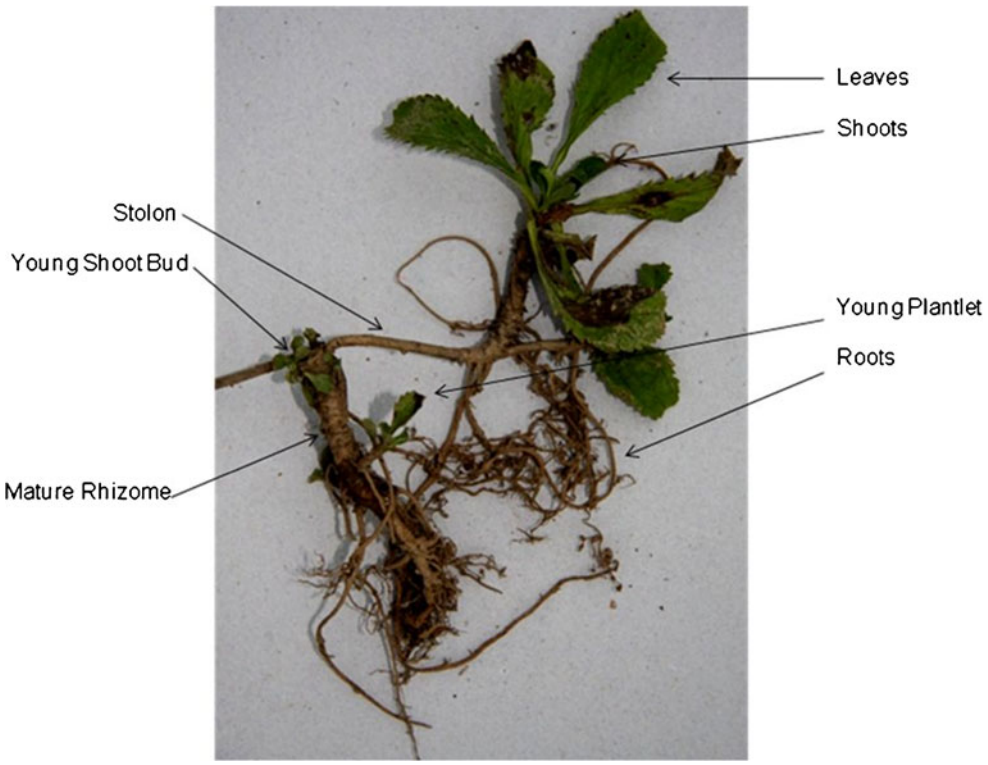


Fig. 1 Mature *P. kurroa* plant (~3 years old)

(Katoch et al. 2011). However, leaves are known to biosynthesize only P-I not P-II, which, therefore, will compromise the quality of herbal drug formulations as well as their pharmacological properties (Sood and Chauhan 2010). The effect of altitude has also been suggested as an important factor influencing P-I and P-II contents in *P. kurroa* (Katoch et al. 2011). However, collections of *P. kurroa* were made from different altitudes which would not only reflect the influence of environment at a particular altitude on the biosynthesis and accumulation of P-I and P-II, but also the collections can be genetically different strains. Therefore, the effect of altitude or other environmental factors on biosynthesis and accumulation of picrosides can be best understood if same strain of *P. kurroa* is grown at different altitudes and then quantified for the P-I and P II contents in tissues of different growth and developmental stages. The current study has explored the status of accumulation and biosynthesis of P-I and P-II in the field grown plant of *P. kurroa* vis-à-vis different growth and developmental stages of *P. kurroa.* The effect of altitude on P-I and P-II contents in different organs and developmental stages of the same strain of *P. kurroa* was also investigated.

Materials and methods

Collection of plant material

P. kurroa plants were procured from the Regional Research Station of Himalayan Forest Research Institute (HFRI), Panthaghati, Shimla, India. *P. kurroa* plants of the same strain were grown at Sairopa (4,500 m altitude, 31°38′–31°54′N and 77° 20′–77°45′ E) and Jagatsukh (1,900 m altitude, 20°35.6′–32° 6.1′N and 78°57.8′–77°33.7′E), Himachal Pradesh (H.P), India.

Selection of plant samples of different growth and developmental stages

The young buds (7–15 days old) were taken as the youngest propagule of *P. kurroa* (Fig. 1). Subsequent growth of shoot buds into fully developed shoots with rhizome and roots was monitored regularly in the mother plants and categorized into different growth stages (Table 1). The samples were taken at different time intervals representing each stage i.e. the shoots, rhizome and root tissue for the analysis of P-I and P-II. Mature plants of *P. kurroa* were further categorized into 3 stages with a yearly difference in their age as determined from the growth of young buds.

Samples from rhizome sections

As mature rhizomes (3–4 year old) are mostly used in the preparation of herbal drug formulations, different sections (top, middle, lower) of a dried mature rhizome were tested for the amounts of P-I and P-II. Each rhizome was cut horizontally into 3 parts and then vertically from the centre so as to provide two replications of each sample from each rhizome (Fig. 2).

Selection of leaf samples of different developmental stages

Each shoot of *P. kurroa* consists of a whorl of leaves wherein the innermost leaves are the youngest and the outermost are the oldest. The leaves were classified into five different development stages named as L1 (inner most) to L5 (outermost) (Fig. 3). Fresh leaves of each stage were analyzed for P-I and P-II. The sampling was done in the month of September.

Preparation of samples and extraction of picrosides

The samples were thoroughly washed under running tap water, segregated as per the classification given above, and ground into a fine powder using a pestle mortar in liquid nitrogen. All samples were used fresh except the mature rhizome sections, which were dried before grinding. Powdered samples (100.0 mg) were extracted in 10.0 ml 80 % HPLC grade methanol, vortexed to mix properly and incubated overnight at room temperature (25 °C). Samples were centrifuged at 10,000 rpm for 15 min, the supernatant was filtered through 0.22-μm filter (Millipore) and used for HPLC analysis.

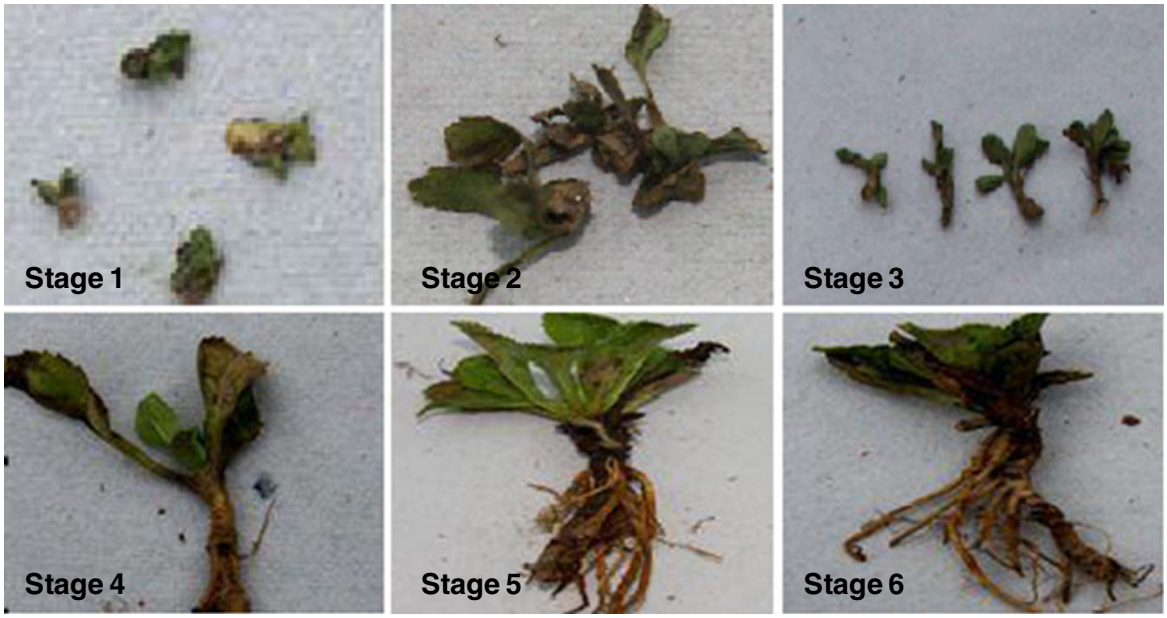
Quantification of P-I and P-II through HPLC analysis

The quantification of P-I and P-II was carried by reverse phase HPLC (Waters 515) through C18 (5 μm) 4.6× 250 mm Waters Symmetry Column using PDA detector (Waters 2996). Two solvent systems were used for running the test samples i.e. Solvent A (0.05 % trifluoroacetic acid) and Solvent B (1:1 methanol/acetonitrile mixture). Solvent A and B were used in the ratio of 70:30 (v/v). The column was eluted in the isocratic mode with a flow rate of 1.0 ml/min. The P-I and P-II were detected at absorbance of 270 nm wavelength. The cycle time of analysis was 30 min at 30 °C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex, Inc.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) to test for significance of differences using MINITAB-14. Significance between the mean values was tested by Tukey's test at the confidence level of $P \leq 0.05$

Table 1 P-I & P-II contents in different growth and developmental stages of *P. kurroa*



Different growth and developmental stages of *Picrorhiza kurroa.*

Stage 1: Shoot Bud 7-15 Days,

Stage 2: Shoot Proliferation 40-45 Days, Stage 3: Fully Developed Shoot 90-100 Days,

Stage 4: Developed Shoot With Rhizome Initiation 110-120 Days,

Stage 5: Developed Shoot With Developed Rhizome and Root Initiation 160-170 Days,

Stage 6: Developed Shoot With Developed Rhizome and Root ~220 Days

	Shoots	Rhizomes		Roots
	PI (%)*	PI (%)*	PII (%)*	PII (%)*
Stage 1	0.05± 0.04	0	0	0
Stage 2	0.32± 0.02	0	0	0
Stage 3	0.62± 0.02	0	0	0
Stage 4	0.68± 0.01	0.15± 0.05	0.1± 0.05	0
Stage 5	0.73± 0.02	0.25± 0.05	0.22± 0.02	0.07± 0.02
Stage 6	0.76± 0.01	0.50± 0.05	0.45± 0.05	0.15± 0.05

Results and discussion

P-I and P-II contents in different growth and developmental stages of *P. kurroa*

P-I was detected in low concentration (0.05 %) in the young shoot buds and increased to 0.76 % (fresh weight basis) in fully developed shoots of stage 6. No significant increase in the P-I content occurred in the shoots of stages 4, 5 & 6 (Table 1). P-II was not detected in the shoots of any of the six developmental stages (S1 to S6), however, it was detected at very low levels (0.07 %) in the roots of stage 5 and reached to maximum (0.15 %) in the fully developed roots of plants at stage 6 (Table 1). The rhizome formation occurred at stage 4 with the accumulation of P-I (0.15 %) and P-II (0.10 %) with an increase in their concentrations to

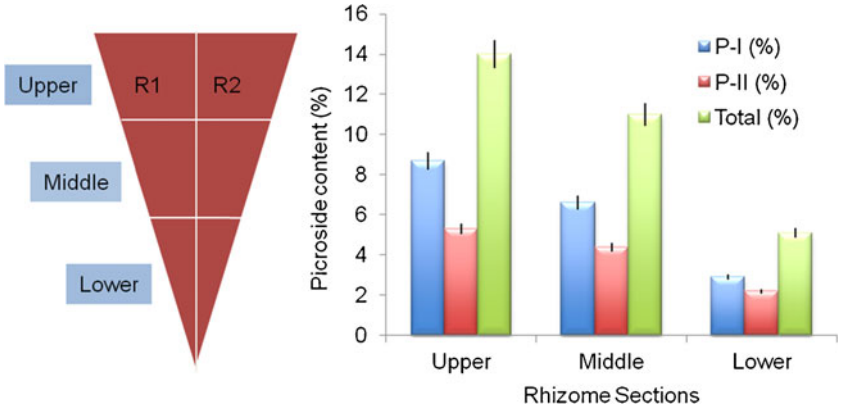


Fig. 2 P-I and P-II contents in different sections of a mature rhizome

0.50 % and 0.45 %, respectively in the fully developed rhizomes of stage 6. P-I has been detected in shoots and P-II in roots of *P. kurroa*. However, varying amounts of P-I and P-II in morphogenically and developmentally different stages of *P. kurroa* suggests that uniformity in the selection of plant material is essential for the preparation of herbal drug formulation of desired efficacy. Understanding P-I and P-II contents vis-à-vis morphogenic and developmental growth of shoots, rhizomes and roots has provided insights about the sites of biosynthesis and storage of picrosides in *P. kurroa*. Differential biosynthesis and accumulation of metabolites occur in different tissues of plants. For example the accumulation of hypercin and hyperforin occurs in leaves and infloroscence of *Hypericum perforatum*, respectively (Kowalski and Wolski 2006). The accumulation of withanolide occurs in shoot tips and leaves of *Withania somnifera*(Praveen et al. 2010). The age and developmental stage of a plant also influences the biosynthesis and accumulation of secondary metabolites. The amounts of vanillin, glucovanillin and other metabolites were higher in the older pods compared to younger pods of *Vanilla planifolia* (Palama et al. 2009). Similarly the biosynthesis of rutin is influenced by the growth and developmental stages of *Fagopyrum* species (Gupta et al. 2011). The biosynthesis and accumulation of picrosides occur in specialized cells of *P. kurroa* in in vitro cultures where no picrosides were detected in the callus cells of *P. kurroa,* however, P-I was detected in the cultures differentiating into shoots (Sood and Chauhan 2011). The present study has, therefore, suggested that the primary site of biosynthesis of P-I is in green leaf cells of young shoot buds, whereas the primary site of biosynthesis for P-II is either in the young rhizome or root cells because it was detected in both the tissues (Dutt et al. 2004; Sood and Chauhan 2010).

P-I and P-II contents in mature *P. kurroa* plants of different age groups

The estimation of P-I and P-II in shoots, roots and rhizomes of *P. kurroa* of different age groups (yr. 1, yr. 2 & yr. 3) also showed a differential pattern of biosynthesis and storage of P-I and P-II such that the P-I was detected in the shoots, P-II in the roots and both in the rhizomes. The P-I content was almost same (0.76–0.8 %) in the shoots of 1 to 3 years old plants, whereas P-II content increased from 0.15 % in the roots of 1 year old plants to 0.2 % in the roots of 3 year old plants. Significant increase in P-I and P-II contents occurred in the

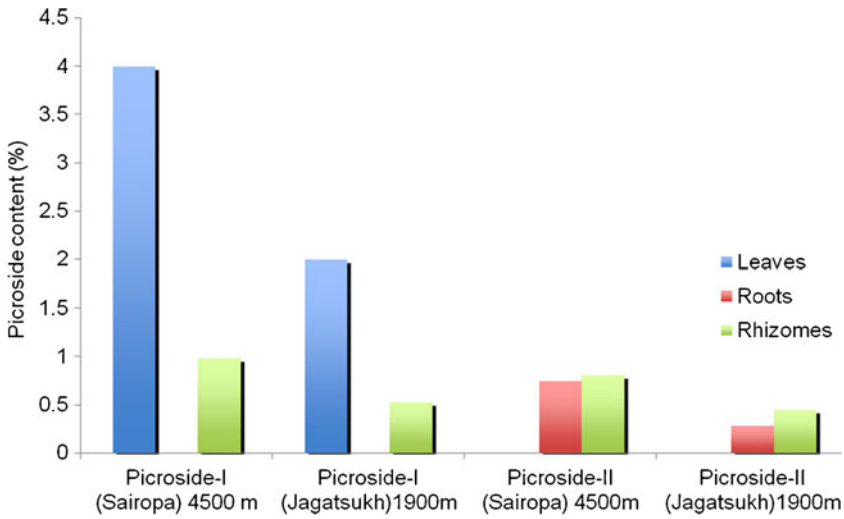


Fig. 3 Effect of altitude on P-I and P-II contents in different parts of *P. kurroa*

Table 2 P-I and P-II contents in mature plants of *P. kurroa* of different age groups

Picrorhiza kurroa plants of different age. A: First Year, B: Second Year, C: Third Year

	Shoots	Rhizome		Root
	PI (%)*	PI (%)*	PII (%)*	PII (%)*
A	0.76±0.05	0.5±0.05	0.45±0.05	0.15±0.05
B	0.78±0.04	0.75±0.05	0.58±0.04	0.17±0.02
C	0.8±0.05	0.98±0.01	0.81±0.01	0.21±0.01

rhizomes from 0.5 % to 0.98 % and 0.45 % to 0.81 %, respectively, with the increase of rhizome age (Table 2). Increase in podophyllotoxin content was reported in the rhizomes of different age groups of *Podophyllum hexandrum* Royle (Pandey et al. 2007). Similarly the amount of berberine increased in the roots and rhizomes of 5 year old *Berberis darwini* compared to 3 year old plants (Cromwell 1933). The same amount of P-I in the shoots of different ages of plants (1 to 3 year) of *P. kurroa,* whereas progressive increase in the P-II contents in the roots and rhizomes of different age group plants is due to the fact that the shoots appear fresh after every winter season of the year whereas the roots and rhizomes of *P. kurroa*, being underground, continue to grow with the age of plants. Mature rhizomes and roots are predominantly used in the preparation of herbal drug formulations from *P. kurroa* because these organs are rich in both the picrosides. It is, therefore, suggested that while uprooting *P. kurroa* plants, mature rhizomes of 3–4 year old be uprooted for herbal drug preparation and the remaining plant parts such as young shoot buds, young rhizomes and growing stolons be replanted so as not only to reclaim natural populations of *P. kurroa* but also to maintain uniformity in the plant material, which would otherwise affect the amounts of P-I and P-II and the herbal drug efficacy.

P-I and P-II contents in different sections of a mature rhizome

The estimation of P-I and P-II in different sections (upper, middle and lower) of a mature rhizome revealed that the upper section contained maximum amount of P-I (8.7 %) and P-II (5.3 %) followed by middle portion with 6.6 % and 4.4 % of P-I and P-II, respectively. The lower most portion of the rhizome contained the least amounts of P-I (2.9 %) and P-II (2.2 %) (Fig. 2). The higher amount of P-I and P-II in the upper sections of the rhizome can be attributed to the fact that those are the oldest parts of a rhizome and, therefore, store higher amounts of picrosides. The lower most part of a rhizome is relatively young, even in a 3–4 year old rhizome, and,

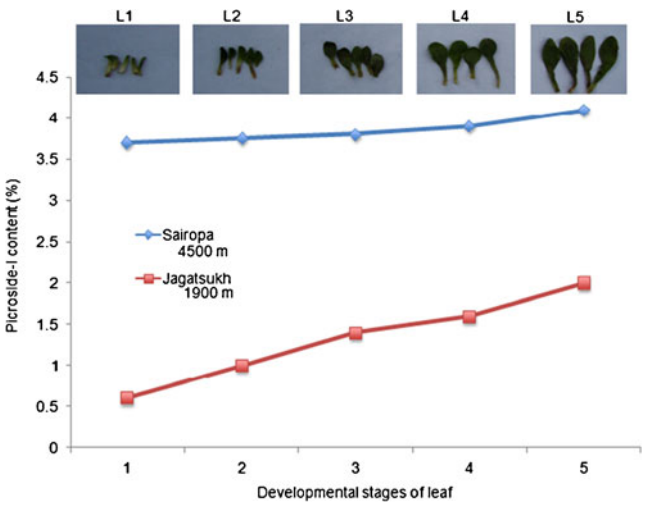


Fig. 4 Effect of altitude on P-I content in leaves of different developmental stages (L1-L5)

therefore, contained less amounts of P-I and P-II. The varying contents of P-I and P-II in different sections of a mature rhizome suggest that the preparation of herbal drug formulations from *P. kurroa* should consider uniformity in the rhizome age and section of a rhizome to be used so as to provide desired amount of picrosides.

Effect of altitude on P-I and P-II contents in different parts of *P. kurroa*

The estimation of P-I and P-II in leaves, roots and rhizomes of the same *P. kurroa* strain planted at two altitudes, Sairopa (4,500 m asl 31°38′–31°54′N and 77°20′–77°45′ E) and Jagatsukh (1,900 m asl 20°35.6′–32°6.1′N and 78°57.8′–77°33.7′E), revealed that the content of picrosides was highest in all plant parts grown at high altitude (4,500 m) compared to low altitude (1,900 m). The concentration of P-I was higher in mature leaves (3.7 %) from Sairopa compared to mature leaves (1.7 %) from Jagatsukh. The content of P-I (0.98 %) and P-II (0.81 %) was also relatively higher in rhizomes of Sairopa compared to plants grown at Jagatsukh with P-I (0.53 %) and P-II (0.45 %), respectively. Similarly the amount of P-II was higher (0.75 %) in the root samples of *P. kurroa* from higher altitude (Sairopa) compared to root samples (0.28 %) from low altitude (Jagatsukh) (Fig. 3).

The P-I content was also estimated in the leaves of different developmental stages (L1 to L5; Fig. 4) of *P. kurroa* plants of the same strain grown at two altitudes (Sairopa and Jagatsukh). The P-I content was significantly high (3.7 %) in L1 to L5 stages of leaves grown at higher altitude of Sairopa (4,500 m) with no significant differences in the leaf stages (Fig. 4). On the contrary the P-I content increased exponentially from 0.5 % to 1.7 % in the leaves of L1 to L5 stages, respectively of *P. kurroa* grown at lower altitude (Jagatsukh, 1,900 m). The average P-I content was 0.9 % in the leaves of plants grown at lower altitude (Fig. 4). Picrosides contents are reported to be influenced by altitude in *P. kurroa*, however, those differences can also be due to occurrence of genetically different strains collected from geographically different locations (Katoch et al. 2011). Morphologically different strains of *P. kurroa* do occur in geographically different locations of varying altitudes (Shitiz, unpublished). The effect of altitude, geographical origins and climatic factors significantly influences the production of secondary metabolites in plants such as hypercin and hyperforin contents in St John's Wort (Nikolic and Zlatkovic 2010). Similarly the contents of different metabolites in *Aconitum heterophyllum* were influenced by the altitude (Nautiyal 1986). However, the role of different genotypes and strains cannot be ruled out in those studies because the metabolites content analyses were done for different populations grown at different altitudes, which can possibly be genetically different strains. The current study has demonstrated that the altitude influences the contents of picrosides in morphologically different organs of the same strain of *P. kurroa*. However, what particular environmental factors influence picrosides content at higher altitudes remain to be investigated. With an altitude change, changes in various other factors such as ecological niche, temperature, UV radiation, biotic and abiotic factors have been reported (Gairola et al. 2010). The resperine content in *Rauwaolfia serpentina* and camptothecin content in *Nothapodytes nimmoniana* are influenced by the climatic factors (Kumar et al. 2010). It is thus concluded that the contents of P-I and P-II are largely influenced by the growth and developmental stages of *P. kurroa*, thereby, implying that uniformity in the selection of plant material (rhizomes and roots) is required for preparing herbal drug formulations of desired efficacy.

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