Original Research Article

Development and validation of HPLC method for analysis of picroside-I and picroside-II in *Picrorhiza kurroa*

ABSTRACT

Aim: In the present investigation, our aim is to develop and validate HPLC method as per ICH guidelines for analyisis of picroside-I and picroside-II in *Picrorhiza kurroa.*

Place and Duration of Study: Investigation was undertaken in Department of Forest Products, University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India and in the period between June 2016 and December 2016.

Methodology: The system used is of Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and Empower II software. Standards of picroside-I and picroside-II were purchased and used for HPLC method development and validation. The developed HPLC method was validated for parameters as linearity, range, accuracy, precision, LOD, LOQ and robustness as mentioned in ICH guidelines.

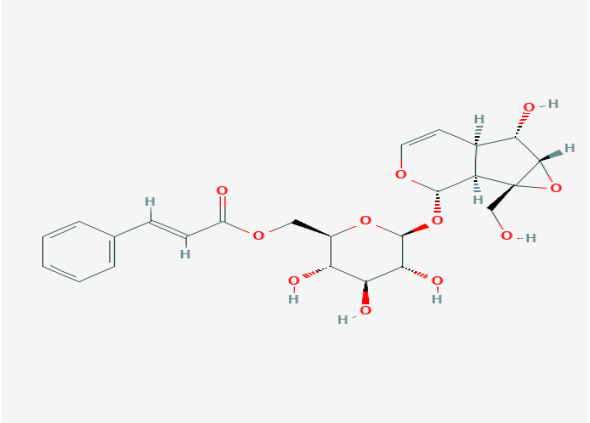
Results: The analytical column, Sunfire C_{18} (4.6×250mm, 5µm) was operated at ambient temperature. Isocratic elution with A methanol and B water (60:40, v/v) at a flow rate of 0.9ml/min was selected. UV detection was done at 270nm and run time was given forty minutes for standard compounds and forty five minutes for samples of *Picrorhiza kurroa*.

Conclusion: Method was found to be satisfactory in terms of linearity, high accuracy and precision. The method was successfully applied to the extracts made of different market samples of *Picrorhiza kurroa.*

Keywords: Picrorhiza kurroa; picroside-I; picroside-II; HPLC; method development; validation

1. INTRODUCTION

Picrorhiza kurroa Royle *ex*. Benth (trade name Kutki), an important member of family Scrophulariaceae, is a perennial herb found in the Himalayan region from Kashmir to Sikkim at an altitude of 3,000-5,000 m above mean sea level in India, China, Pakistan and Bhutan [1,2,3]. In Himachal Pradesh, it is found in the higher reaches of Chamba, Kangra, Mandi, Shimla, Kinnaur and Lahaul and Spiti districts of Himachal Pradesh [4]. Due to extensive harvesting from wild and absence of organized cultivation, the plant is listed as 'endangered' species by IUCN [5,6] and is listed in CITES [7]. Rhizomes of *Picrorhiza kurroa* has been used traditionally for asthma, bronchitis, malaria, chronic dysentery, viral hepatitis, upset stomach, scorpion sting, as a bitter tonic (stimulating the appetite and improving digestion) and as a liver protectant [8,9]. Also, it has been used in the treatment of skin conditions, peptic ulcer and neuralgia, vitiligo and rheumatic arthritis [10]. *Picrorhiza kurroa* has been commonly used and well investigated for the treatment of jaundice [11]. Picroliv- a hepatoprotective drug formulation, is prepared from a standardized iridoid fraction containing Picroside-I and Kutkoside in a 1:1.5 ratio [12,13]. Kutki is the main ingredient in many Ayurvedic preparations and formulations like Arogyavardhini, Tiktadya ghrita, Jatyadi ghrita, Arogya, Livocare, Vimliv, Kutaki *etc.* [14,15].



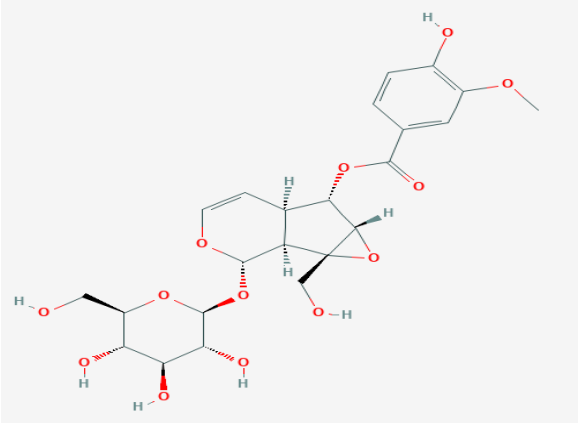


Fig. 1a. Chemical structure of Picroside-I **Fig. 1b. Chemical structure of Picroside-II**

(Fig. 1a & 1b source Pubchem)

In the present study, objective was to develop and validate a HPLC method as per ICH guidelines for picroside-I and picroside-II in samples of *Picrorhiza kurroa*. This can be successfully applied in industries for standardization purpose and for further chemical evaluation studies of the species.

2. MATERIAL AND METHODS

Material and Methods:

Material: The standard compound picroside-I was purchased from Chromadex (Catalogue no. ACB00016819-005) and picroside-II was purchased from Sigma Aldrich (Catalogue no. G0174). Solvents (methanol and water) of HPLC grade were used for HPLC sample preparation and as mobile phase. Solvents used for extraction were of analytical grade.

Methods

Instrumentation and chromatographic conditions: The system used is of Waters binary HPLC unit with Waters HPLC pump 515, dual λ absorbance detector 2487 and program used for data analysis was Empower II software. Numerous optimization experiments on type of column, solvent system, flow rate and wavelengths *etc*. allowed the establishment of best chromatographic conditions to analytical separations of the components.
Optimized chromatographic separation was found in Sunfire C-18 (4.6 x 250mm, 5µm) column with isocratic mode of separation with Methanol : Water :: 60 : 40, v/v) mobile phase and flow rate of 0.9ml/min. The mobile phase was filtered through 0.45µm Millipore membrane filter and degassed with sonicator for 10 minutes before use. The determinations were performed with UV detector set at 270nm.
Picroside-I and picroside-II standards-analytical curve: standard stock solution of mixed Picroside-I (225.00µg/ml) and picroside-II (237.50µg/ml) was freshly prepared by transferring 2.5 mg of both standards, accurately weighed, to a 10 mL volumetric flask, using mobile phase to transfer the sample and to complete the volume. Working solutions, (3.510µg/ml, 7.031µg/ml, 14.062µg/ml, 28.125µg/ml 56.250µg/ml and 112.500µg/ml) of picroside-I and (3.710µg/ml, 7.421µg/ml, 14.843µg/ml, 29.687µg/ml 59.375µg/ml and 118.750µg/ml) of picroside-II were prepared by diluting the stock solution in mobile phase. To obtain the analytical curve, 20 µL of each concentration was injected into the HPLC system (Fig.2) and the area under curve (AUC) for each peak was plotted versus standard concentration. The analysis was carried out in triplicate and a straight line standard curve for both picroside-I and picroside-II was obtained by linear regression of the experimental data (Fig. 3 & 4).

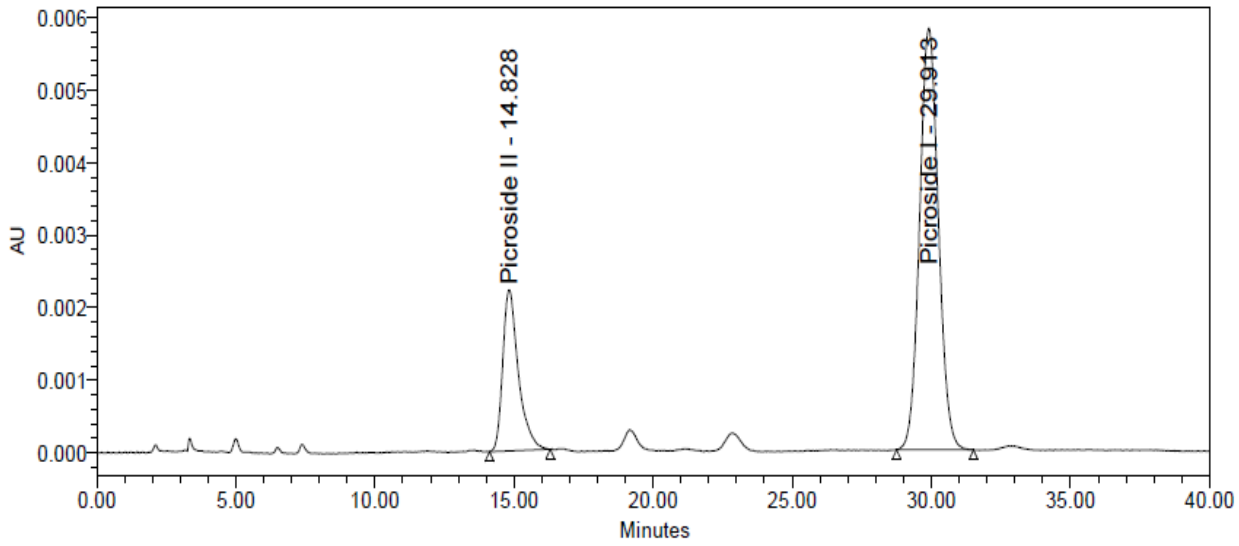


Fig. 2. Chromatogram of Picroside-I (112.5 µg/ml) and Picroside-II (118.75 µg/ml) (reference compounds)

Sample preparation for testing of developed method: The developed HPLC method was used for quantification of picroside-I and picroside-II in market samples of drug kutki procured from different markets of H.P. Accurately weighed samples (2gm each) were extracted with cold extraction method for 8 hours, after that extract filtered with whatmann filter paper, distilled off completely to obtain dry extract for HPLC estimation.

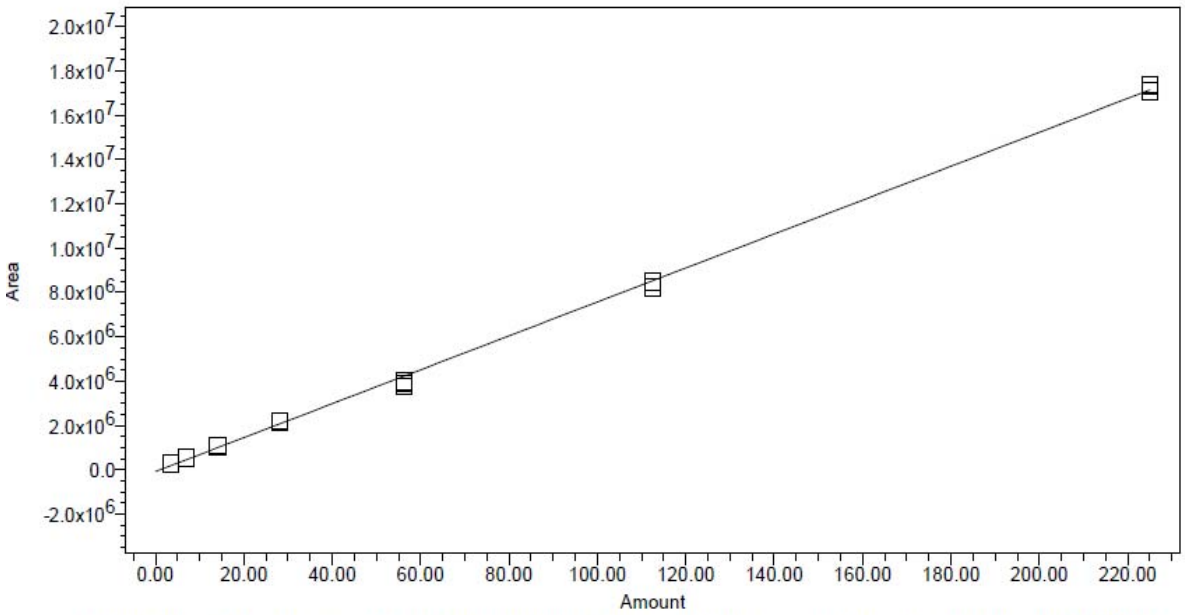
HPLC assay of picroside-I and picroside-II in market samples: The well dried extracted samples were diluted with mobile phase (methanol : water, 60 : 40, v/v) up to 1000 times, centrifuged at 3500rpm then filtered through 0.2µm membrane prior to injection in the HPLC system. This well prepared sample was then analyzed by developed HPLC method.

(ii) Method Validation

The developed HPLC method was validated for seven parameters as mentioned in ICH guidelines and procedure followed for testing these parameters was also as per ICH guidelines (ICH Q2(R1), (2005)). Different parameters used for validation were Linearity and range, Accuracy, Precision, Limit of detection (LOD), Limit of quantitation (LOQ) and Robustness.

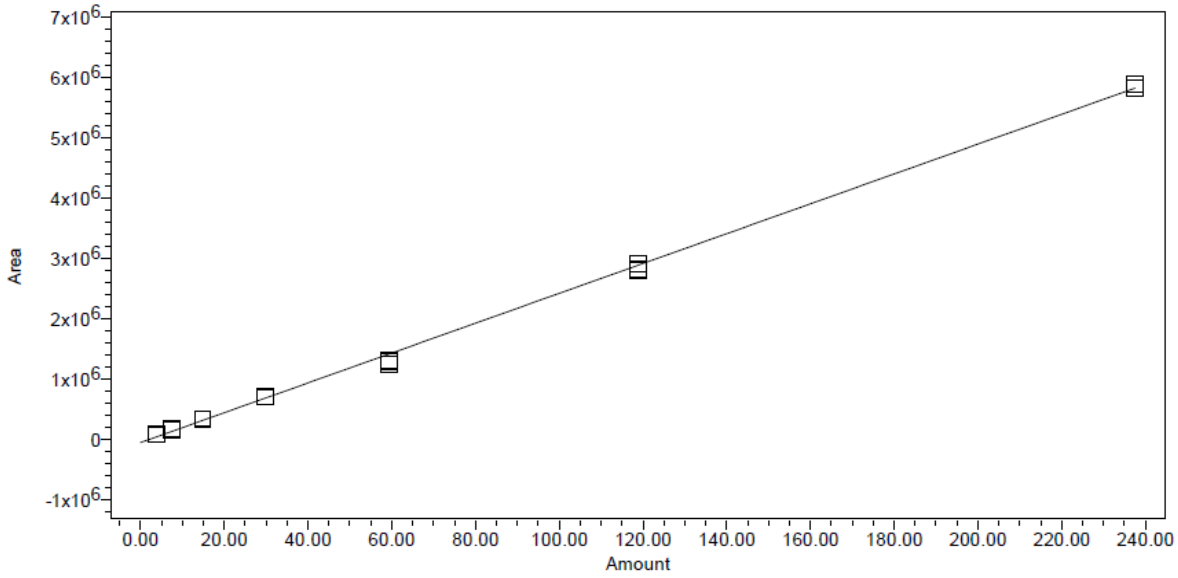
1) Linearity and range

Linearity was determined from triplicate analytical curves obtained by HPLC analysis of picroside-I and picroside-II standard solutions. The concentration range of the method was derived from interval between upper and lower values (including these values) of linearity.



Peak Name: Picroside I; RT: 29.913; Fit Type: Linear (1st Order); Cal Curve Id: 11636; R: 0.999515; R^2: 0.999030; Weighting: None; Equation: Y = 7.66e+004 X - 7.78e+004; Normalized Intercept/Slope: -0.008889; RSD(E): 3.896356

Fig. 3. Calibration curve of Picroside-I (Reference compound)



Peak Name: Picroside II; RT: 14.828; Fit Type: Linear (1st Order); Cal Curve Id: 11635; R: 0.999428; R^2: 0.998856; Weighting: None; Equation: Y = 2.47e+004 X - 4.91e+004; Normalized Intercept/Slope: -0.016452; RSD(E): 4.290940

Fig. 4. Calibration curve of Picroside-II (Reference compound)

2) Accuracy

The accuracy of the method was studied by recovery studies. The accuracy of the method was determined by percentage recovery of picroside-I and picroside-II in the spiked sample at three concentration levels (i. sample with known quantity of picroside-I (7.031µg/ml) and picroside-II (7.421µg/ml) + picroside-I 14.063µg/ml + picroside-II 14.844µg/ml; ii. sample with known quantity of picroside-I (7.031µg/ml) and picroside-II (7.421µg/ml) + picroside-I 28.125µg/ml + picroside-II 29.688µg/ml; iii. sample with known quantity of picroside-I (7.031µg/ml) and picroside-II (7.421µg/ml) + picroside-I 56.250µg/ml + picroside-II 59.375µg/ml). The resultant samples were then analyzed (replicated three times) and the average percentage recoveries were calculated as:

$$\text{Recovery (\%)} = \frac{\text{Observed amount of compound(µg/ml)}}{\text{Actual amount of compound (µg/ml)}} \times 100$$

3) Precision

To study the precision of the method, inter-day and intra-day precisions were determined as below:

i. Intra-day precision: The intra-day precision was measured by injecting same concentration of standard mixture (28.125µg/ml of picroside-I, 29.687µg/ml picroside-II) for six times in a day and measuring their response. The relative standard deviation (%R.S.D.) of response was taken as measurement of intra-day precision.

ii. Inter-day precision: The inter-day precision was measured by injecting same concentration of standard mixture (28.125µg/ml of picroside-I, 29.687µg/ml of picroside-II) for six consecutive days and measuring their response. The relative standard deviation (%R.S.D.) of response was taken as measurement of inter-day precision.

4) Limit of Detection (LOD)

The lowest concentration of working solution of the analyte was further diluted with mobile phase (methanol : water :: 40:60, v/v) to yield a series of appropriate concentrations. Limit of detection (LOD) of the developed method was determined by injecting progressively low concentrations of the standard solutions and S/N ratio for each concentration was observed. The concentration having signal to noise ratio nearly 3 has been found as LOD.

5) Limits of Quantitation (LOQ)

The lowest concentration of working solution of the analyte was further diluted with mobile phase (methanol : water :: 40:60, v/v) to yield a series of appropriate concentrations. Limit of quantitation (LOQ) of the developed method was determined by injecting progressively low concentrations of the standard solutions and observed S/N ratio of each concentration. The LOQ for each investigated compounds was established at signal to noise ratio approaching nearly to 10.

6) Robustness

Robustness of the developed method was investigated by testing the influence of small changes in HPLC conditions as change in flow rate (±0.05%) and change in mobile phase composition (±1%). A fixed standard concentration (112.500µg/ml picroside-I and 118.750µg/ml picroside-II) was selected for robustness study. The selected concentration was injected in triplicate, with standard HPLC conditions, with change in flow rate from standard 0.9ml/min. to 0.85 ml/min. and 0.95 ml/min. and with change in mobile phase composition from standard methanol : water (40:60, v/v) to methanol : water (39:61, v/v) and methanol : water (41:59, v/v). The % RSD of the retention time was calculated for mean value of each factor.

(iii) Testing of the developed method

The developed HPLC method was used for quantification of picroside-I and picroside-II in different market samples of drug kutki (*Picrorhiza kurroa*) procured from different markets of H.P. Well dried, finely powdered and accurately weighed samples (2gm each) were extracted and analyzed by HPLC as described above in this section.

3. RESULTS AND DISCUSSION

Method validation: Developed method was validated for the following parameters:

1) Linearity and range

The results obtained for linearity and range for both picroside-I and picroside-II presented in Table 1. Linearity of picroside-I was established for seven concentrations ranging from 3.515µg/ml – 225.000µg/ml. Regression equation obtained was linear with correlation coefficient (R) value 0.999. The regression equation derived from the linearity data was Y = 7.66e+004 X - 7.78e+004. The retention time of picroside-I was 29.913±0.344 minutes.

Table 1: Linearity data of Picroside-I and Picroside-II

Sr. No.	Phyto-constituent	Linearity range (µg/ml)	Regression equation	Correlation coefficient (R)	Retention Time (minutes)	
					Mean[a]	% RSD
1.	Picroside -I	3.515 – 225.000	Y = 7.66e+004 X - 7.78e+004	0.999	29.913±0.344	1.15
2.	Picroside -II	3.710 - 237.500	Y = 2.47e+004 X - 4.91e+004	0.999	14.828±0.157	1.06

[a]Mean ± SD (n=21)

Linearity of picroside-II was established for seven concentrations ranging from 3.710µg/ml - 237.500µg/ml. Regression equation obtained was linear with correlation coefficient (R) value 0.999. The Regression equation of the calibration curve was 2.47e+004 X - 4.91e+004. The retention time of picroside–II was 14.828±0.157 minutes. The calibration curve was constructed by plotting the mean peak area versus the concentration of each analyte.

2) Accuracy

The results showed that the recovery percentage for picroside-I ranged from 100.52± 0.756% to 101.001 ± 0.453% with RSD% ranged from 0.189 to 0.752. The recovery percentage for picroside-II ranged from 100.766 ± 0.362% to 102.595 ± 0.404% with% RSD ranged from 0.359 to 0.720%. The overall recovery percentage for picroside-I was found 100.804 ±

0.084% and for picroside-II was 101.876 ± 0.325%. The results presented in Table 2 showed that the method has good recovery as the % RSD was less than 1.

Table 2: Recovery studies of Picroside-I and Picroside-II

Phytoconstituent	Initial Quantity (µg/ml)	Added Quantity (µg/ml)	Total Quantity (µg/ml)	Recovery			Overall recovery[b] (%)
				Mean recovery [a] (µg/ml)	Mean recovery (%)	% RSD	
Picroside I	7.0312	14.063	21.093	21.305 ± 0.096	101.001 ± 0.453	0.448	100.804±0.084
	7.0312	28.125	35.156	35.339 ± 0.266	100.52 ± 0.756	0.752	
	7.0312	56.250	63.281	63.845 ± 0.121	100.89 ± 0.191	0.189	
Picroside II	7.421	14.844	22.265	22.436 ± 0.081	100.766± 0.362	0.359	101.876±0.325
	7.421	29.688	37.109	37.951 ± 0.273	102.269± 0.736	0.720	
	7.421	59.375	66.796	68.530 ± 0.270	102.595± 0.404	0.394	

[a]Mean±SD (n=3)
[b]Mean±SD (n=9)

3) Precision

The intraday precision was evaluated by analyzing same sample six times during the day. The intra-day precision evaluated on the basis of % RSD (coefficient of variation) for picroside-I was 0.48% and for picroside-II as 0.61%. The interday precision was evaluated by analyzing same sample for consecutive six days. The %RSD for interday precision for picroside-I was found as 1.85% and for picroside-II 1.37% (Table 3).

Table 3: Precision, Limit of detection and Limit of quantitation data of Picroside-I and Picroside-II

Phyto-constituent	Precision		LOD (µg/ml)	LOQ (µg/ml)
	Intra-day (%RSD)[a]	Inter-day (%RSD)[b]		
Picroside – I	0.48	1.85	0.043	0.175
Picroside - II	0.61	1.37	0.185	0.618

[a]Intra-day precision : data expressed as mean (n=6)

[b]Inter-day precision: data expressed as mean (n=6)

4) Limit of detection (LOD)

The limit of detection for picroside-I and picroside-II were found 0.043µg/ml and 0.185µg/ml respectively which has an average S/N ratio of 3 (Table 3).

5) Limit of quantititation (LOQ)

The limit of quantitation for picroside-I and picroside-II were found 0.175µg/ml and 0.618µg/ml respectively which has an average S/N ratio of 10 (Table 3).

6) Robustness

The developed method had flow rate of 0.9 ml/min. and with this flow rate picroside-I and picroside-II elutes at 29.866 minutes and 14.800 minutes. When the flow rate of mobile phase was slightly decreased to 0.85 ml/min., the elution time of picroside-I and picroside-II increased to 31.683 minutes and 15.643 minutes. With the increase in flow rate to 0.95ml/min. from 0.9ml/min., the elution time of picroside-I and picroside-II decreased to 28.526 minutes and 14.096 minutes. The %RSD for retention time of picroside-I and picroside-II was 1.795% and 1.739% respectively (Table 4).

Table 4: Robustness studies of Picroside-I and Picroside-II

Factor I - Flow rate (ml/min); Mobile phase (methanol :water::40:60, v/v)	**Picroside-I (Retention Time, minutes)[a]**	**Picroside-II (Retention Time, minutes)[a]**
0.85	31.683±0.037	15.643±0.029
0.9	29.866±0.088	14.800±0.057
0.95	28.526±0.123	14.096±0.076
Mean	30.026	14.847
S.D.[b]	0.528	0.258
% RSD	1.795	1.739
Factor II- Mobile phase (methanol :water, v/v); Flow rate (1 ml/min)		
39:61	34.196±0.088	16.600±0.041
40:60	29.866±0.088	14.800±0.057
41:59	26.850±0.028	13.470±0.017
Mean	30.304	14.957
S.D.[b]	1.231	0.524
% RSD	4.062	3.501

[a]Mean±SD (n=3)

[b]Mean±SD (n=9)

The developed method had mobile phase of (methanol : water ::40 : 60, v/v) and with this mobile phase picroside-I and picroside-II elutes at 28.866 minutes and 14.800 minutes. When the mobile phase was slightly altered to methanol : water :: 39 : 61 the elution time of picroside-I and picroside-II increased to 34.196 minutes and 16.600 minutes. With the alteration in mobile phase as methanol : water :: 41 : 59 the elution time of picroside-I and picroside-II decreased to 26.850 minutes and 13.470 minutes. The %RSD for retention time of picroside-I and picroside-II was 4.062% and 3.501% respectively (Table 4).

Testing of the developed method:

The developed method was used for quantification *Picrorhiza kurroa* rootstock samples from five different market sources cited as market-I to market-V in Table 5. The peaks of picroside-I and picroside-II were clearly identifiable, well resolved and without any fronting and tailing (Fig. 5).

Table 5: Quantification of Picroside-I and Picroside-II in market samples of *Picrorhiza kurroa*

Sr. No.	Samples Source	Picroside-I content (%)	Picroside-II Content (%)
1	Market-I	2.047±0.103	2.920±0.094
2	Market-II	1.069±0.104	2.217±0.060
3	Market-III	0.823±0.047	2.692±0.061
4	Market-IV	0.711±0.023	1.477±0.012
5	Market-V	2.737±0.041	5.885±0.017

Picroside-I content ranged from 0.711% to 2.737% and picroside-II content ranged from 1.477% to 5.885% in market samples collected from five different sources. The results are presented in Table 5 and chromatogram is presented in Fig. 5.

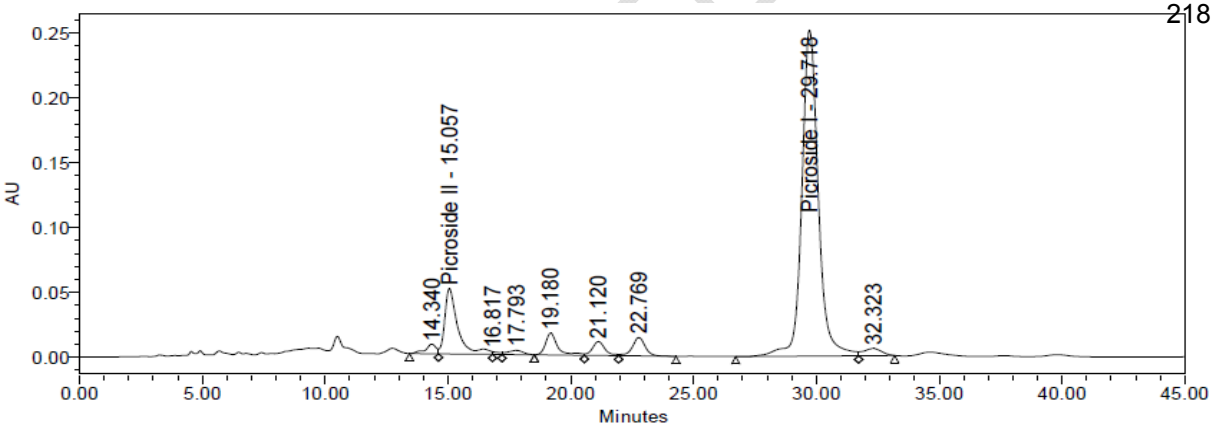


Fig. 5 Chromatogram of *Picrorhiza kurroa* samples

4. CONCLUSION

The results shows that the HPLC method presented here can be considered suitable for the analytical determination of picroside-I and picroside-II in underground part of *Picrorhiza kurroa* samples, owing to its high recovery, linearity in the concentration ranged, adequate accuracy in the concentrations studied.

References:

[1] Hooker J D. 1885. *Flora of British India*. Bishen Singh Mahendra Pal Singh Delhi.

[2] Kaul M K and Kaul K. 1996. Studies on medico- ethnobotany, diversity, domestication and utilization of *Picrorhiza kurroa* Royle *ex.* Benth. *In*: Supplement to cultivation and utilization of medicinal plants, edited by S S Handa and M K Kaul: Regional Research Laboratory. CSIR. pp. 333-348.

[3] Chettri N, Sharma E, Lama S D. 2005. Non-timber forest produces utilization, distribution and status in a trekking corridor of Sikkim, India. *Lyonia: J Eco. Appl.* **8**: 89-101.

[4] Uniyal, S K, Singh, K N, Jamwal P and Lal B. 2006. Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalaya. *J Ethnobio Ethnomed*. **2**: 2-14.

[5] Nayar M P and Sastry A P K.1990. Red Data Book of Indian Plants. Botanical Survey of India, Kolkata.

[6] Samant S S, Dhar U and Palni L M S. 1998. Medicinal plants of Himalaya: diversity distribution and potential values. Himvikas, Gyanodaya Prakashan: Nanital.

[7] Shitiz K, Pandit S, Chauhan R S and Sood H. 2013. Picrosides content in the rhizomes of *Picrorhiza kurroa* Royle *ex*. Benth. traded for herbal drugs in the markets of North India. *Int. J. Med. Arom. Plant Sc.* **3**: 226-233.

[8] Boros C A and Stermitz F R.1990. Iridoids-an updated review. *J Nat. Prod.*. **53**, 1055.

[9] Wang N, Zhao B and Shao B. 1993. Preparation and application of colchicine sensor. *Huaxue Chuanganai*. **13**: 71–74.
[10] Stuppner H and Wagner H. 1989. New cucurbitacin glycosides from *Picrorhiza kurroa*. *Planta Med.* **55**: 559- 563.

[11] Vaidya A B, Antarkar D S, Doshi JC, Bhatt A D, Ramesh V, Vora P V, Perissond D, Baxi A J and Kale P M. 1996. *Picrorhiza kourroa ex* Benth. as a hepatoprotective agent experimental and clinical studies. *J. Postgrad. Med.* **42**: 105-108.

[12] Ansari R A, Tripathi S C, Patnaik G K and Dhawan B N. 1991. Antihepatotoxic properties of Picroliv, an active fraction from the rhizome of *Picrorhiza kurroa*. *J. Ethnopharm.*. **34**:61-68.

[13] Dwivedi Y, Rastogi R, Gerg N K and Dhawan B N.1992. Effects of picroliv, the active principle of *Picrorrhiza kurroa*, on biochemical changes in rat liver poisoned by *Amanita phalloidess*. *Chung Kuo Yao Li Hsueh Pao.***13**:197-200.

[14] Billare KV, Yelne MB, Dennis TJ, Chaudhari BG.2005. Database of medicinal plants used in Ayurveda. New Delhi: Central Council for Research in Ayurveda and Siddha. p. 180–182.

[15] Bhandari P, Kumar N, Singh B, Gupta A P, Kaul V K and Ahuja P S.2009. Stability indicating LC-PDA method for determination of picrosides in hepatoprotective Indian herbal preparations of *Picrorhiza kurroa*. *Chromatographia*. **69**: 221-227.