

# Optimization of a preparative RP-HPLC method for isolation and purification of picrosides in *Picrorhiza kurroa*

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**Abstract** Preparative reversed phase high pressure liquid chromatography (prep-RP-HPLC) coupled with photodiode array (PDA) and evaporative light scattering (ELSD) detectors was employed to isolate picrosides present in *Picrorhiza kurroa* Royle ex Benth. A binary gradient method (water and acetonitrile) was optimized on Water Spherisorb S10 ODS2 20 mm × 250 mm Semiprep Column with a 20 mL/min flow rate at ambient temperature with linear binary gradient conditions; at 0 min 15 % acetonitrile hold for 15 min; 15 to 22 % acetonitrile in next 2 min, hold for 13 min; 22 to 15 % acetonitrile in 5 min hold for 5 min to equilibrate column for next injection. The picroside-I and picroside-II fractions were 98.6 and 99.7 % pure with 13.9 mg and 9.8 mg yield per 200 mg of crude extract, respectively from mature dry rhizomes. Structures of isolated iridoids were confirmed with UV scan, <sup>1</sup>H-NMR and direct infusion ESI-Q-TOF-MS/MS data.

**Keywords** Isolation · Purification · Picroside-I · Picroside-II and prep-HPLC

## Abbreviations

ELSD	Evaporative Light Scattering Detector
P-I	Picroside-I
P-II	Picroside-II
Q-TOF	Quadrupole-time of flight
ESI	Electrospray ionization
NI	Negative ionization
LCMS	Liquid chromatography–mass spectrometry
PVDF	Polyvinylidene Fluoride
MS <sup>2</sup>	Tandem mass spectrometry
CID	Collision induced dissociation
RT	Retention time

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## Introduction

*Picrorhiza kurroa* Royle ex Benth (Family: Scrophulariaceae) is an endangered herb of the North-Western Himalayan regions of India grown at altitudes of 3000–4300 m (Sood and Chauhan 2010). The herb is of a high medicinal value and used in various formulations such as Picroliv, Katuki, Arogya, Livomap and Kutaki (Bhandari et al. 2009). The plants is primarily used for its hepatoprotective effect (Saraswat et al. 1999) and also possess other pharmacological activities like anti-tumour and anti-carcinogenic (Joy et al. 2000), antioxidant (Gangola et al. 2013; Rajkumar et al. 2011; Tiwari et al. 2012), Immunomodulatory (Gupta et al. 2006), anti-allergic (Baruah et al. 1998), anti-asthmatic, scavengers of superoxide (Chander et al. 1992), antiviral (Kapadia et al. 1996; Medasani and Divi 2011) and anti-diabetic (Joy and Kuttan 1999). These medicinal activities have been implicated due to the presence of two major iridoids,

picroside-I (P-I) and picroside-II (P-II) (Fig. 1) (Ansari et al. 1991). The plant is mainly used either as a raw powder or as alcoholic extracts comprising both compounds, P-I and P-II which do have individual activities such as P-I is active against breast cancer, has stimulatory effect on P-glycoprotein ATPase activity (Najar et al. 2010; Rathee et al. 2013) and P-II is effective in cerebral ischemia, hepatocyte apoptosis liver fibrosis and anti-lipid peroxidation (Gao 2005a; Gao and Zhou 2005b; Guo et al. 2010; Guo et al. 2011; Lee et al. 2008; Lee and Ku 2008). Acylated analogs of picroside-II have been tested for immune-adjuvant activity in the presence of weak antigen ovalbumin (Chen et al. 2011; Kumar et al. 2010). Hence it is important to isolate these compounds from the plant to develop new formulations that are specific in their pharmacological action. Previously these compounds were isolated with counter-current chromatography from *Picrorhiza scrophulariiflora* but the method is tedious, time consuming and uses large amount of high quantity of solvents (Chen et al. 2011). Therefore, optimization of a rapid, time and cost effective method for isolation and purification of P-I and P-II is required.

## Experimental

### Instruments used

The Waters Preparative HPLC (2545 Binary Gradient Module, SFO, 2998 Photodiode Array Detector, 2424 Evaporative Light Scattering Detector and 2767 Sample Manager and fraction collector) controlled with MassLynx 4.0 was employed for isolation of P-I and P-II and Waters HPLC (515 pump,

717plus Autosampler, 2996 Photodiode Array Detector) controlled with Empower was used for purity analysis of fractions obtained. Waters Micromass Q-TOF MS controlled by MassLynx 4.0 and Bruker compact<sup>®</sup> Q-ToF were used for molecular mass determination and mass fragmentation. Heidolph 2 G3 Rotary evaporator was used for concentrating the crude extract and evaporation of fractions.

### Chemicals and reagents

Picroside-I and picroside-II standards were procured from ChromaDex, India. Methanol and acetonitrile solvents (HPLC Grade, Merck, India) were used for method optimization, extraction and sample preparation. Trifluoroacetic acid and acetic acid were procured from Merck, India. Milli Q Plus ultra-pure water was used in all the experiments (Millipore, Milford, MA).

### Sample

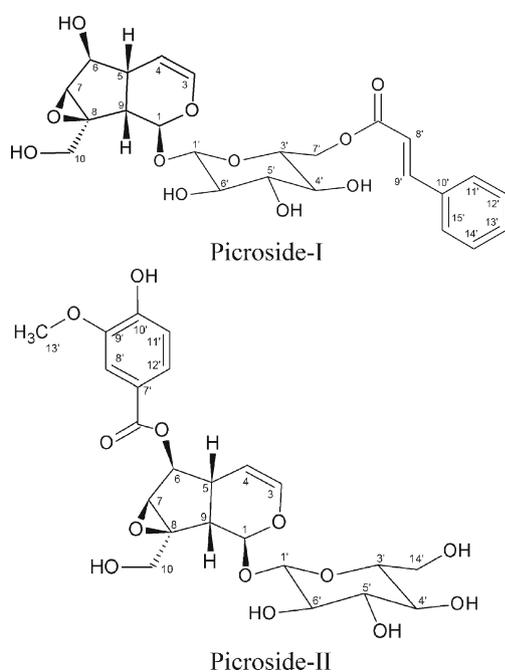
*P. kurroa* tissue samples were collected from plants grown in nursery (Sairopa, Himachal Pradesh, 4500 m altitude, 31°38' -31°54' N and 77°20'-77°45' E) and a voucher sample was submitted to HFRI (Himalayan Forest Research Institute, Shimla, HP, India) with Herbarium Acc. No. 0670.

### LC-qtof-HRMS/MS method

All analyses were acquired using internal calibration before starting each LCMS run from 0.0 to 0.7 min of each chromatogram. The internal calibration was performed using a syringe pump (kd Scientific, Holliston, MA USA.) connected to interface with divert valve, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide and 2 % formic acid in water: isopropyl alcohol 1:1 (v/v). A calibration curve based on numerous cluster masses, each differing by 68 Da (NaCHO<sub>2</sub>) was plotted. It compensates the temperature drift in the TOF with each run and provides accurate mass values for each LCMS run. LC-HRMS analysis was carried out on a reversed phase Waters Spherisorb<sup>®</sup> 5µm ODS2 (250 mm × 4.6 mm i. d.) reverse phase column with 0.8 mL/min flow rate at 35 °C column temperature. The mobile phase was 0.2 % formic acid in water and mixed with acetonitrile in 80:20 ratios. Bruker compact<sup>®</sup> Q-ToF was operated in negative ionization (NI) mode for 10 min during LC run at 4500 capillary voltage, -500 V end plate offset, 2000 V charging voltage, nebulizer at 1.5 Bar, dry heater at 200 °C, dry gas at 7.0 L/min, 10.0 source collision energy and mass scan range of m/z 80 to 1300 for MS and autoMS<sup>2</sup> with collision energy 30 up to m/z 1000.

### Preparation of crude extract

Leaves, roots and rhizomes were collected and stored in -20 °C till utilized for extraction to prevent degradation of metabolites.



**Fig. 1** Chemical structures of iridoids present in *Picrorhiza kurroa*

2 g of sample was powdered finely in liquid nitrogen with the help of a pestle mortar. The powder was transferred immediately to a 500 mL beaker; 100 mL of methanol was added and sonicated for 20 min. The mixture was shaken periodically to increase the extraction efficiency. The mixture was centrifuged at 3000 rpm to settle down the plant material. Solvent was decanted and again extracted with 100 mL of methanol; this step was repeated for 2 times. All three fractions were mixed, filtered with whatman filter paper and concentrated under reduced pressure at 30 °C up to 1/20th of the original volume. The solution was stored at 4 °C until used. The extracted sample was filtered through 0.45 µm PVDF filter (Millipore) before injecting HPLC column.

### Preparative RP-HPLC method

A binary gradient method was optimized after several isocratic and gradient trials of different compositions of mobile phase, volatile buffers trifluoroacetic acid and acetic acid but the picrosides were not stable in acidic medium hence water was used as mobile phase with acetonitrile and methanol in different compositions. For getting sharp peaks and desired resolution, methanol was replaced with acetonitrile. MilliQ water was used as a mobile phase A, the water was degassed by sonication for 5 min in ultra-sonication bath by applying vacuum on water. The filtered and degassed acetonitrile was used as mobile phase B. For achieving separation, Water Spherisorb S10 ODS2 20 × 250 mm Semiprep Column (SN. 0125111571KK00) was used as Prep HPLC Column with a 20 mL/min flow rate at ambient temperature with linear binary gradient conditions; at 0 min 15 % B hold for 15 min; 15 % to 22 % B in next 2 min, hold for 13 min; 22 to 15 % B in 5 min hold for 5 min to equilibrate column for next injection. Injector was set at 900 µL with partial loop fill with PDA detection from 200 to 400 nm UVscan and the fraction collector was set at the retention time and UV spectra of peaks obtained from chromatogram matched with that of peaks of picroside-I and picroside-II. The ELS detector was also used to see the mass ratio of these two compounds which was found to be 1.7 (Fig. 2). ELS detector conditions were optimized at 40 psi gas pressure, nebulizer heating at 90 % and

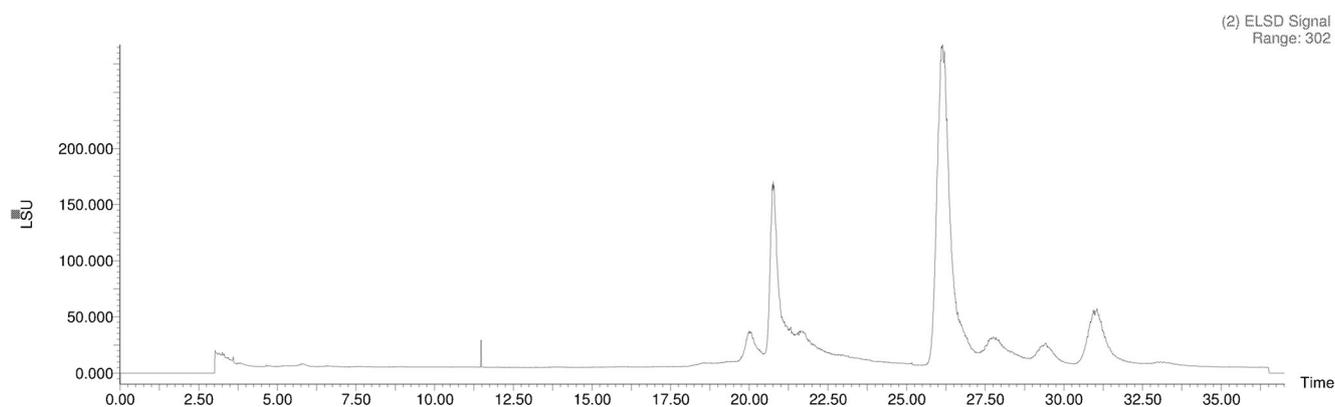
drift tube temperature  $90 \pm 25$  °C with timed event on gain at 0 min gain on 1, 3 min gain change to 100 which remained up to 36 min, again changed to 1 at 36.5 min. The time based collection of the peaks were done, fractions at 20.2–20.8 min interval for picroside-II and fraction at 25.1–25.7 min interval for picroside-I were collected at room temperature and stored at  $-20$  °C for further processing. The fractions stored at  $-20$  °C were evaporated under reduced pressure at 30 °C to evaporate organic part of fractions. The concentrated aqueous fractions were kept at  $-80$  °C for 4 h to freeze. The frozen fractions were lyophilized to get solid and purified picroside-I and picroside-II.

### HPLC analysis for purity and identification of fractions collected

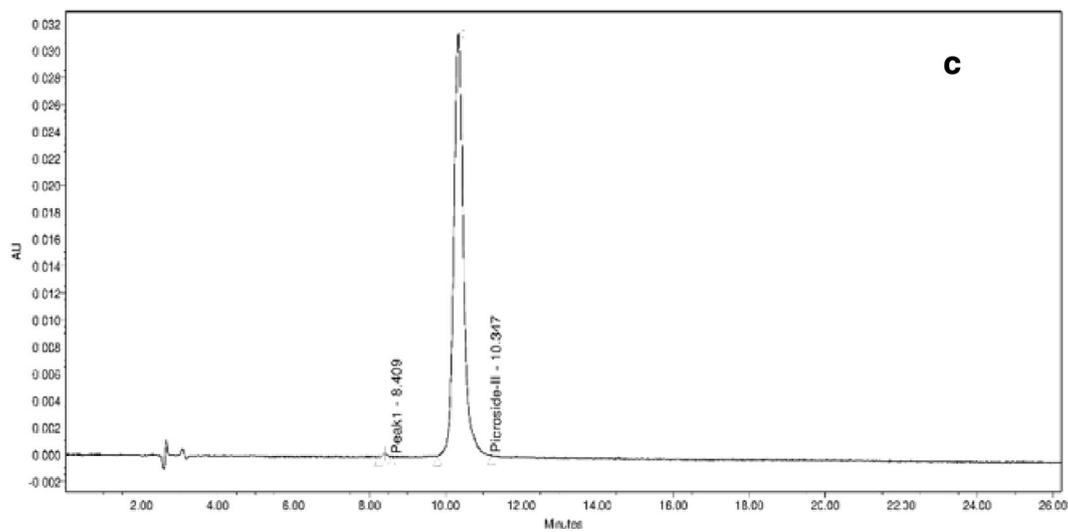
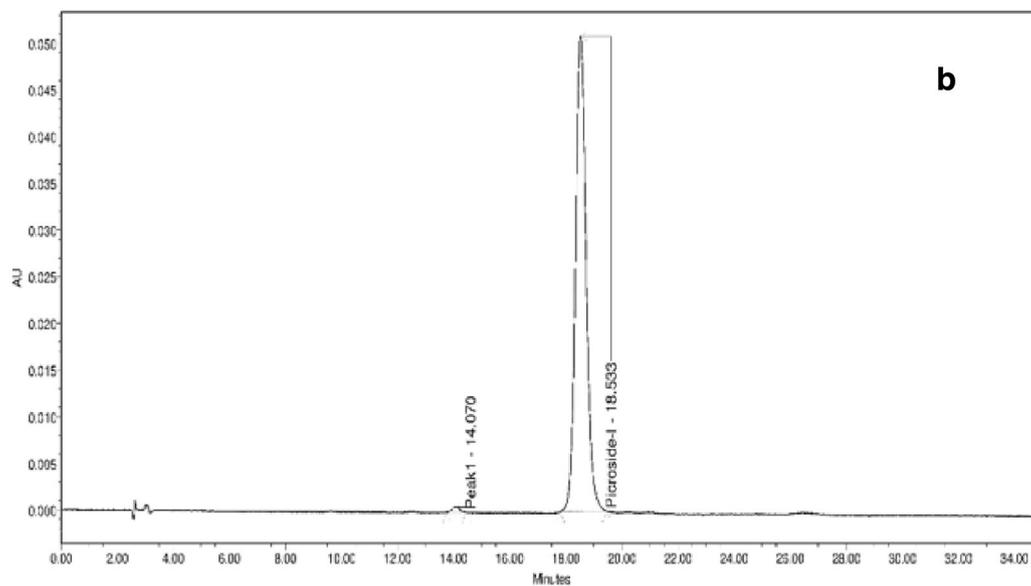
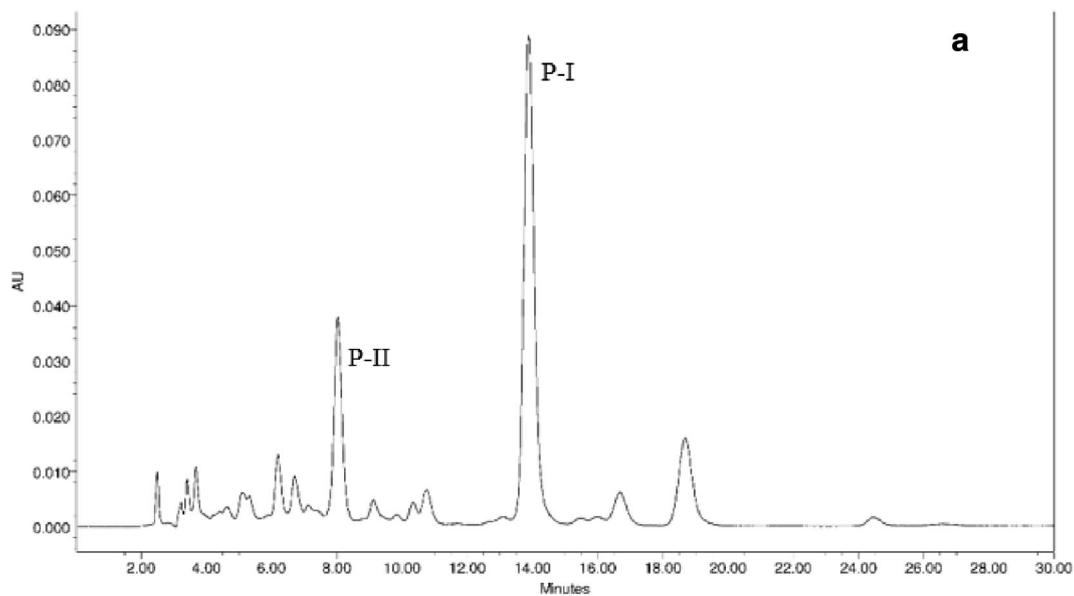
HPLC analysis was carried out on a reversed phase Waters Spherisorb® 5µmm ODS2 (250 × 4.6 mm i.d.) reverse phase column with 1 mL/min flow rate at ambient temperature. The mobile phase A was 0.05 % trifluoroacetic acid in HPLC grade water and mobile phase B were acetonitrile/methanol in 1:1 ratio. The mobile phase A and mobile phase B were used in 70:30 ratio as isocratic mode with a 1 mL/min flow rate. A 20 µL sample was injected to column maintained at 30 °C temperature and data were acquired at 280 nm UV wavelength after matching spectra and retention time with authentic sample (Pandit et al. 2013). MS and MS<sup>2</sup> Micromass Q-TOF was operated in negative ionization (NI) mode at 2731.0 capillary voltage, 30.0 sample cone voltage, 2.0 extraction cone voltage, 300 °C desolvation temperature, 100 °C source temperature, 7.0 collision energy and mass scan range of  $m/z$  100 to 1000 for MS and  $m/z$  60 to 600 for MS<sup>2</sup>.

### Results and discussion

The P-I and P-II were collectively quantified in crude methanolic extract and analysed by RP-HPLC and LC-MS to check the quality of sample (Fig. 3a). High picrosides content samples were selected and extracted with methanol. The buffers



**Fig. 2** Prep-RP-HPLC chromatogram for determination of mass ratio of P-I and P-II using ELSD



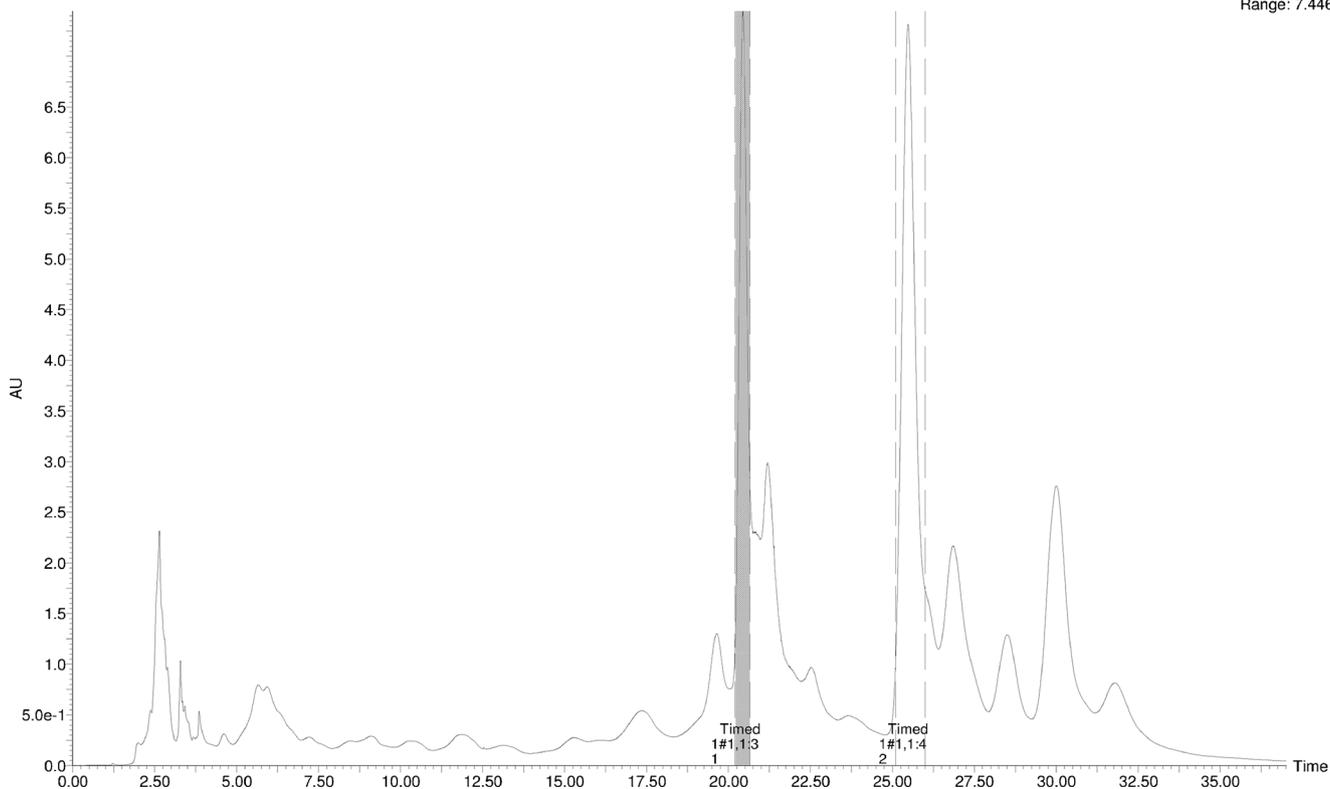
**Fig. 3** a. HPLC chromatogram of crude methanolic extract of *Picrorhiza kurroa*. b. HPLC chromatogram of fraction collected from prep-RP-HPLC between 25.1 to 25.7 min for P-I. c. HPLC chromatogram of fraction collected from prep-RP-HPLC between 20.2 to 20.8 min for P-II

used in the prep-RP-HPLC should be volatile so that in rotary evaporator and lyophilization those can be removed easily, therefore, we had a limited choice of buffers such as ammonium salts (carbonate, bicarbonate, formate and acetate etc.) and acids like formic acid, acetic acid and trifluoroacetic acid (TFA). Methanolic extract of *Picrorhiza kurroa* was analyzed by HPLC and a preliminary method was prepared after several isocratic and gradient trials with formic acid, acetic acid and trifluoroacetic acid. An isocratic method at 1 mL/min flow rate containing water/acetonitrile/TFA (70:30:0.05 %) for 30 min was finalized for short run time and better resolution. The optimized method was then transferred to the prep-RP-HPLC using volume and mass loading formulas with slight modification in flow. This gave a better chromatogram but on increasing the injection loadings, the peaks merged thus giving less purity of compounds. However, the isolated fraction of P-I was degraded, giving less pure fractions whereas the fractions of P-II were stable. It was further investigated and found that P-I was acid sensitive and degrades under mild acidic conditions whereas P-II was stable. Therefore the TFA was removed from the mobile phase and again method was optimized. After removing TFA from mobile phase the peak

was broad and gave a low resolution. Therefore, acetonitrile concentration was decreased to 15 from 30 % which gave a long run time and broad peaks. The method was changed to gradient elution program which started from 15 % acetonitrile and went up to 22 % linearly. The gradient elution program gave better peak shape and required resolution with a short run time (Fig. 4). The fraction collector was set as time based from time 20.2 to 20.8 min for P-II and 25.1 to 25.7 min for P-I. The purity of fractions were analyzed by HPLC and found to be highly pure and identified by retention time (Fig. 3b, c) with reference standard and MS<sup>2</sup> spectra by CID. The P-I pseudo molecular [M-H]<sup>-</sup> ion i.e.  $m/z$  491 gave daughter ions in MS<sup>2</sup> spectra at  $m/z$  199 [M-H-292]<sup>-</sup> by the loss of cinnamic acid and glucose moiety,  $m/z$  147 and  $m/z$  103 ions showed the presence of cinnamic acid. Daughter ion at  $m/z$  169 [M-H-292-CH<sub>2</sub>OH]<sup>-</sup> was due to the loss of CH<sub>2</sub>OH group from  $m/z$  199 ion. The MS<sup>2</sup> of mass  $m/z$  511 which corresponds to P-II gave daughter ions at  $m/z$  167 and  $m/z$  123 for vanilic acid,  $m/z$  349 due to aglycone moiety [M-H-162]<sup>-</sup>.

### Structure identification

The chemical structures of the peaks in Fig. 4 were identified according to their HR-MS and <sup>1</sup>H-NMR data. (Supplementary data).



**Fig. 4** Prep-RP-HPLC-UV chromatogram for time based fraction collection for isolation 25.1 min to 25.7 min for P-I and 20.2 to 20.8 min for P-II

Peak 1: High resolution Negative electrospray ionization (HR-ESI-Q-TOF) MS,  $m/z$  511.1463 (calc. 511.1457) [M-H]<sup>-</sup>. <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  ppm 2.59 (m, 1H, 9-H) 2.65 (d,  $J$  = 8.44 Hz, 1H, 5-H) 3.25–3.66 (m, 4H, 3'-6'-H) 3.73 (d,  $J$  = 8.27 Hz, 1H, 7-H) 3.78–3.88 (m, 2H, 14'-H) 3.91 (s, 3H, 13'-H) 4.07 (d,  $J$  = 13.57 Hz, 1H, 10-H) 4.78 (d,  $J$  = 7.70 Hz, 1H, 10-H) 4.89–5.03 (m, 1H, 1'-H) 5.03–5.18 (m, 2H, 1-H, 4-H) 6.34 (m, 1H, 6-H) 6.90 (d,  $J$  = 8.07 Hz, 1H, 3-H) 7.51 (d, 1H, 11'-H) 7.53–7.62 (d, 1H, 12'-H) 7.78 (s, 1H, 8'-H). The obtained product was identified as P-II.

Peak 2: High resolution Negative electrospray ionization (HR-ESI-Q-TOF) MS,  $m/z$  491.1556 (calc. 491.1558) [M-H]<sup>-</sup>. <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  ppm 2.17–2.30 (dd,  $J$  = 4.22, 1.65 Hz, 1H, 9-H) 2.54 (dd,  $J$  = 9.72, 7.89 Hz, 1H, 5-H) 3.25–3.58 (m, 4H, 3'-6'-H) 3.80 (d,  $J$  = 8.07 Hz, 1H, 7-H) 4.10 (d,  $J$  = 13.20 Hz, 1H, 10-H) 4.40 (dd,  $J$  = 11.92, 5.32 Hz, 1H, 6-H) 4.45–4.57 (d,  $J$  = 13.20 Hz, 1H, 10-H) 4.78 (d,  $J$  = 8.07 Hz, 1H, 7'-H) 4.84 (d,  $J$  = 9.90 Hz, 1H, 7'-H) 4.99–5.11 (m, 1H, 1'-H) 6.19–6.34 (m, 2H, 1-H, 4-H) 6.48 (d,  $J$  = 15.77 Hz, 1H, 3-H) 7.27–7.47 (m, 4H, 12'-14'-H, 7'-H) 7.57 (dd,  $J$  = 6.60, 2.93 Hz, 2H, 11'-H, 15'-H) 7.66 (d,  $J$  = 15.77 Hz, 1H, 9'-H). The obtained product was identified as P-I.

Two main bioactive iridoids (picroside-I and picroside-II) from *Picrorhiza kurroa* Royle ex Benth were successfully isolated and purified with prep-RP-HPLC using binary gradient water and acetonitrile. From 200 mg of crude methanolic extract 13.9 P-I and 9.8 mg P-II was isolated with purities of 98.6 and 99.7 %, respectively in one step separation.

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