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Chemoprofiling and tissue culture studies on *Picrorhiza kurroa* Royle ex Benth. for production of picroside II

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Abstract

Picrorhiza kurrooa Royle ex Benth. (Kutki), is an important medicinal herb, endemic to alpine Himalaya, serves as an alternative source of medicine. This paper reports results of its quality control by picroside II analysis, rapid isolation protocol for picroside by flash chromatography and potential of its *in vitro* cultures for production of secondary metabolites. High performance thin layer chromatographic (HPTLC) method was developed for estimation of picroside II in *Picrorhiza kurroa* samples collected/purchased from different places of India. The separation was achieved by using chloroform: methanol: formic acid (8:2:0.1, v/v/v) as solvent system on precoated silica gel 60 F_{254} TLC plates. The densitometric quantification of picroside II was carried out at wavelength 265 nm, giving well resolved peak of picroside II at R_f0.38± 0.2. An ultrafast flash chromatography method was developed for isolation of picroside-II from extract of *Picrorrhiza kurroa*. Chromatogram and PDA-spectra of isolated picroside R_T (10.278), was matched with spectra and chromatogram of standard picroside-II. Since the isolated picroside (purity > 80%) can be used as a bulk drug or as a potential marker for quality control analysis.The callus cultures and regenerated cultures with embryogenic calli were developed and maintained up to 220 days. *In vitro*, tissue cultured samples were tested for their potential for production of picroside, which was quantified by validated HPLC method.

Key words: *Picrorhiza kurroa* Royle ex Benth., densitometriy, HPTLC, picroside II, HPLC, flash chromatographic, *in vitro* culture

1. Introduction

Picrorhiza kurrooa Royle ex Benth. (Family: Scrophulariaceae; local/ trade name: Kutki), is an important medicinal herb, endemic to alpine Himalaya (Thakur *et al.*, 1989), is distributed between 2800-4800 m altitude.The plant is self-regenerating but unregulated over-harvesting, has caused it to be threatened to near extinction (Subedi, 2000).

The plant has been listed as an 'endangered' due to reckless collection and indiscriminate exploitationfrom its natural habitat(Rai *et al.*, 2000).Over exploitation, consequent degradation from natural habitat, narrow distribution range, small population size and high economical value were major threats for its survival (Kala, 2000). Current research on *Picrorhiza kurroa* has focused on its hepatoprotective, anticholestatic, antioxidant, and immune-modulating activity (Atal *et al*., 1986; Rastogi *et al*., 1948; Kitagawa *et al*., 1969; Weinges*et al*., 1972; Jia *et al*., 1999; Pushpangadan *et al*., 2014). *P. kurroa* is a prosperous source of hepatoprotective picrosides like picroside I, picroside II and other metabolites like picroside III, picroside IV, apocynin, androsin, catechol, kutkoside, *etc*. The roots of the plants have been used to treat disorders of the liver, chronic diarrhea, scorpion sting (Jameel *et al.*, 2005; Subramoniam, 2014) as well as bitter tonic, antiperiodic, cholagouge, stomatic, laxative in small doses and cathartic in large doses. Pharmacological evidences revealed antioxidant and antihepatotoxic effect (Jeyakumar *et al.*, 2008)due to picroside II (Gao and Zhou, 2005) and also reported for antimicrobial potential against several bacterial strains (Kumar *et al.*, 2010). There are different types of samples (collected from different places), available in herbal drug market, which were used by pharmaceutical industries for preparation and formulations which may lead to differential bioactivity if their chemical makeup is not same. Hence, chemoprofiling (HPTLC fingerprint) is essential part of quality control to check variability in bioactivity.In present investigation, we attempted to carry out HPTLC fingerprint of *P.kurroa* samples, collected from different markets of India and sample having highest picroside content was used for flash chromatographic isolation, which may prove to be a quick, cost effective and easy method for isolation of marker for academic and research purposes. An attempt has also been made to developed *in vitro* culture of *P. kurroa* as an alternative source of medicine and for *in vitro* production of picroside. The estimation of picroside II in cultures was carried out, using validated HPLC method.

2. Materials and Methods

2.1 Material

Picrorhiza kurroa Royle ex Benth. (leaves and rhizomes) collected from the plant, growing in IHBT (Institute of Himalayan

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Bioresource Technology, Palampur), identified by Dr. Anil Sood (Head, Department of Biotechnology, CSIR, IHBT Palampur), whereas fresh leaves sample for tissue culture studies were collected from HNB (High Altitude Plant Physiology Research Centre) under guidance of Professor M. C. Nautiyal, HAPPRC, Hemwati Nandan Bahuguna Garhwal University (Medicinal Plants), Srinagar, Uttaranchal. Similarly, rhizomes of *Picrorhiza kurroa* were collected from different markets in India: Rajasthan (Sri Ganganagar), Karnataka (Bangalore), Kerala, UP (Aligarh), HP (Palampur), Punjab (Amritsar), Delhi, J and K (Jammu). Samples were authenticated by comparing with the specimen available at Department of Pharmacognosy and Phytochemistry, Hamdard University, New Delhi by running TLC, using marker compounds of plant as per Indian Pharmacopeia

HPLC grade acetonitrile was purchased from Merck, India. Milli Q water was used throughout the experiment, which was prepared using Millipore water purification system.

The reference compound was purchased from Sigma-Aldrich (Bangalore, Karnataka, India) and other chemicals and reagents used were of analytical grade and procured from Merck Ltd., India.

2.2 HPTLC analysis of *Picrorhiza kurroa* rhizome collected from different places

2.2.1 Preparation of standard and sample solution

The stock solution of reference standard picroside II (99.5%) was prepared in methanol (1.0 mg mL^{-1}). The samples were prepared by weighing 5.0 g of each of dried powdered drug and macerating them separately in 100 mL of methanol for two hour, followed by sonication for 30 min. Extracts were concentrated to dryness under reduced pressure, using rotavapor and residue obtained were reconstituted in 5.0 ml of chromatographic grade methanol which, was further, used for sample analysis using HPTLC.

2.2.2 HPTLC conditions for fingerprinting and quantification

The samples were spotted in the form of band (5.0 mm) with a Camag microlitre syringe on TLC aluminium plate precoated with silica gel $60F_{254}$ (20 x 10 cm with 0.2 mm thickness, E. Merck, Germany), using a CamagLinomat V sample applicator. A constant application rate of 120 $nLsec^{-1}$ was employed and space between two bands was 6.6 mm. The slit dimension was kept at 4.0 × 0.45 mm and 20 mm sec^{-1} scanning speed was employed for scanning. When tried in different solvents and ratios but mobile phase composed of chloroform: methanol: formic acid (8:2:0.1, v/v/v) gave good resolution with compact spot of picroside II at R_f0.38±0.2. The development was carried out in linear ascending manner in twin trough glass chamber (20 x 10 cm), saturated with mobile phase, composed of chloroform:methanol: formic acid (8:2:0.1, v/v/v). The optimized chamber saturation time for mobile phase was 20 min at room temperature and the chromatogram was developed up to the length of 85 mm. Developed plate was dried by the current of hot air, and further, scanned using densitometer at 265 nm D_2 lamp in absorbance mode at 27 ± 2 ºC and 45% relative humidity for quantification whereas scanned at 254 and 366 nm for fingerprinting.

2.2.3 Calibration curve

The calibration curve was prepared by applying 0.5, 1.0, 2.0, 4.0, 5.0 and 10.0 µL of stock solution ofpicroside II in triplicate on TLC plate in order to obtain concentrations of 500,1000, 2000, 4000, 5000 and 10000 ng $spot^{-1}$. The data of peak area vs. drug concentration was treated, using linear least-square regression, and thus, equation obtained was used for quantification in samples.

2.2.4 Flash chromatographic isolation of picrosideII

Best variety of rhizome of *Picrorhiza kurroa* was chosen among rhizomes, sample collected from different places of Indian subcontinent on the basis of HPTLC analysis. Dried rhizomes of *P. kurroa* were powdered, passed through the sieve no 60-80 and 30 gm of powder was extracted, using soxhlet with ethanol (500 mL) for 5 h and then it was evaporated to dryness in rotary evaporator. The residue of ethanol extract dissolved in aqueous methanol 200 mL, (1:1,v/v), and further, partitioned successively with chloroform (100 mL) thrice. Chloroform layer was discarded and aqueous methanolic layer was partitioned thrice with ethyl acetate (100 mL). The ethyl acetate fractions were concentrated on rotary evaporator.Ethyl acetate extract (1.0 gm) was dissolved in 3mL of methanol and adsorbed on to 4.0 gm of silicagel 230-400 mesh (0.037 mm-0.063 mm). Methanol was removed by rota evaporator and the adsorbed sample was placed in an empty 4.0 g cartridge. It was then eluted on a normal phase silica column. Teledyne IscoCombiFlash companion system (35.8 cm x 43.2 cm x 61 cm (D x W x H)), consisting of dual pumping system (200 PSI Precision), UV detector,automated fraction collector pre-packed silica gel 230-400 mesh (0.037 mm-0.063 mm) and Teledyne IscoRedi*Sep* 4gm normal phase column at a flow rate 15 $mLmin^{-1}$ (mobile phase chloroform and methanol) at a wavelength 265 nm was used for separation.

2.3 Development of *in vitro* cultures

2.3.1 Surface sterilization

Surface sterilization of explants (immature leaves) were first treated with very dilute detergent solution in a conical flask and then washed with running tap water for 10 min. They were then washed with double distilled water and transferred to beakers containing varying concentration of different surface sterilizing reagents. Best surface sterilization of explants of *Picrorhiza kurroa* Royle ex. Benth. has been observed, using 0.1% mercuric chloride treatment for 5 min.

2.3.2 Initiation of callus from leaf explants

(Murashige and Skoog, 1962) basal nutrient medium (MS) has been used in the present investigation. The fresh explants (leaves) were transferred aseptically into sterilized MS media, supplemented with optimised hormonal combinations; 2,4-D(2mgL^{-1}) + IBA(0.5mgL^{-1}), 6-BA(2 mgL^{-1})+ NAA(1 mgL^{-1}), Kinetin (3 mgL^{-1}) + 6-BA(2 mgL^{-1}) and Kinetin(4 mgL^{-1}) + IAA(1 mgL^{-1}). It was kept in BOD Incubator (Yorco Delhi) under light and dark cycle (16 h light and 8 h dark) at 16 ± 2ºC with 60-70% relative humidity. The initiated calli were observed for growth up to 70 days in same media. The growth was observed with hormonal combination 2,4-D(2 mgL^{-1}) + IBA(0.5 mgL^{-1}) and 6-BA(2 mgL^{-1}) + NAA (1 mgL^{-1}) on leaf explant. Independent calli on the MS media supplemented were maintained up to 126 days, which showed growth in following order- 2,4-D (2 mgL^{-1}) + IBA(0.5 mgL^{-1}), 6-BA (2 mgL^{-1}) + Kinetin (3 mgL^{-1}) and 2,4-D (0.5 mgL^{-1}) + Kinetin (2 mgL^{-1}).

Subculturing of the developed calli on the MS media, supplemented with 6-BA (2 mgL^{-1}) + Kinetin (3 mgL^{-1})and 6-BA (2 mgL^{-1}) + NAA (1 mgL^{-1}), showed regeneration of shoots from embryogenic callus developed from leaf explant.

2.4 Development of HPLC method for analysis of picroside II in *in vitro* culture

2.4.1 Preparation of sample

Leaf callus (500 mg) each was extracted twice, using ultrasonication in methanol 7.5 mL each for 20 min; the methanolic extract obtained was filtered through whattmanfilter paper (No. 42) and evaporated to dryness under stream of nitrogen.

The natural leaves of *P. kurroa* were dried at 40°C for three hour in hot air oven and crushed in grinder and powdered. Accurately weighed 1.0 g of powdered samples were extracted twice in a conical flask, containing 15 mL of methanol. It was then filtered and washed with fresh methanol. The filtrate and washings were pooled and evaporated to dryness under the stream of nitrogen. The residue obtained from natural leaf and *in vitro culture* was reconstituted with 25 mL of HPLC grade methanol and filtered through 0.22 µm syringe filter before injecting to HPLC column.

2.4.2 HPLC instrumentation and general conditions

The HPLC analysis was carried out on a Waters Alliance e2695 separating module (Waters Co., MA and USA), using photo diode array detector (waters 2998) with autosampler and column oven. The instrument was controlled by use of Empower software installed with equipment for data collection and acquisition. Compounds were separated on a C18 reverse phase column (25 x 4.6mm, particle size 5 µm, Merck, Germany), maintained at room temperature. The mobile phase consisted of solvent A (Water) and solvent B (Acetonitrile) with the elution profile as follows: 10–20% B at 0–5 min, 20–25% B at 5–7 min, 25–30% B at 7–10 min, 30–60% B at 10–15 min, and the flow rate was 1.0 mLmin.$^{-1}$

2.4.3 Calibration curves, limit of detection and quantification

The stock solution containing 1000 µgmL^{-1}of reference compounds (picroside-II) was prepared and diluted to six appropriate concentrations and 20 µL of each solution was injected in triplicate for the construction of calibration curves. The limit of detection (LOD) and limit of quantification (LOQ) under the present chromatographic condition was determined on the basis of signal-to-noise ratio. The concentration of sample giving signal to noise ratio three was fixed as the LOD. The concentration of the sample giving signal to noise ratio ten was fixed as LOQ.

2.4.4 Accuracy as recovery

The accuracy of the method was determined by recovery studies, using standard addition method. Pre-analyzed samples were spiked with standard picroside-II at three different concentration levels *i.e.*, 50,100 and 150% and the mixtures were reanalyzed by the proposed method and for % recovery.

2.4.5 Precision

The precision of the method was carried out by doing repeatability and intermediate precision.

In repeatability, three different injections of same standard sample (three concentrations) were injected and calculated the assay. In intermediate precision, intraday, interday, and inter analyst system precisions were carried out. Intraday and interday precisions were done by preparing and applying three different concentrations of standard in triplicate three times a day and similarly on three different days, respectively.

2.4.6 Robustness of the method

Robustness of the method was determined by introducing very small changes in the analytical methodology at single concentration level (200 µgmL^{-1}). In two different ways, *i.e.*, by making deliberate change in the flow rate and by changing the detection wavelength of analysis. The % RSD of the experiment was calculated to assess the robustness of the method.

3. Results and Discussion

3.1 HPTLC chemoprofiling

The solvent system composed of chloroform: methanol: formic acid (8:2:0.1, v/v/v) gave good resolution with compact spots of components with picroside II at R_f0.38±0.2. This solvent system was used for fingerprinting and quantitative study of different samples.

The samples were applied in triplicate and developed chromatogram (Figure1) scanned at 254 nm and 366 nm. The samples showed a large variation in between different samples with respect to presence /absence of particular constituent as well as their relative abundance (Tables 1 and 2), (Figures 2 and 3).

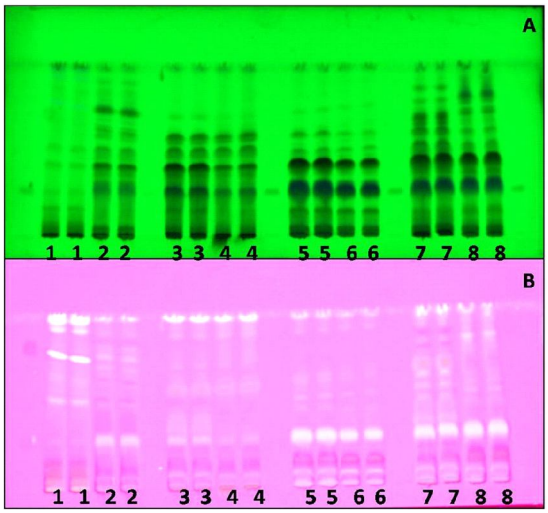

Figure 1: Developed HPTLC plate of methanol extract of various picrorhiza samples collected from 1-Rajasthan (Sri ganganagar), 2-Kerala, 3-UP (Aligarh), 4-JK (Jammu), 5-Karnataka (Banglore), 6-HP (Palampur), 7-Delhi, 8-Punjab (Amritsar) at **A**- 254 nm and **B**- 366 nm.

HPTLC fingerprinting at 254 nm:The rhizomes samples collected from Kerala, Punjab showed presence of highest number of compound (12 each), followed by sample collected from H.P. (10), while Rajasthan and Karnataka showed (9 each). The samples collected from U.P. (8), Delhi (8) and Jammu (7) showed presence of least number of metabolites.

In HPTLC fingerprinting using 254 nm, the compounds with R_f value 0.19± 0.03, 0.34± 0.03 (picroside isomers) were found common in all samples. Unlikely, compound B and E with R_f value 0.12± 0.03 and 0.42± 0.03, respectively were rarely present. Kerala and Punjab comprise of maximum number of compounds, except compound B with R_f value 0.12± 0.02, which was not present. In regions like Rajasthan and Karnataka, all compounds were present except compound B, E, G and L in Rajasthan, whereas and compound E, F, H and I in Karnataka (Table 1).

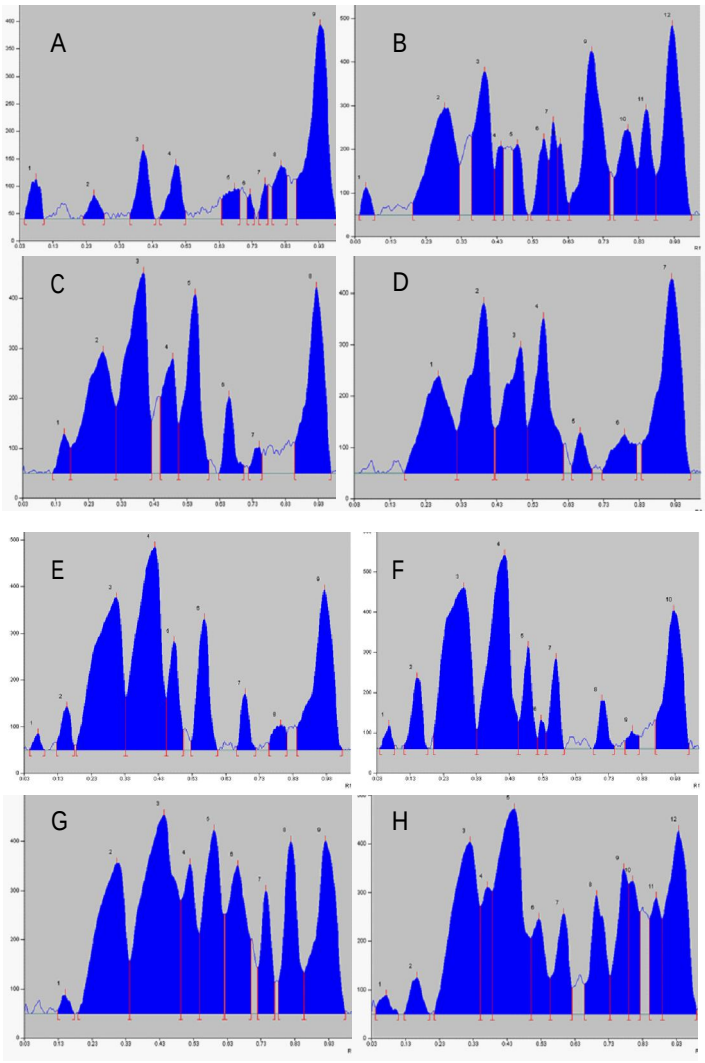

Figure 2: HPTLC chromatogram of different picrorhiza samples at 254 nm**, A**- Rajasthan (Sri ganganagar), **B**- Kerala, **C**- UP (Aligarh), **D**- JK (Jammu), **E**- Karnataka (Banglore), **F**- HP (Palampur), **G**- Delhi, **H**- Punjab (Amritsar)

Substances (R_f)	Samples							
	Rajasthan	Kerla	UP (Aligarh)	J and K (Jammu)	Karnataka (Banglore)	HP (Palampur)	Delhi	Punjab (Amritsar)
A (0.04)	+	+	-	-	+	+	-	+
B (0.12)	-	-	+	-	+	+	+	-
C (0.19)	+	+	+	+	+	+	+	+
D (0.34)	+	+	+	+	+	+	+	+
E (0.42)	-	+		+	-	-	-	+
F (0.46)	+	+	+	-	-	+	+	+
G (0.52	-	+	+	+	+	+	-	+
H (0.57)	+	+	-	-	-	+	+	+
I (0.62)	+	+	+	+	-	-	+	+
J (0.7)	+	+	+	+	+	+	-	+
K (0.76)	+	+	-	-	+	+	+	+
L (0.84)	-	+	+	+	+	-	+	+
M (0.88)	+	+	-	-	+	+	-	+

Table 1 : HPTLC fingerprint data of samples at 254 nm

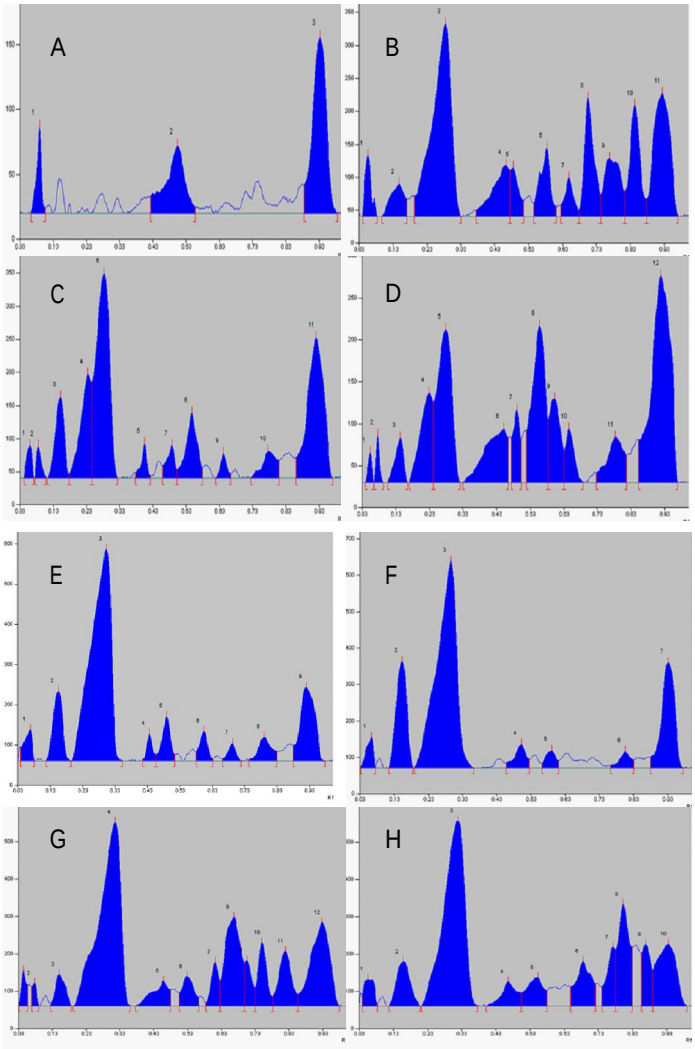

Figure 3: HPTLC chromatogram of different picrorhiza samples at 366 nm**, A**- Rajasthan (Sri ganganagar), **B**- Kerala, **C**- UP (Aligarh), **D**- JK (Jammu), **E**- Karnataka (Banglore), **F**- HP (Palampur), **G**- Delhi, **H**- Punjab (Amritsar)

Substances (R_f)	Samples							
	Rajasthan	Kerla	UP (Aligarh)	J and K (Jammu)	Karnataka (Banglore)	HP (Palampur)	Delhi	Punjab (Amritsar)
A (0.04)	+	+	+	+	+	+	+	+
B (0.12)	-	+	+	+	+	+	+	+
C (0.19)	-	+	+	+	+	-	+	+
D (0.34)	-	+	+	+	-	-	-	-
E (0.42)	+	-	-	-	+	-	-	+
F (0.46)	-	+	+	+	+	+	-	-
G (0.52	-	+	+	+	-	-	+	+
H (0.57)	-	-	-	+	+	+	+	-
I (0.62)	-	+	+	+	+	-	+	+
J (0.7)	-	+	-	+	+	-	+	+
K (0.76)	-	+	-	-	-	+	+	+
L (0.84)	-	+	-	+	-	-	+	+
M (0.88)	+	-	+	-	+	+	-	+

Table 2 : HPTLC fingerprint data of samples at 366 nm

HPTLC fingerprinting at 366 nm: The rhizomes samples collected from Punjab and Jammu showed presence of highest number of compounds (10 each), followed by sample collected from Delhi (9) and Karnataka (9) each at 366 nm analysis. The sample from UP showed 8 compound whereas sample from H.P. showed 6 compound. The least number of metabolites at 366 nm was found present in sample collected from Rajasthan (3).

In HPTLC fingerprinting using 366 nm,the compound with $R_f$value 0.4± 0.02 (picroside isomers) were found common in all samples. Regions like Punjab and Jammu contain maximum number of compound except compound D, F, H in case of Punjab and compound E, K and M in Jammu. Delhi and Karnataka samples contain all compounds except compound D, E, F, M and D, G, K and L, respectively. Similarly, in UP, sample compounds E, H, J, K and L whereas in HP sample compound C, D, E, G, I, J and L were absent. In Rajasthan, only three compounds are present, namely; A, E and M having $R_f$value0.04±0.02, 0.42±0.02, 0.88± 0.02, respectively (Table 2).

3.2 Estimation of picroside II in samples

The samples were applied in triplicate and developed chromatograms, were scanned as per the method. The developed method was found to have good linearity range (500 - 4000 ng for picroside II). Densitometric analysis of picrosideII was carried out in the absorbance mode at 265 nm. The regression equation obtained Area Y = 1944.370 + 1.735* X with r = 0.98322, was used for analysis of picroside II in samples (Table 3).

S.No.	Sample	Content % w/w in extract
1	Punjab	5.28
2	Delhi	4.59
3	Kerla	3.8
4	UP	3.86
5	Rajasthan (Sri Ganganagar)	Not quantifiable
6	Jammu	1.82
7	HP	5.66
8	Karnataka (Banglore)	6.85

[a] Standard deviation,
[b]Relative standard deviation,
n = 3, *i.e.*, results are means from three observations

Table 3 : Results of HPTLC estimation of picroside II in samples of *Picrorhiza kurroa* collected from different places of Indian subcontinent

The maximum amount of picroside II was found in sample collected from Karnataka, *i.e.* 6.85% w/w. The sample collected from HP and Punjab was having almost same amount of picroside II. Sample collected from Jammu market contains minimum amount of picroside II (1.82 % w/w), whereas samples collected from Rajasthan contain very less amount of picroside II, which have not been quantified using present method.

3.3 Flash chromatographic isolation

We investigated the chromatographic behaviour of reference compound on TLC and on normal phase ISCO combiflash silica column. Method was developed using 4.0 gm normal phase silica column, using chloroform and methanol as a mobile phase in gradient (0.0-20.2% of methanol) at $R_T$of 15 min, using wavelength of 265 nm**.** It was found that chloroform and methanol showed best separation (Figure 4).Developed method was reproducible and can be used for the isolation of compounds up to 120 gm column in one time. The purity of isolated picroside II was confirmed by TLC, HPLC and mass spectroscopy which showed its purity above 85%. A total of 5.5 % picroside II was recovered from methanolic extract which can be used as bulk drug for industrial purpose

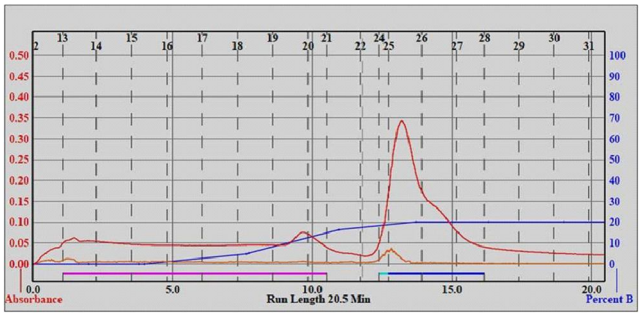

Figure 4: Description of eluted fractions from flash chromatography

3.4 Development *in vitro* culture

Initiation of callus was observed on MS basal media, supplemented with different hormonal combination. Best hormonal combination was observed as 2, 4-D (2 mgL^{-1}) + IBA(0.5 mgL^{-1}), followed by 6-BA(2 mgL^{-1})+ NAA(1 mgL^{-1}), Kinetin (3 mgL^{-1}) + 6-BA(2 mgL^{-1}) and Kinetin(4 mgL^{-1}) + IAA(1 mgL^{-1}) for leaf explants. The initiated calli were observed for growth up to 70 days in same media which shows good growth, *i.e.*,2,4-D(2 mgL^{-1}) + IBA(0.5 mgL^{-1}), 6-BA(2 mgL^{-1}) + NAA (1 mgL^{-1}), for leaf calli. Further, explant independent calli were maintained up to 126 days, which showed growth in following order: 2,4-D (2 mgL^{-1}) + IBA(0.5 mgL^{-1}), 6-BA (2 mgL^{-1}) + Kinetin (3 mgL^{-1}) and 2,4-D (0.5 mgL^{-1}) + Kinetin (2 mgL^{-1}). Sub culturing of the developed callus on the MS media, containing hormonal combination 6-BA (2 mgL^{-1}) + Kinetin (3 mgL^{-1})and 6-BA(2 mgL^{-1}) + NAA (1 mgL^{-1}), shows regeneration of shoots from embryogenic callus, developed from leaf explant. During maintenance, the subculturing of embryogenic regenerated callus (callus 2) was routinely done at every three week in same environmental condition of BOD, which is mentioned above up to 220 days (Figure 5).

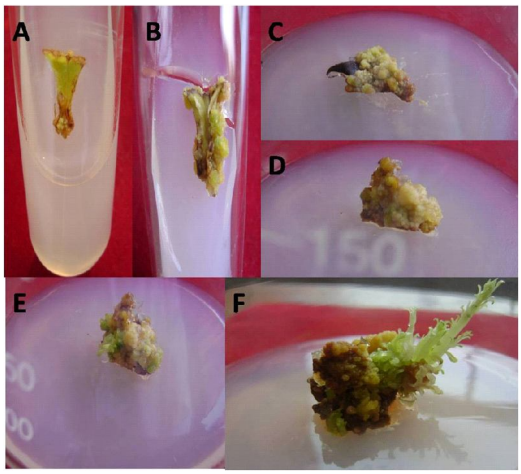

Figure 5 : Induction and establishment of callus from leaf explants of *Picrorhiza kurroa* and regeneration of microshoots from callus; **A**- Callus initiation after 35 days On MS+ 2,4-D (2 mg/L) + IBA (0.5 mg/L), **B**- Growth of callus

after 68 days on MS + Kinetin (2 mg/L) + IAA (3 mg/L), **C**- Developed callus after 91 days on MS + 6-BA + Kinetin + (2 mg/L +3 mg/L), **D**- Maintained callus after 115 days on MS + 6-BA + Kinetin + (2 mg/L+3 mg/L),**E**- 156 days old leaf callus on MS + 6-BA(2 mg/L) + Kinetin(3 mg/L) shows emergence of microshoots, **F**- 215 days old leaf callus on MS + 6-BA (2 mg/L) + Kinetin (3 mg/L) showed growth of shoots.

3.5 HPLC analysis of picroside II in *in vitro* culture

3.5.1 Optimization of mobile phase

The mobile phase was chosen after several trials with acetonitrile and water. The mobile phase consisting of acetonitrile and water in the ratio of 40:60 v/v. The standard picroside-II showed well defined peak but separation of picroside-II from the very immediate constituents was not resolved well in samples. Hence, it was decided to run a gradient elution system consisting of water and acetonitrile. Changing organic phase from 10% to 60% of the mobile phase within 15 min helped in achieving optimal separation with well defined, well resolved sharp peak in both standard and samples at R_t10.00 ± 0.28 (Figure 6).

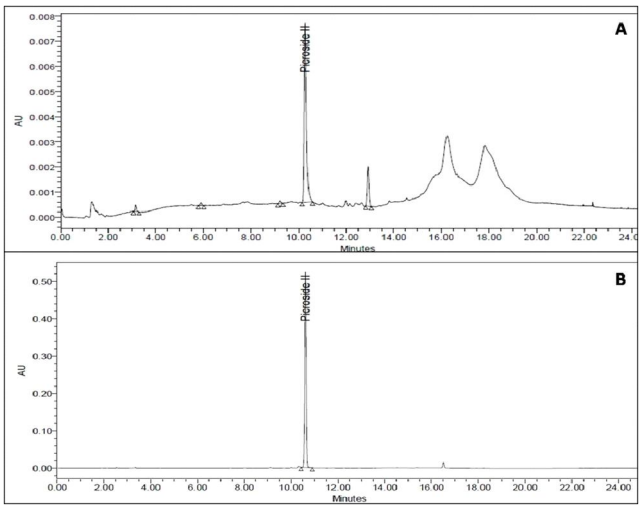

Figure 6 : HPLCchromatogram of **A**- *in vitro* culture**B**- standard picroside-II at 265 nm

3.5.2 Calibration curve

The linear regression calibration curve was plotted by using peak area against concentration and was found linear in the range of 10 µgmL^{-1} to 1000 µgmL^{-1}with a good linear relationship r^2 = 0.993 ± 0.0011.

3.5.3 Method validation

The method proposed was validated as per ICH guidelines as well as similar to the methods reported by author (Rabea *et al.*, 2010; Jha *et al.*, 2010; Singh *et al.*, 2011).

Recovery study for the proposed method was conducted by spiking previously analyzed test solution with the standard picroside-II. The recovery of the method was found in the range of 99.36 – 99.98 %. The values of % recovery, standard deviation and % RSD have been listed in (Table 4).

Parameters	Observations
Linearity range	10-1000µg/Ml
Regression equation	$y^a = 449.9x^b - 9925$
Correlation coefficient	0.993±0.0011
Slope ± SD	448.63±1.20
Intercept ± SD	9938.34±42.73

Table 4: HPLC linear regression data for calibration plot (n=3)

The repeatability and intermediate precisions were calculated and reported in terms of % RSD in Tables 5 and 6. Intermediate precision include data of intraday, interday, and inter system precision. The low values of % RSD indicate the reproducibility of the method, which can be adopted in any lab for the routine analysis of picroside-II in crude drug as well as in callus.

% of standard spiked to the sample	Theoretical content (µg/mL)	Amount of drug recovered (µg ±SD)	% of drug recovered
0	745	740.2167 ± 0.10	99.36
50	1117	1117.333 ± 1.52	99.97
100	1490	1492 ± 2.0	99.87
150	18625	18628 ± 2.64	99.98

Table 5 : Accuracy of the HPLC method (n=3)

Conc (µg/mL)	Mean peak Area ± SD	% RSD
100	38568.33 ± 1102.21	2.86
200	83010.33 ± 1232.04	1.49
500	19111 ± 112.30	0.59

Table 6: Repeatability of the HPLC method (n=3)

Robustness was studied by introducing small changes in flow rate and detection wavelength. The standard deviation and % RSD of R_T and area were calculated and the low values of the % RSD showedthe robustness of the method (Table 7).

Parameter	Tested	Mean area ± S.D	% RSD
Flow rate (mLmin^{-1})	0.8	83140.33 ± 357.4274	0.43
	1	82848.67 ± 434.4702	0.52
	1.2	82873.33 ± 1434.092	1.73
Detection wavelength	263	83070 ± 885.21	1.07
	265	82195.33 ± 1725.38	2.1
	267	82762.67 ± 885.21	0.80

Table 7 : Robustness of the HPLC method by changing flow rate as well as wavelength (concentration 200 µg/mL)

The LOD and LOQ were determined by signal to noise ratio method and found to be 3.3 µg/ ml and 9.8 µgmL,$^{-1}$respectively.

3.5.4 Analysis of picroside-II in natural leaf and *in vitro* cultures

The proposed developed and validated analytical method was applied for analysis of picroside-II in *P. kurroa* natural leaf and

in vitro cultures. The peak areas of triplicate samples were analysed by regression equation obtained from calibration plot to get the content of picroside-II in samples. It was found to contain 0.7% and 5.4% w/w of picroside-II in leaves and *in vitro* culturs callus of *P. kurroa*, respectively. It was also demonstrated that peaks of picroside-II in samples were well resolved and did not merged with any impurity or any other constituent of drug.

Recent reported protocol on callus induction and plantlet regeneration from different explants of *P. kurroa* by (Sood *et al*., 2010) and protocol on micropropagation by (Jan *et al*., 2010) were devoid of quantitative estimation of active metabolite. However, present report has been found promising for *in vitro* production and quantitative estimation of picroside by HPLC.

Similar results have also been reported for production of asciaticoside from callus culture of *Centella asiatica* (Gandi and Giri, 2013) as well as for development of regenerated shoots using MS basal media in *Bacopa monniera* (Rao *et al*., 2012). The picroside isolated and produced from *in vitro* cultures is the major and unique constituent of *P. kurroa*, and hence, it can work as a chemical marker (Rasheed *et al*., 2012) for the quality checking, detection of adulterants of crude drugs present in herbal drug market as well as for quality control and stability testing of herbal formulations containing it as an ingredient including picroliv, Liv-52, *etc*. (Rasheed *et al*., 2013; Subramoniam, 2014).

4. Conclusion

The results of analytical HPTLC method show good linearity range and recovery, which prove its application for herbaldrug analysis, where as comparable content of picroside II proved use of *in vitro* callus as an alternative source of medicine and opens an avenue for further research.The flash chromatographic method for isolation of picroside II is rapid isolation protocol and may be very useful for academic and industrial researches. A rapid, simple and reliable HPLC method has been developed for the determination of picroside-II in rhizomes, leaf and *in vitro* cultured callus, which will be add on the scientific knowledge of the field as well as can be used for determination of picroside-II in herbal and pharmaceutical formulations. The biomass generated through *in vitro* culture was found very potent for production of secondary metabolite. This protocol can be used for large scale industrial production of picroside.

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Conflict of interest

We declare that we have no conflict of interest.

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