Online HPLC-DPPH method for antioxidant activity of *Picrorhiza kurroa* Royle ex Benth. and characterization of kutkoside by Ultra-Performance LC-electrospray ionization quadrupole time-of-flight mass spectrometry

Pamita Bhandari, Neeraj Kumar, Bikram Singh* & Paramvir S Ahuja
Natural Plant Products Division, Institute of Himalayan Bioresource Technology (CSIR), Palampur, Himachal Pradesh, 176 061, India

Received 4 November 2009; revised 11 December 2009

*Picrorhiza kurroa* Royle ex Benth., is widely used in the Indian systems of medicine for the treatment of various liver ailments. Since, the role of oxidative stress in the pathogenesis of liver injury has become generally recognized, in present study the free radical scavenging effect of *P. kurroa* was assessed by on-line HPLC-DPPH and colorimetric DPPH methods. The comparative study on antioxidant activity of *P. kurroa* extracts by both methods revealed that colorimetric method showed very less free radical scavenging effect while HPLC-DPPH method showed high activity. Further, the kutkoside, an important ingredient of a potent hepatoprotective formulation “kutkin/ picroliv” was investigated for its chemical composition by ultra-performance liquid chromatography coupled with diode array detection/electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD/ESI-QTOF-MS). Kutkoside was considered to be a single compound and reported as picroside-II or kutkoside, however, present investigation illustrated that kutkoside is a mixture of iridoid glycosides namely, picroside II, picroside IV and 6-ferulloylcatalpol.

**Keywords:** Antioxidant activity, 6-Ferulloylcatalpol, *Picrorhiza kurroa*, Picroside-I, Picroside-II, Picroside-IV, Ultra-performance liquid chromatography

Excessive production of reactive oxygen species (ROS) plays an important role in the pathogenesis and progression of various diseases involving different organs. Lipid peroxidases produced from unsaturated fatty acids via free radicals cause toxic effects and promote the formation of additional free radicals in a chain reaction. If the *in vivo* activity of enzymes or scavengers is not adequate to neutralize these radicals, oxidative stress develops and leads to various diseases such as cancer, cardiovascular diseases, liver diseases, brain dysfunctions etc. The rationale for the use of antioxidants is well established in prevention and treatment of diseases where antioxidant plays a major role.

*Picrorhiza kurroa* Royle ex Benth., (Scrophulariaceae) is a small reputed alpine herb, endemic to Himalayan region of Pakistan, India, Nepal and China. In India, *P. kurroa* is distributed from Kashmir to Sikkim at altitudes ranging from 2700 to 5000 m. It is an important medicinal plant used in traditional as well as modern medicines, for the treatment of liver disorders, fever, asthma, and jaundice caused by environmental pollution, industrial toxicants, food adulteration, malnutrition, and injudicious use of drug doses, excessive consumption of alcohol and certain infections. It is also useful in gastrointestinal and urinary disorders, leukoderma, snake bite, scorpion sting and inflammatory affections. Other activities such as antiperiodic, cholagogue, stomachic, laxative, cathartic, immunomodulator and antiasthmatic have also been reported for the plant. Medicinal values mentioned above have been attributed due to the presence of the iridoid glycosides, which mainly includes picroside-I and picroside-II. More than 50 secondary metabolites have been reported from the plant which includes iridoid glycosides, cucurbitacins and phenolic compounds. The kutkin/ picroliv (an important herbal formulation) mainly, responsible for hepatoprotective activity in hepatic damage induced by galactosamine in rats and *Plasmodium berghei* (mamstoms) contains two major iridoid glycosides (50-70%) and mixture of cucurbitacins (4-5%) with

*Correspondent author
Telephone: +91-1894-230426;
Fax: +91-1894-230433
E-mail: bikram_npp@rediffmail.com
IHBT communication No. 0892
some uncharacterized minor compounds\textsuperscript{14}. Among the iridoid glycosides present in the kutkin, one iridoid glycoside was identified as picroside-I, while the identity of second iridoid glycoside is still controversial. However, it was treated as a single compound and mentioned as kutkoside\textsuperscript{15-17} or picroside-II\textsuperscript{18-20} in earlier reports.

Thus, in the present study \textit{P. kurroa} was investigated for antioxidant activity evaluated by on-line HPLC-DPPH and colorimetric methods and simultaneously, the composition of kutkoside has been studied by thin layer chromatography and ultra performance liquid chromatography coupled with diode array detector/electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-QTOF-MS).

Materials and methods

The plant material of \textit{P. kurroa} was collected during September, 2007 from the Chamba District of Himachal Pradesh, India, growing wild at an altitude of 2500-3000 m. Plant material was identified at Biodiversity Division of IHBT, Palampur. Voucher specimen was deposited in IHBT Palampur Herbarium. The rhizomes of the plants were dried, powdered and extracted with methanol: water (80:20, v/v). The combined percolations were concentrated to dryness under vacuum at 45° C.

All the solvents (methanol, acetonitrile and water) were of analytical grade obtained from J.T. Baker (USA). 1, 1-diphenyl-2-picryl hydrazyl (DPPH) and reference compounds such as gallic acid, L-ascorbic acid, picrosides-I and picroside-II were purchased from Sigma-Aldrich (Delhi, India) and Life Technologies, India.

Sample preparation—Fresh DPPH stock solution (5 ml) at a concentration of 2.5 mM/ml was prepared on each day of analysis. The stock solution of L-ascorbic acid (1 mM/ml) was prepared in methanol and stored at -20°C. The samples of \textit{P. kurroa} were prepared in methanol.

Colorimetric DPPH scavenging analysis—Free radical scavenging capacity of methanolic and ethylacetate extracts was evaluated with the DPPH stable radical. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to 0.3 ml of different extract concentrations (1-100 µg/ml) and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm against the blank, which did not contain the extract. The L-ascorbic acid was used as the positive control. The radical scavenging activity (% inhibition) was expressed as percentage of DPPH radical elimination calculated according to the following equation:-

\[
\% \text{ inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where \(A_{\text{control}}\) is the absorbance of the negative control and \(A_{\text{sample}}\) is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates and the average value was calculated. The discoloration was plotted against the sample concentration in order to calculate the IC\textsubscript{50} value, which is the amount of sample necessary to decrease the absorbance of DPPH by 50%.

HPLC-DPPH analysis—HPLC analysis was performed on a Shimadzu Prominance HPLC system, equipped with LC-20AT quaternary gradient pump, SPD-M20A diode array detector (DAD), CBM-20A communication bus module, CTO-10AS VP column oven, rheodyne injector and Shimadzu LC solution (ver. 1.21 SP1) software. The chromatography was carried out on a Zorbax Extend C-18 column (250 mm × 4.6 mm, 5 µm particle size) from Agilent at a column temperature 27°C using MeOH/water (80:20) as mobile phase in isocratic elution with flow rate 1 ml/min. The DPPH peaks were monitored at 517 nm. The difference in the reduction of peak area of DPPH (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample.

\[
\% \text{ inhibition} = \left( \frac{PA_{\text{control}} - PA_{\text{sample}}}{PA_{\text{control}}} \right) \times 100
\]

Isolation of kutkoside—Methanolic extract (15 g) of \textit{P. kurroa} was subjected to dry column chromatography over silica gel-H and sequentially eluted with n-hexane, chloroform and methanol. The fractions eluted with methanol/chloroform (5-10%) afforded picroside-I on crystallization with methanol. The fractions eluted with methanol/chloroform (15-25%) afforded kutkoside fraction which appears to be a single spot in normal phase thin layer chromatography however, it resolved into two compounds when analysed by reversed phase F\textsubscript{254} TLC (Fig. 1).

UPLC-DAD-electrospray ionization-quadrupole time-of-flight mass spectrometry—Kutkoside fraction was studied by ultra performance liquid chromatography coupled with diode array
detector/electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-DAD-ESI-QTOF-MS) in positive ion mode. The chromatography was performed on an ACQUITY UPLC System (Waters, Milford MA, USA) with diode array detector. The picrosides were separated with mobile phase consisting of acetonitrile: water (75:25, v/v) in isocratic elution on ACQUITY UPLC® BEH C-18 column (100 mm × 2.1 mm I.D., 1.7 µm particle size; Waters). The flow rate used for the analysis was 0.25 µl/min and column temperature was kept at 30°C. The samples were filtered through 0.22 µm (Millipore) filters and 0.2-0.5 µl were injected for each analysis. The diode array detector was set to an acquisition range of 200-500 nm at a spectral acquisition rate of 1.0 scan/s. The general conditions for ESI-QTOF-MS study of kutkoside fraction were: source temperature of 80°C, capillary voltage of 2.1 kV and cone voltage of 23 V. Mass spectra were acquired and accumulated over 60 s and spectra were scanned in the range between 50 and 1000 m/z. MassLynx 4.1 (Waters, Manchester, UK) was used for data analysis.

Results

Antioxidant activity—Optimization of HPLC-DPPH method for determination of DPPH was carried out by employing different compositions of mobile phase, methanol/water. Finally, 80:20 (v/v) composition of methanol/water was selected for the present study (Fig. 2). Colorimetric-UV analysis of methanol and ethylacetate extract with positive control i.e. L-ascorbic acid, showed that the scavenging effect of positive control on the DPPH radical is 95.2% at 100 µg/ml of concentration while it was very low in case of P. kurroa extracts (32.8 and 29.3% at 100 µg/ml for methanolic and ethylacetate extracts, respectively). When these extracts were analyzed by developed online HPLC-DPPH method the scavenging effect of methanol and ethylacetate extracts was increased significantly (88.3 and 84.6% at 100 µg/ml for methanolic and ethylacetate extracts, respectively). The activity of L-ascorbic acid to scavenge DPPH radical became almost stable after 80 µg/ml (Fig. 3). In contrast to positive control, there was significant difference in the IC₅₀ values of the extracts when determined by both methods. The IC₅₀ values for the methanolic and ethylacetate extracts was found relatively lower (35.6 and 32.9 µg/ml) in HPLC-DPPH method. Overall results revealed that colorimetric estimation showed very less free 

Fig. 1—LC chromatogram of picroside-I, picroside-II, kutkoside and 6-ferulloylcatalpol.

Fig. 2—LC chromatograms of (A) free; and (B) scavenged DPPH.

Fig. 3—Free radical scavenging activities of (A) methanolic; and (B) ethylacetate extracts and L-ascorbic acid (positive control), measured at 517 nm using HPLC-DPPH and colorimetric-DPPH method.
Fig. 4—LC chromatogram of (A) standard picroside-I and picroside-II; (B) kutkoside; (C) TIC chromatogram of standard picroside-I and picroside-II; and (D) TIC chromatogram of kutkoside [P-I: picroside-I; P-II: picroside-II; P-IV: picroside-IV and 6FC: 6-feruloyl catalpol].
radical scavenging effect for *P. kurroa* whereas the HPLC method had significant free radical scavenging effect Table 1. The results clearly indicated some interference in colorimetric-UV method which diminishes the antioxidant activity of the extracts. The plant extracts contain several kinds of interfering colored compounds along with antioxidant compounds which might interfere with the absorbance of DPPH in the colorimetric method hence, leading to the failure in detection of small changes in DPPH absorbance. Therefore, HPLC method proved to be superior for the detection of small changes in the DPPH absorbance reflected by peak area even in the presence of other interfering colored compounds.

**UPLC-DAD-ESI-QTOF-MS/MS characterization of kutkoside**—UPLC analysis (λ<sub>max</sub> 280) of standard picroside-II and I showed their retention at 1.48 and 2.57, respectively. The ESI-Mass spectra of standards displayed molecular ion peaks at *m/z* 513 [M+ H]⁺ for picroside-II and 493 [M+ H]⁺ for picroside-I. The UPLC analysis of kutkoside fraction displayed five peaks at RT 1.04, 1.26, 1.50, 1.98 and 2.29 min (Fig. 4). The peak observed at RT 1.50 min was identified as picroside-II by comparison of its retention time and UV spectrum with authentic reference. The mass study of kutkoside fraction with authentic picrosides (F4) further confirmed the identity of peak (RT 1.50 min) as picroside-II (m/z 513 [M+ H]⁺). Other peaks at RT 1.98 and 2.29 min showed λ<sub>max</sub> 312 and 328 nm, respectively and indicated their identity as iridoids. Mass spectra of these peaks showed protonated molecules at m/z 509 [M+ H]⁺ and 539 [M+ H]⁺ and were characterized as picroside-IV and 6-ferulloylcatalpol on the basis of earlier reports<sup>16,20</sup>. The DPPH colorimetric method for evaluation of antioxidant activity is generally recorded to have certain shortcomings like failure to indicate antioxidant activity of some plant extracts due to the interference of other colored constituents. A simple and sensitive HPLC method was developed for screening antioxidant activity of plant extracts. The method was specific for DPPH with an acceptable reproducibility and short run time allowing rapid determination of radical scavenging activity of several extract samples. The HPLC method was successfully applied for the determination of antioxidant activity of plants/formulations and can serve as a quality control tool. The present method is more specific, accurate and less time consuming. Therefore, it can directly be applied to carry out antioxidant activity of active UV sensitive constituents.

The characterization of major constituents present in the kutkoside fraction is a significant finding. In the present study, three main picrosides i.e. picroside-II, picroside-IV and 6-ferulloylcatalpol have conclusively been identified in kutkoside. Owing to the importance of ongoing research on *P. kurroa*, the findings will be very useful for commercial and academic purposes.

**Acknowledgement**

One of the authors PB is grateful to CSIR-EMR for awarding Senior Research Fellowship.

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| Table 1—Antioxidant free radical scavenging activity of standard and plant extracts  
| [Values are mean ± SE of 3 experiments]  |
| **Extracts** | DPPH method | DPPH-HPLC method |
| | DPPH (IC<sub>50</sub>) | DPPH (IC<sub>50</sub>) |
| | µg/ml | µg/ml |
| L-Ascorbic acid | 40.2 ± 0.11 | 42.3 ± 0.06 |
| Methanol *P. kurroa* | 47.4 ± 0.75 | 35.3 ± 0.66 |
| Ethylacetate *P. kurroa* | 44.5 ± 0.52 | 32.9 ± 0.45 |


