Chemical composition, antioxidant and macromolecule damage protective effects of *Picrorhiza kurroa* Royle ex Benth

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**Abstract**

In the present study, we identified the chemical constituents of 70% hydroalcoholic fraction of *Picrorhiza kurroa* by LC–ESI–MS/MS which showed the presence of iridoid glucosides such as picroside I, picroside II, picroside III, picroside IV, kutkoside, pikuroside and flavonoids like apocynin and vanillic acid. *P. kurroa* exhibited DPPH radical scavenging and metal chelating activities with IC50 of 75.16 ± 3.2 and 55.5 ± 4.8 μg/mL and also showed potent reducing power and total antioxidant activities. The extract inhibited macromolecule damage such as H2O2 induced plasmid DNA damage and AAPH induced oxidation of bovine serum albumin and lipid peroxidation of rat hepatic tissues.

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**1. Introduction**

Oxidative stress is caused due to the imbalance between reactive oxygen species (ROS) generation and antioxidant defence of the body. Increasing levels of ROS like hydroxyl radical (•OH), superoxide anion (O2•−) and hydrogen peroxide (H2O2) reduce the antioxidant levels such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) and glutathione (GSH) and further damage the cellular components like DNA, proteins and lipids (Díaz-Castro et al., 2013; Kandikattu et al., 2013). Oxidative stress is well reported in ischemia, hypoxia, Parkinson’s, Huntington’s and Alzheimer’s diseases (Halliwell, 2006; Swomley et al., 2013). Supplementation of a diet rich with antioxidant principles such as polyphenols and flavonoids can protect the cell from damage of ROS. Herbal supplements rich in flavonoids, polyphenols and terpenoids are used as a source of natural antioxidants to reduce or control symptoms associated with chronic or stress related illnesses (Saeidnia and Abdollahi, 2013). Therefore, it is worth to investigate the phytochemical constituents of herbs and their antioxidant mechanism at the cellular level.

*Picrorhiza kurroa* Royle ex Benth. (family: Scrophulariaceae) commonly known as “kutki” is a traditional medicinal herb which grows at an elevation of 3000–5000 m in the Himalayan region. *P. kurroa* includes chemical components such as picroside I, II, o-mannitol, kutkiol, kutki sterol and apocynin (Upadhyay et al., 2013). *P. kurroa* has many medicinal benefits such as immunomodulatory, anti-allergic, anti-anaphylactic and anti-neoplastic activities (Bhandari et al., 2008; Rajkumar et al., 2011). The flavonoid apocynin is one of the active metabolites of *P. kurroa* and has been reported to attenuate Parkinson’s, hypoxia and ischemia-reperfusion by its inhibitory action on NADH oxidase; expressed during oxidative stress (Wang et al., 2006; Hui-guo et al., 2010; Philippens et al., 2013).

The objectives of the present study are to identify the chemical composition of 70% ethanolic fraction of *P. kurroa* roots by LC–ESI–MS/MS and to evaluate its antioxidant, as well as its protective effects against oxidative damage of macromolecules such as DNA, protein and lipids.

**2. Materials and methods**

**2.1. Plant material**

*P. kurroa* Royle ex Benth. root material was purchased from the local market and identified by Dr. K. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara University, Tirupati, India. A voucher specimen (herbarium accession number 801) was deposited in the herbarium, Department of Botany, S.V. University, Tirupati, India.

**2.2. Preparation of 70% ethanolic fraction of P. kurroa (PKE)**

*P. kurroa* roots were washed thoroughly, shade dried and finely powdered. The root powder was macerated with 70% ethanol in a...
shaker for 2 days. The extract was filtered using micropore membrane and concentrated using flash evaporator followed by lyophilization to remove the residual water. The yield of the extract was recorded as 8%.

2.3. Chemicals and reagents

AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride), gallic acid and quercetin were purchased from Sigma (India, Bangalore), whereas FC (Folin–Ciocalteu) reagent was procured from Merck (Bangalore, India). DPPH (2,2-diphenyl-1-picrylhydrazyl) and TPTZ (2,4,6-tripyridyl-s-triazine) were procured from Hi-media (Bangalore, India) and the other chemicals used for the experiments were high quality grade and procured from SRL (Bangalore, India).

2.3.1. Metabolite analysis of P. kurroa root extract

2.3.1.1. LC–ESI–MS/MS analysis of 70% ethanolic fraction P. kurroa. LC–ESI–MS/MS analysis of 70% ethanolic fraction of P. kurroa was performed on 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC equipped with UV–Vis detector. The column was Zorbax SB C18 Rapid resolution, 4.6 × 150 mm, 3.5 µm particle size and the conditions were: (A) Formic acid (0.1% v/v) and 5 mM ammonium (B) acetonitrile gradient (in solvent B): (i) 35%, from 0 to 25 min, (ii) 90%, from 25 min, (iii) 90%, from 25 to 32 min, and (iv) 35%, from 32 min with a total run time of 40 min; flow rate: 0.25 mL/min; injection volume 1 µL; ESI parameters: Both negative and positive ion mode; mass range of 100–1200 m/z; spray voltage of 4 kV; gas temperature of 325 °C; gas flow of 10 L/min; and nebulizer of 40 psi.

2.3.1.2. Estimation of polyphenols and flavonoids. The total phenolic content was determined using FC reagent (Kujala et al., 2000). Various concentrations of extracts (20, 40, 60, 80 and 100 µg/mL) diluted in 1 mL of distilled water were mixed with FC reagent and incubated at room temperature for 10 min. After incubation, 7% Na2CO3 (2 mL) solution was added and the absorbance was recorded at 650 nm. Gallic acid was used as a standard.

The flavonoid content of the extract was determined as described by Delcourt and Varebeke (1985). To 1 mL of appropriately diluted extracts/quercetin (standard), 5 mL of chromogen reagent (0.1% cinnamaldehyde solution in a cooled mixture of 75 mL methanol and 25 mL concentrated HCl) was added. After incubation for 10 min, the absorbance of the samples was recorded at 640 nm.

2.3.2. In vitro antioxidant assays

2.3.2.1. DPPH assay. The DPPH radical scavenging assay is commonly employed to evaluate the activity of antioxidants to scavenge free radicals as described by Blois (1958). The ethanolic extract of P. kurroa was dissolved in methanol to get test solution of 1 mg/mL. Different concentrations of root extract (20, 40, 60, 80 and 100 µg/mL) were mixed with DPPH solution (500 µM) and incubated in dark for 45 min at room temperature. The control was prepared without the test compound where as BHA was used as standard. The absorbance was recorded at 515 nm and the results were expressed in terms of IC50 µg/mL.

2.3.2.2. Metal chelation. The chelating effect of extract/EDTA (standard) was estimated as described by Dinis et al. (1994). Different concentrations of diluted extracts were mixed with 5 µL of 2 mM FeCl2 and the reaction was initiated by the addition of 200 µL of ferrozine (5 mM) followed by incubation for 10 min at room temperature. The absorbance of the contents was measured at 562 nm and the results were expressed in terms of IC50 µg/mL.

2.3.2.3. Ferric reducing antioxidant power (FRAP) assay. This assay was used to evaluate the reducing capacity of P. kurroa as described by Benzie and Strain (1996). To 30 µL of extract in different concentrations (20, 40, 60, 80 and 100 µg) FeSO4 (standard), 900 µL of FRAP reagent (2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM FeCl3·6H2O and 25 mL of 300 mM acetate buffer (pH 3.6)) and 70 µL of water were added. The reaction mixture was incubated at 37 °C for 30 min. The absorbance was measured at 593 nm and the results were expressed as IC50 µg/mL.

2.3.2.4. Total antioxidant activity. Total antioxidant activity of extracts was determined using ammonium molybdate reagent, where as gallic acid was used as a standard. To 3 mL of reagent, different concentrations of plant extract were added and the final concentration was made up to 300 µL with water followed by incubation at 95 °C for 90 min. The absorbance was recorded at 695 nm and the results were expressed as gallic acid equivalents (GAE)/mg of extract (Prieto et al., 1999).

2.3.3. Macromolecule damage protective activity

2.3.3.1. Plasmid DNA nick assay. pUC19 plasmid DNA was treated with AAPH to induce DNA damage and the DNA damage inhibitory activity of P. kurroa was analyzed by agarose gel electrophoresis. Plasmid DNA (200 ng) was incubated for 1 h with 10 mM AAPH with or without different concentrations of PKE (2.5 µg, 5 µg and 10 µg) for 30 min. The DNA samples were analyzed on 1% agarose gel in TBE buffer pH: 8 (Kalita et al., 2012).

2.3.3.2. Protein oxidation. Cellular proteins are subjected to oxidative stress in the presence of a variety of ROS. In the present work bovine serum albumin (BSA) was used as a source of protein and was oxidatively challenged with AAPH which decomposes the oxygen and generates peroxyl radicals. BSA (5 µg) was dissolved in phosphate buffer (pH 7.3) and incubated in the presence or absence of P. kurroa extract for 15 min followed by 1 h treatment with 10 mM AAPH. After incubations, the protein samples were subjected to SDS-PAGE electrophoresis. The gels were stained with 0.15% coomassie brilliant blue R-250 and the amount of protein damage was quantified by measuring the density of each band using NIH Image J software (Mayo et al., 2003).

2.3.3.3. Lipid peroxidation. The quantitative estimation of lipid peroxidation products in the liver homogenate subjected to AAPH treatment was measured according to the method of Wright et al. (1981). Male wistar albino rats (3–4 month old) were sacrificed and liver tissues were collected followed by homogenization with ice-cold buffer (3 mM Tris buffer containing 250 mM sucrose and 0.1 mM EDTA pH 7.4). The reaction mixture contains 0.5 mL liver homogenate (10%, w/v), 0–100 µL extract made up to 1 mL with phosphate buffer (0.1 M, pH 7.4). To initiate the peroxidation 200 µM AAPH was added and incubated at 37 °C for 2 h. The reaction was terminated by the addition of 1.0 mL TCA (10%, w/v). To this 1.0 mL of TBA (0.67% w/v) was added and kept in boiling water for 20 min. The samples were centrifuged at 2500 × g for 10 min and the absorbance of the supernatants was recorded at 535 nm against a reagent blank. BHA was used as a standard antioxidant.

2.3.3.4. Statistical analysis. The in vitro antioxidant and free radical tests were performed in triplicate and standard deviation was expressed.

3. Results and discussion

3.1. Metabolite analysis of P. kurroa by LC–ESI–MS/MS analysis

Phytochemical analysis was carried out by LC–Q–TOF–MS/MS to determine the chemical composition of hydroalcoholic fraction of P. kurroa. The identity of compounds was confirmed by mass fragmentation analysis and the + ESI, − ESI, DAD chromatograms and MS–MS spectra of individual compounds are shown in Figs. 1 and 2. A total of
8 metabolites were identified. The chemical formulae and mass of the detected compounds are listed in Table 1.

*P. kurroa* possesses various groups of compounds. It contains iridoid glucosides like veronicoside, 6-feruloylcatalpol and minecoside (Stuppner and Wagner, 1989). The cucurbitacins were identified by Stuppner and Moller (1993). Further Baruah et al. (1998) isolated an iridoid glucoside picroliv from the rhizomes of *P. kurroa* and demonstrated its anti-anaphylactic activity. In another study Zhang et al. (2005) had identified the terpenoids from the seeds of *P. kurroa* and also demonstrated the cyclooxygenase inhibitory activity. The antimicrobial and anti-cancerous effects of kutkin, picroside I and kutkoside of *P. kurroa* were demonstrated by Rathee et al. (2012) and Rathee et al. (2013). Of late Upadhay et al. (2013) have reported the bioavailability of picrosides I and II in rat plasma.

### 3.2. Total phenolic and flavonoid content

Natural antioxidants such as polyphenols and flavonoids provide health benefits by preventing biological damage through free radical scavenging by their hydrogen donating ability (Benariba et al., 2013). In general, antioxidant activities of plants are often explained with respect to their total phenol and flavonoid contents which are considered as the most important antioxidant substances. Flavonoids known for their antioxidant activity are implicated in maintenance of health by...
various properties. Flavonoids inhibit the low density lipoprotein oxidation and impart cardio protective effects (Lecour and Lamont, 2011). The phenolic content of *P. kurroa* root ethanolic extract was 222 ± 11.4 μg GAE/mg extract, whereas the flavonoid content was 197 ± 9.8 μg quercetin equivalents/mg extract. In an earlier study Rastogi et al. (1949) and Basu et al. (1971) identified the presence of phenolic compounds such as vanillic acid and apocynin in *P. kurroa*.

### 3.3. In vitro antioxidant and free radical scavenging activity of *P. kurroa*

The effect of antioxidants on DPPH radical scavenging is determined by the hydrogen-donating ability. DPPH assay indicates that the antioxidants acting as a hydrogen donor could terminate the oxidation process by converting the free radicals to their stable forms. DPPH assay was used to determine the radical scavenging activity of *P. kurroa*. DPPH, a purple color solution, reacts with antioxidant compounds present in the test extract and is reduced to yield a light-yellow color. In the present study, the DPPH radical scavenging ability of *P. kurroa* root extract was found to be 75.16 ± 3.2 μg/mL (Table 2).

The method of metal chelating activity is based on chelating of Fe²⁺ ions by the reagent ferrozine which is a quantitative product, and forms complex with Fe²⁺ ions. In the presence of other chelating agents, the complex formation is disrupted with the result that the absorbance of the complex is decreased. Measurement of the color reduction allows estimation of the chelating activity of the coexisting chelator. The absorbance of ferrozine–Fe²⁺ complex decreased linearly in a dose dependent manner with an IC₅₀ value of 55.5 ± 4.8 μg/mL (Table 2).

The principle of FRAP assay is based on the reduction of colorless Ferr–TPTZ complex to blue colored Ferr–TPTZ complex, by action of electron donating antioxidants in biological samples (Sadeghnia et al., 2013). In this study, the capacity of the extract to reduce iron (III) to iron (II) was determined and compared to FeSO₄, which is known for its strong reducing properties and the IC₅₀ of *P. kurroa* was found to be 41 ± 2.4 μg/mL (Table 2).

### Table 1

| S. No. | RT (min) | Compound name  | Mass  | Formula          |
|--------|----------|----------------|-------|------------------|
| 1      | 2.345    | Picroside III  | 538.1521 | C₂₅H₃₀O₁₃ |
| 2      | 3.075    | Apocynin       | 166.0651 | C₉H₁₀O₃   |
| 3      | 3.6661   | Pikuroside     | 530.1596 | C₂₃H₃₀O₁₄  |
| 4      | 5.413    | Picroside IV   | 508.1772 | C₁₄H₂₄O₁₂ |
| 5      | 6.915    | Vanillic acid  | 168.0441 | C₈H₈O₄   |
| 6      | 7.342    | Picroside II   | 512.1859 | C₂₃H₂₈O₁₃  |
| 7      | 11.127   | Kutkoside      | 512.1522 | C₂₃H₂₈O₁₃  |
| 8      | 16.914   | Picroside I    | 492.1619 | C₂₄H₂₈O₁₁  |

### Table 2

| Assay                          | 70% Ethanolic Extract (PKE) (μg/mL) |
|-------------------------------|-----------------------------------|
| Total polyphenolic content    | 222 ± 11.4 μg GAE/mg               |
| Total flavonoids              | 197 ± 9.8 μg QE/mg                 |
| Total antioxidant activity     | 113 ± 7.6 μg GAE/mg                |
| FRAP                           | 41 ± 2.4 IC₅₀ (μg/ml)              |
| DPPH                          | 75.16 ± 3.2 IC₅₀ (μg/ml)           |
| Metal chelating               | 55.5 ± 4.8 IC₅₀ (μg/ml)            |
| Anti-lipid peroxidation        | 40 ± 3.8 IC₅₀ (μg/ml)              |
3.5. DNA damage protective activity of P. kurroa

The protective efficacy of P. kurroa was determined by inducing damage on the plasmid DNA using H2O2. The pUC18 plasmid DNA damaged with H2O2 showed nicked/open circular pattern (lane 2). The plasmid DNA treated with P. kurroa root extract (2.5, 5, 10 μg/mL) and gallic acid (5 μg/mL) showed significant DNA damage protective effect (Fig. 3). The observed results corroborate with our recent study which demonstrated that Cyperus rotundus inhibits H2O2 induced DNA damage (Hemanth Kumar et al., 2014).

3.6. Protein oxidation protection

The protection of the hydroxyl-mediated oxidation of BSA takes place by reducing/scavenging the peroxy radicals formed during protein oxidation (Njayou et al., 2008). These effects suggest that plant extracts are able to scavenge free radicals induced damage of cellular components. The inhibitory effect against the free radical-mediated degradation of BSA by plant extracts as mentioned above may also be attributed to the content of flavonoids and polyphenols which are known to be antioxidants. The protein oxidation bands were observed in Fig. 4. The PKE showed dose dependent inhibition of protein oxidation with 90% protection at 10 μg/mL dose compared with the oxidized BSA band (lane-2). In an earlier study Ilaiyaraja and Khanum (2011) have also demonstrated the protective effects of Tinospora cordifolia against AAPH induced oxidative damage of BSA.

3.7. Anti-lipid peroxidation

In the present study, we also investigated the lipid peroxidation activity of PKE by thiobarbituric acid method. The extract inhibited AAPH induced peroxidation of liver tissue in a dose-dependent manner with an IC50 of 40 ± 3.8 μg/mL (Table 2). Our observed lipid peroxidation inhibitory activity is in line with the recent study which also showed that P. kurroa exhibits lipid peroxidation inhibitory activity against ferric chloride induced rat liver tissue damage (Rajkumar et al., 2011).

4. Conclusion

The present study demonstrates antioxidant and protective effects of P. kurroa against oxidative damage of macromolecules such as DNA, protein and lipids. The observed effects could be due to its phytochemical components identified by LC–ESI–MS/MS and high content of polyphenols and flavonoids. However further in vitro and in vivo studies are necessary to better clarify the antioxidant activity of P. kurroa at the cellular level.

Conflict of interest

We declare that we do not have any conflict of interest.

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| GA (μg/ml) | PKE (μg/ml) |
|-----------|-------------|
| Control   | 2.5         | 5           | 10          |
| Control   | 2.5         | 5           | 10          |

Fig. 3. The protective effect of P. kurroa on H2O2 induced plasmid DNA damage analysis by agarose gel electrophoresis.

Fig. 4. (a) The protective effect of P. kurroa on AAPH induced protein oxidation of BSA analyzed by polyacrylamide gel electrophoresis. (b) The densitometric analysis of protein oxidation was analyzed by NIH Image J software.
Life Sciences, University of Hyderabad, Hyderabad for LC–ESI–MS/MS analysis.

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