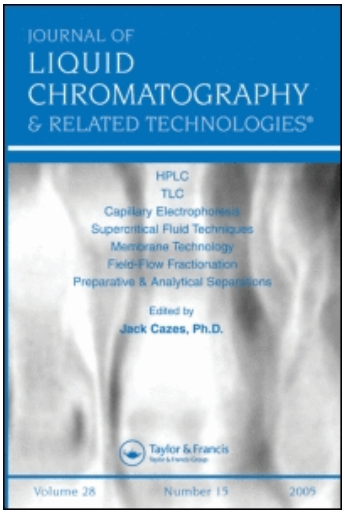

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Quantification of Picroside-I and Picroside-II in *Picrorhiza kurroa* by HPTLC

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Abstract: A new high performance thin layer chromatography (HPTLC) method for the simultaneous quantification of picroside-I and picroside-II in *P. kurroa* is described. Separation of picroside-I and picroside-II was achieved by mobile phase of $CHCl_3$:MeOH (82:18, v/v) on precoated silica gel 60 F_{254} aluminum plate. The densitometric determination of picrosides was carried out at 290 nm, in absorption-reflection mode. The calibration curves were linear in the range of (2–5 μg). The method is simple, specific, rapid, and reliable for simultaneous determination of P-I and P-II in *P. kurroa.* The proposed method was applied for accurate quantification of large number of samples collected from different altitudes of western Himalaya.

Keywords: Picroside-I, Picroside-II, *Picrorhiza kurroa*, HPTLC

INTRODUCTION

Picrorhiza kurroa Royle ex Benth. (family-Scrophulariaceae)[1] is a small important alpine herb, having its habitat in the western Himalaya from Kashmir to Sikkim and growing at an altitude of 3000–5000 m. Locally, it is known as "Kutki". In the Chinese system of medicine it is known as "Hung-hunhlien". It is an important medicinal plant used in traditional and modern medicine, for liver disorders, fever, asthma, and jaundice,[2–5] and is known to possess hepatoprotective,[6] immunomodulator, and antiasthamatic activities.[7–9] It is highly useful in fever and dyspepsia, and is one of

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the important ingredients of various purgative combinations.[10] It is also reported to have bile flow enhancer properties.[11]

The main iridoid glycoside reported from rhizomes of *P. kurroa* is "kutkin", which is a mixture of picroside-I and picroside-II (Fig. 1) and is responsible for hepatoprotective activity.[12] *P. kurroa* root extract scavenges oxygen free radicals, such as superoxides and hydroxy radicals, and inhibit lipid peroxidation induced by the $Fe2^{+}$ ascorbate system in rat liver homogenata.[13]

A number of high performance liquid chromatography (HPLC) methods[14–18] have been reported for the quantification of picroside-I and picroside-II, which need sample cleanup to remove the interfering constituents in the plant extracts, making the procedure more tedious and unsuitable for screening large number of samples. Recently, high performance thin layer chromatography (HPTLC) has been widely employed for the quantification of secondary metabolites.[19–24] The technique has been applied for the first time for the estimation of picroside-I and picroside-II in *P. kurroa.* HPTLC has the advantage of providing visualization of the separated constituents of the sample. It also provides on line identification of the analyte by in-situ spectrum scanning and post chromatographic derivatization, along with R_f comparison with the standard. It requires very little sample clean up since

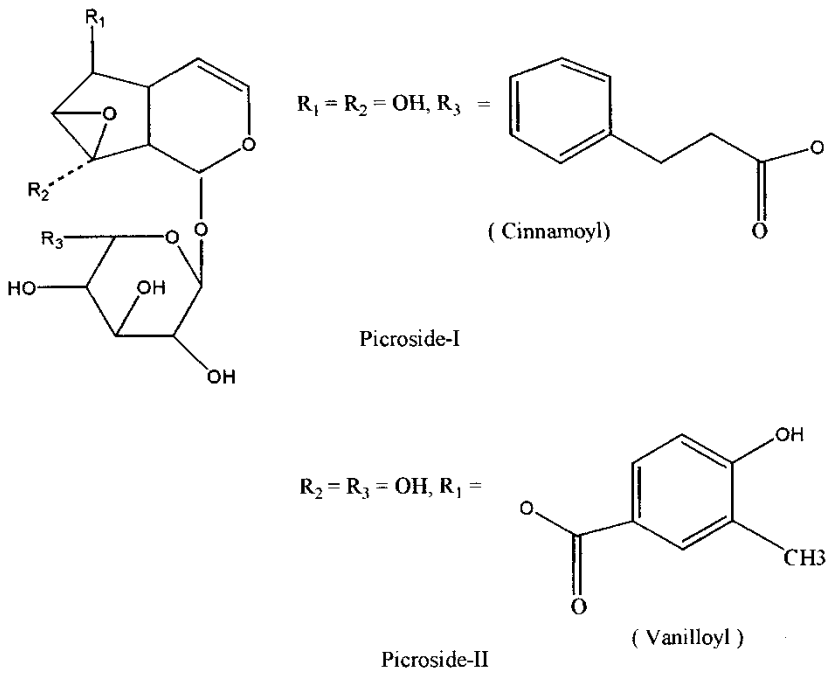

Figure 1. Structure of Picroside-I and Picroside-II.

the layer is disposable. Several samples can be run simultaneously using a small quantity of mobile phase, thus reducing the time and cost per analysis. Due to low consumption of solvent the methodology is eco-friendly. The present work describes a simple yet sensitive, specific, and reproducible HPTLC method for the quantification of P-I and P-II in *P. kurroa.*

EXPERIMENTAL

Chemicals

All the chemicals, including solvents, were of analytical grade from E. Merck, India. The HPTLC plates Si $60F_{254}$ (20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany).

Plant Material

Samples of *P. kurroa* were collected in the month of September–October 2003 from different altitudes (ranging from 2600–4500 m) from the western Himalayan region. They were authenticated by the Biodiversity Department, IHBT, Palampur, H.P. Rhizomes of the plants were air dried at room temperature (25°C ± 2°C).

Preparation of Crude Extract

The air dried (25 ± 2°C) rhizomes of *P. kurroa* (0.2 gm) were extracted in 10 mL of methanol. After 12 hours, they were filtered and dried. Dried extracts were re-dissolved in 1 mL of methanol and 5 μL of each sample was spotted for quantification.

Preparation of Standard Solutions

Stock solutions of Picroside-I and Picroside-II (1 mg/mL) were prepared in methanol, and different amounts (2–5 μL) of these were loaded onto a TLC plate, using ATS 4 for preparing four point calibration curves.

High Performance Thin-Layer Chromatography

A Camag HPTLC system equipped with an automatic TLC sampler ATS 4, TLC scanner 3, and integrated software WinCATS version 1.2.3 was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC

$60F_{254}$ (20 × 20 cm) plate of 0.20 mm layer thickness. The samples and the standards were applied on the plate as 6 mm wide bands with an automatic TLC sampler (ATS 4) under a flow of N_2 gas, 10 mm from the bottom, 10 mm from the side, and the space between two spots was 6 mm of the plate.

Detection and Estimation of Picroside I and Picroside II

The linear ascending development was carried out in a CAMAG twin trough chamber (20 cm × 20 cm), which was pre-saturated with 25 mL mobile phase chloroform:methanol (82:18) for 30 min at room temperature (25°C ± 2°C) and 50% ± 5 relative humidity. The length of the chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in a current of air, with the help of an air dryer, in a wooden chamber with adequate ventilation. The flow of air in the laboratory was maintained unidirectional (laminar flow, towards exhaust). Quantitative evaluation of the plate was performed in the absorption-reflection mode at 290 nm, using a slit width 6 × 0.4 mm, data resolution 100 μm/step, and scanning speed 20 mm/s. The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum 190 and 400 nm. Determination of the content of picrosides in extracts was performed by the external standard method, using pure P-I and P-II as standards. Each sample was carried out in triplicate. A Camag Video Documentation system in conjunction with the Reprostar 3 was used for imaging and archiving the thin layer chromatograms. The object was captured by means of a high sensitive digital camera with 4.0 M pixel CCD sensor and 3x optical zoom, model Power shot G2 (Canon, Singapore) Fig. 2. A special digitizing board (frame grabber) assisted in rapid processing

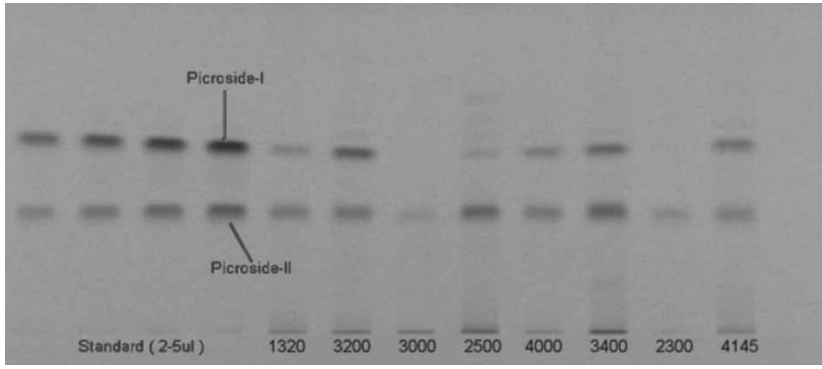

Figure 2. CCD image of TLC plate of *Picrorhiza kurroa* at 254 nm. Lanes: 1–4 = standards 2, 3, 4, and 5 μg of picroside-I and picroside-II, 5–12 samples from different altitude.

via the personal computer system. Image acquisition processing and archiving were controlled via win CATS software.

Calibration Curve

Stock solution of picroside-I and picroside-II (1 mg/mL) were prepared in methanol and 2–5 μL of these solutions were loaded on a TLC plate, using ATS4 for preparing four points calibration graphs.

Validation of HPTLC Densitometry Method

Selectivity

Each compound was separated with baseline return as shown in Fig. 3.

Accuracy and Recovery

To the pre-analyzed sample, 1.0 mg each of standard P-I and P-II were added and the mixture was analyzed by the proposed method. The experiment was conducted in triplicate to check, recovery and accuracy of the system. The results are summarized in Table 1, Table 2a, and 2b showing the accuracy and recovery of the method as the mean values and the %CV values of P-I and P-II.

Limits of Detection and Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantified as exact value. The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), the blank methanol was spotted six times following the same method as explained. The signal to noise ratio was determined as 3:1 and 10:1 considered for LOD and LOQ, respectively. The limit of detection for P-I and P-II were 1.5 and 1.7 μg, respectively, and limit of quantification was 2 μg each.

Precision Validation

System precision was performed by spotting 6 samples each from the stock solution of P-I and P-II, (2000 ng) and spotted on the silica gel 60F 254 plate and analyzed with the proposed method. The results are given in Table 3.

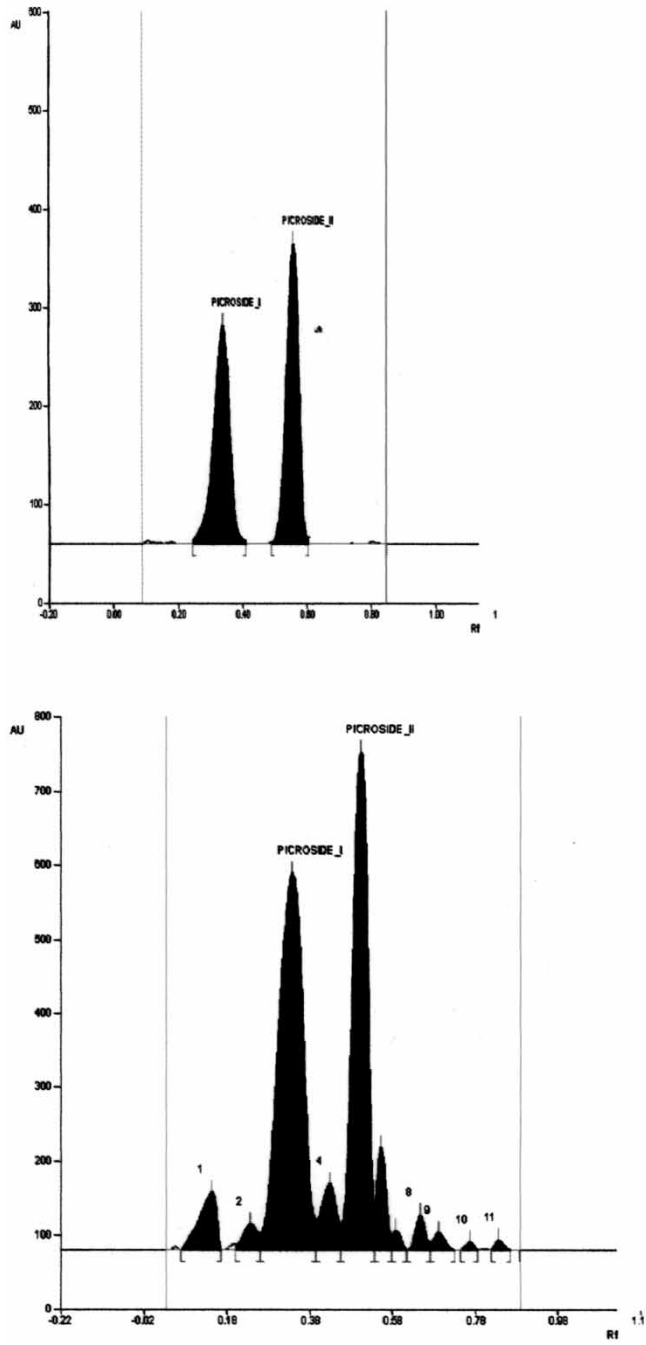

Figure 3. HPTLC chromatogram of (a) standards P-I & P-II; (b) resolution of P-I and P-II in the sample.

Table 1. Picroside-I and picroside-II contents in different locations of *P. kurroa* with altitude in triplicate by HPTLC method

S. no.	Altitude (mts)		HPTLC values in triplicate (%)			Mean (%)	Mean ± SE (%)	CV (%)
1	3200	P-I	1.668	1.675	1.669	1.672	1.672 ± 0.003	0.246
		P-II	1.530	1.478	1.493	1.501	1.501 ± 0.016	5.720
2	3400	P-I	1.653	1.648	1.679	1.660	1.660 ± 0.013	1.003
		P-II	0.563	0.570	0.564	0.566	0.566 ± 0.003	0.673
3	3000	P-I	0.023	0.018	0.021	0.021	0.021 ± 0.002	3.839
		P-II	—	—	—	—	—	—
4	2300	P-I	0.144	0.147	0.148	0.146	0.146 ± 0.0016	0.484
		P-II	0.018	0.017	0.019	0.018	0.018 ± 0.0006	5.555
5	4000	P-I	0.546	0.539	0.531	0.538	0.538 ± 0.005	1.403
		P-II	0.431	0.432	0.428	0.430	0.430 ± 0.0016	0.493
6	2500	P-I	0.176	0.186	0.179	0.180	0.180 ± 0.004	2.859
		P-II	0.031	0.033	0.032	0.032	0.032 ± 0.0006	4.941
7	4145	P-I	0.684	0.692	0.697	0.691	0.691 ± 0.005	0.948
		P-II	1.073	1.068	1.075	1.072	1.072 ± 0.003	0.336
8	1350	P-I	0.376	0.381	0.370	0.375	0.375 ± 0.004	1.484
		P-II	0.379	0.368	0.375	0.374	0.374 ± 0.004	1.488

Table 2a. Recovery study of P-I by the HPTLC method ($n = 3$)

S. no.	Amount of picroside I in rhizome powder (mg)	Amount of picroside I added (mg)	Amount found in mixture (mg)	Recovery (%)
1	1.67	1.00	2.56 ± 0.003	96.00

Table 2b. Recovery study of P-II by the HPTLC method ($n = 3$)

S. no.	Amount of picroside II in rhizomes powder (mg)	Amount of picroside II added (mg)	Amount found in mixture (mg)	Recovery (%)
1	1.50	1.00	2.42 ± 0.001	97.01

RESULTS AND DISCUSSION

Different compositions of the mobile phase were tested and desired resolution of picroside-I and II, with symmetrical and reproducible peaks, was achieved by using mobile phase chloroform-methanol (82:18) (Fig. 3). Peaks corresponding to picroside-I and picroside-II were at R_f 0.60 and 0.43, respectively.

The methanolic extract, when subjected to HPTLC, showed the presence of P-I and P-II peaks. Comparison of the spectral characteristic of the peaks for standards of P-I and P-II, and that of all the samples, revealed the identity of P-I and P-II present in all the samples. The same sample of *P. kurroa* was used for the HPTLC analysis. The calibration curves (Fig. 4) were linear in the range of 2 μg to 5 μg for P-I and P-II, respectively. Linear regression equation, R_f, and standard deviation are given in Table 4. Peak purity tests of P-I and P-II were done by comparing UV-visible spectra of P-I & P-II in standard and sample track (Fig 5a, 5b). For the examination of recovery

Table 3. System precision studies of the developed method

	System precision	
S. no.	Area picroside-I	Area picroside-II
1	2929.54	13317.98
2	2984.36	13348.14
3	2897.15	13457.32
4	2915.04	13486.47
5	2934.46	13397.17
6	2963.41	13387.65
STD DEV	60.78	63.79
RSD	2.069	0.476

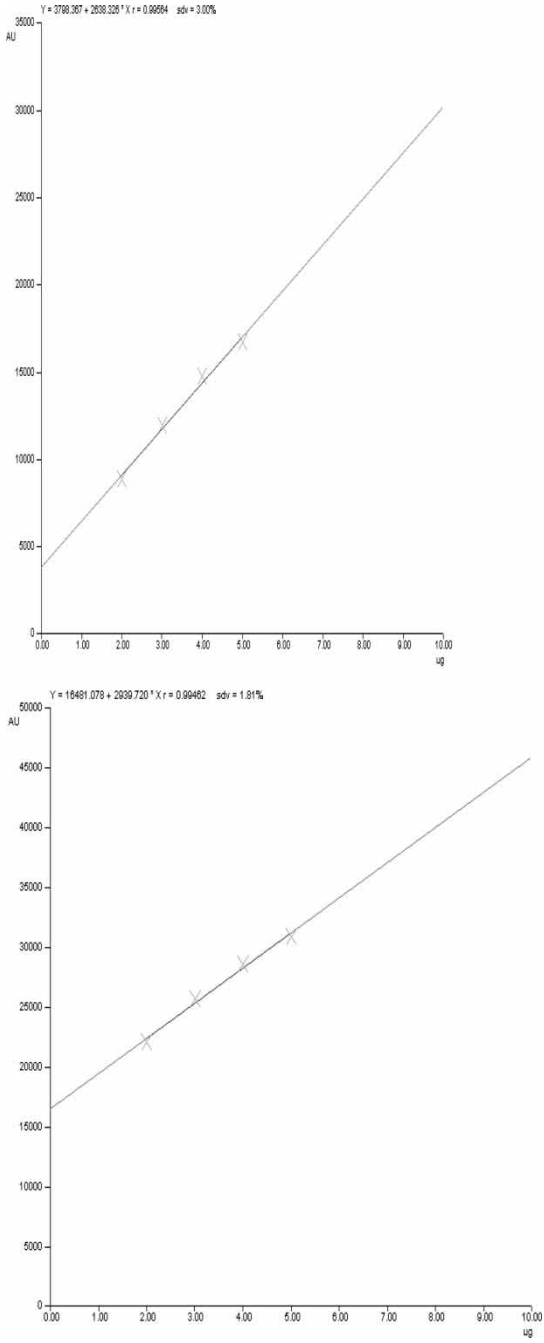

Figure 4. Linear Calibration curve of P-I for 4 points, Linear Calibration curve of P-II for 4 points.

Table 4. Linear regression equation and R_f for picrosides

Picrosides	R_f	Regression equation	r[a]	RSD	LOD (μg)	LOQ (μg)
Picroside I	0.60	Y = 3788.367 + 2638.326	0.99564	3.00%	1.5	2
Picroside II	0.43	Y = 16481.078 + 2939.720	0.99462	1.81%	1.7	2

[a]Correlation coefficient.

rates, 1.0 mg each of stock solutions of pure P-I and P-II were added in one *Picrorhiza kurroa* extract and quantitative analysis was repeated three times. The average values of recovery were 96% and 97% for P-I and P-II, respectively (Table 2a and 2b). In the method applied here, peaks corresponding to P-I & P-II were symmetrical and well separated from other spots.

After analyzing all samples collected from different altitudes in the month of October when the plant undergoes dormancy, it was found that %mean

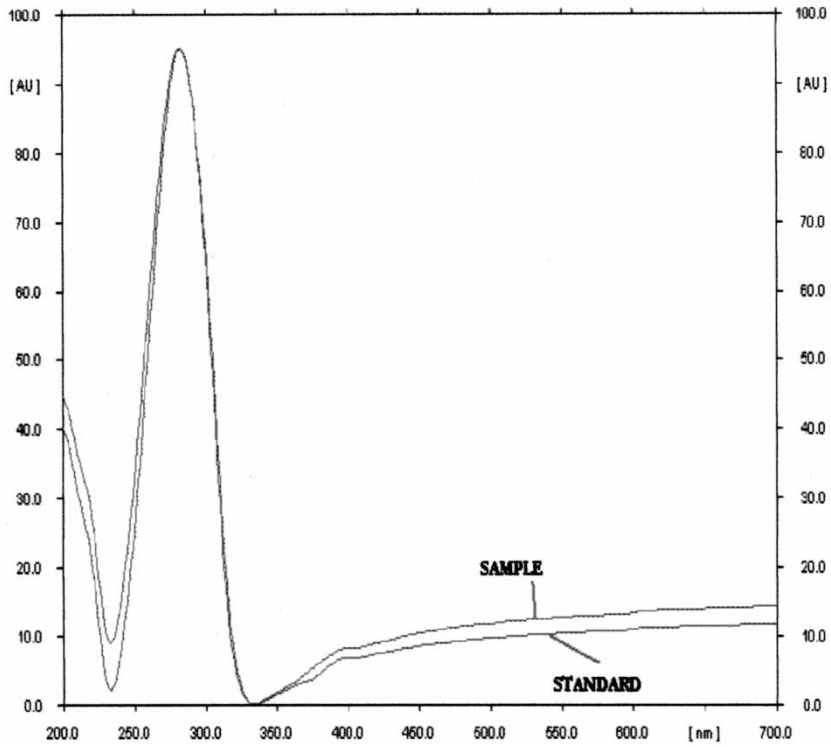

Figure 5. a) UV-Vis spectrum of standard P-I. b) UV-Vis spectrum of standard P-II. (*continued*)

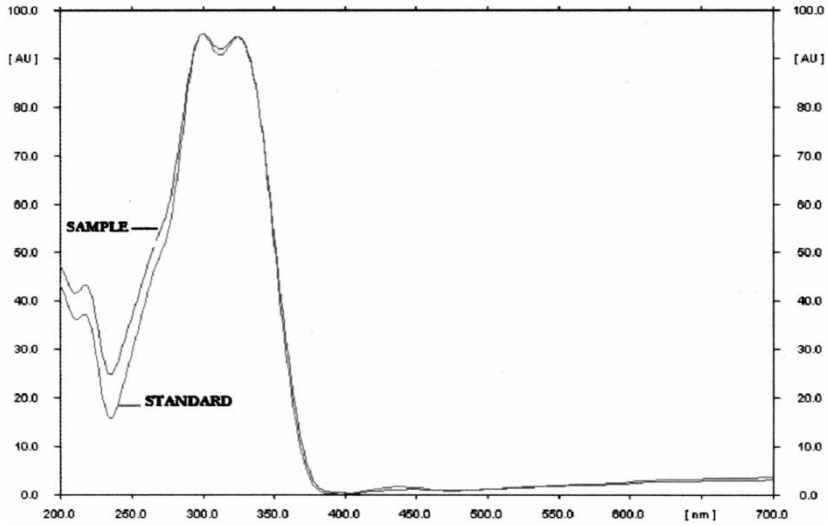

Figure 5. Continued.

values of picroside-I and II were maximum at an altitude of 3200 m (P-I, 1.67% and P-II 1.50%). Further, it was observed that at an altitude of 3000 m no P-II was present. However, further studies are needed to ascertain the role of age and stage of harvest on picroside contents. Estimation of picroside content of all samples collected from different altitudes is given in Table 1 and bar diagram (Fig. 6).

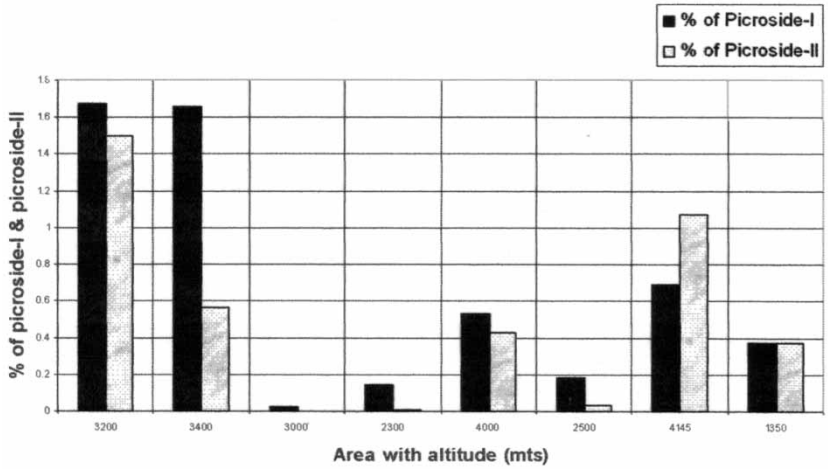

Figure 6. Typical bar diagram of P-I and P-II in *P. kurroa* collected from different locations with different altitudes.

CONCLUSION

The HPTLC method developed here for the quantification of picroside-I and picroside-II in *P. kurroa* is simple, rapid, cost-effective, and easily adaptable for screening and quantitative determination than any other analytical technique. Fifteen samples can be analyzed on a 20 × 20 cm TLC plate in about a 45 minute time duration after extraction of the samples. The present method is suitable for rapid screening of large numbers of plant samples for crop improvement under the plant-breeding program.

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