Research Paper

Evaluation of Antioxidant Activity of *Picrorhiza kurroa* (Leaves) Extracts

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Picrorhiza kurroa **is a well-known herb in Ayurvedic medicine. Although it shows antioxidant, antiinflammatory and immunomodulatory activities, it is most valued for its hepatoprotective effect. The rhizomes are widely used against indigestion problems since ancient times due to improper digestive secretions. Aim of this study was to explore antioxidant study of *P. kurroa* leaves for a new source of naturally occurring antioxidants. Two pure compounds, luteolin-5-O-glucopyranoside (1) and picein (2) were isolated from butanol extract through column chromatography. Different extracts of *P. kurroa* leaves (ethanol, ethyl acetate, butanol) were quantified for isolated compound (2) by high-performance liquid chromatography. All the extracts and isolated compounds were evaluated for its antioxidant activity using two assays, 2,2-diphenyl-1-picrylhydrazyl radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay. The linear detection range was 1.56-200 μg/ml for picein. The limit of detection and limit of quantification for picein were 2.34 and 7.81 μg/ml, respectively. Butanol and ethyl acetate extract showed greater antioxidant activity as compare to ethanol extract. Compound 1 and ascorbic acid showed nearly similar antioxidant activity where as 2 showed no activity at standard concentration. The IC_{50} values for 2,2-diphenyl-1-picrylhydrazyl radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay for ascorbic acid, compound 1, ethanol extract and its different fractions (ethyl acetate and butanol) were found to be 0.81, 1.04, 67.48, 39.58, 37.12 and 2.59, 4.02, 48.36, 33.24, 29.48 μg, respectively.**

Key words: ***Picrorhiza kurroa,*** **leaves, HPLC, antioxidant activity, DPPH, ABTS**

Herbals are still used widely by world population, because of better compatibility with the human body and lesser side effects[1]. Therefore, world has now turned its attention to natural products. *Picrorhiza kurroa* Royle ex Benth. (Scrophulariaceae), is a small perennial herb found mainly in the Himalayan region growing at an elevation of 3,000-5,000 m[2,3]. The leaves of the plant are flat, oval and sharply serrated. The leaf, bark and the underground parts of the plant, mainly rhizomes are widely used in the traditional Indian systems of medicine (Ayurved) since ancient times. Although it shows antioxidant, antiinflammatory and immunomodulatory activities, it is most valued for its hepatoprotective effect. *P. kurroa* rhizomes are widely used against indigestion problems since ancient times due to improper digestive secretions[4]. The major glycoside is 'Kutkin', which is a mixture of (picroside-I and II) and possess significant hepatoprotective action[5]. The major uses of the plant are due to its hepatoprotective, anticholestatic, antioxidant, and immunomodulatory activity[6-9]. Other reported activities in the plant are against leucoderma, antiinflammatory, jaundice, fever and urinary diseases[10]. Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and free radicals, such as the hydroxyl radical ($^{\bullet}OH$) and superoxide anion (O_2^{-}), are produced as normal products of cellular metabolism. Rapid production of free radicals can act as a precursor for oxidative damage to biomolecules and may cause disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular diseases, neurodegenerative diseases, and premature aging[11]. Antioxidants have also shown their vital role in food industry to prevent deterioration and nutritional losses. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods because of their promising activity in health promotion, disease prevention, high safety, and consumer acceptability[12,13].

Previous reports on antioxidant activity of *P. kurroa* shows that root extract scavenges oxygen-free radicals, such as superoxides and hydroxyl radicals, and inhibits lipid peroxidation induced by the Fe^{2+} ascorbate system in rat liver homogenate[14].

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Medicinally, in rhizomes extracts of *P. kurroa*, antioxidant and antineoplastic activities have also been reported[15]. Comparative antioxidant activity of two different species i.e., *P. kurroa* and *P. scrophulariiflora* has also been studied[16]. Recently it has reported that the active principles of Kutkin (picroside I and II) are also present in aerial part of the plant[17]. Till date, only roots and rhizomes were explored for their activity; however, presence of picroside I and II in leaves and inflorescence strongly support that the leaves can also be an alternative of the roots and rhizomes. Scientific data on the biological activities of leaves as compare to roots and rhizomes is limited[18]. In the present study the objective was to evaluate antioxidant activity of extract, different fractions and isolated compounds of *P. kurroa* (leaves) by using 2,2'-diphenyl-l-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays.

MATERIALS AND METHODS

The fresh leaves of *P. kurroa* were dried and used in this investigation were collected in the months of August and September 2011, from Holi-Nala village, district Chamba situated at an elevation of 2800-3200 m above sea level located in the mid-hills of the Western Himalayas (Dhauladhar range). The plant material was authenticated in house by a taxonomist of the institute and voucher specimen deposited in herbarium of the CSIR-IHBT, Palampur, India (voucher # PLP 11694). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid were purchased from Sigma-Aldrich Chemie, Steinheim, Germany. HPLC grade solvents (acetonitrile and water) were purchased from J. T. Baker, USA. All other solvents and chemicals were of analytical grade and obtained from S. D. Fine-Chem. Ltd., Mumbai, India.

Extraction, fractionation and isolation:

The fresh plant material was dried at 40±5° and crushed properly. One kilogram of powdered material was extracted with ethanol:water (95:5, v/v) (1×7 l, 1×3.5 l, 6×1 l). The ethanol solutions were combined and dried in a rota evaporator at 40±5° (130 g). The crude ethanol extract (100 g) was suspended in water and successively extracted with hexane (3×250 ml), chloroform (3×250 ml), ethyl acetate (3×250 ml), and *n*-butanol (3×250 ml) and evaporation of the solvents at reduced pressure gave 6.6 g of *n*-hexane, 7.0 g of chloroform, 1.5 g of ethyl acetate and 23.2 g of *n*-butanol extract. These extracts were lyophilized and kept in the dark at +4° until tested. Isolation of compounds through column chromatography was started by using 20.0 g of extract from the mixed obtained fractions of *n*-butanol. A slurry of the extract was prepared by dissolving it in a minimum volume of methanol followed by adsorbing the extract over silica gel (mesh size 230-400, 20.0 g). The slurry of the extract was uniformly packed over dry silica gel column (mesh size 230-400, 455.0 g, 35×7.0 cm) for column chromatography. Elution of components was started through column chromatography using isocratic solvent system (ethyl acetate:chloroform: methanol:water; 15:8:2:0.5). Fractions of 100 ml each were collected in a conical flask. TLC (silica gel F_{254}) of all individual fractions were developed using solvent system (ethyl acetate:chloroform: methanol:water; 15:8:4:1; 12:8:8:2) and then viewed under UV chamber at both the wavelengths (254 and 366 nm) followed by spraying with iodine in iodine chamber and finally sprayed with vanillin sulfuric acid as visualizing reagent. Based on the TLC profile of the fractions, similar fractions were pooled and then dried in rotavapor under reduced pressure at a temperature of about 40±5°. After drying of all the fractions, pooled fractions were obtained. Fraction no. 70-80 were precipitated to obtain luteolin-5-O-glucoside (compound 1, fig 1a). Fraction no. 30-40 were again rechromatographed on RP-18 column (RP-18, 45.0 g, 25×2.0 cm) using solvent system methanol:water (2:3) to obtain picein (compound 2, fig 1b).

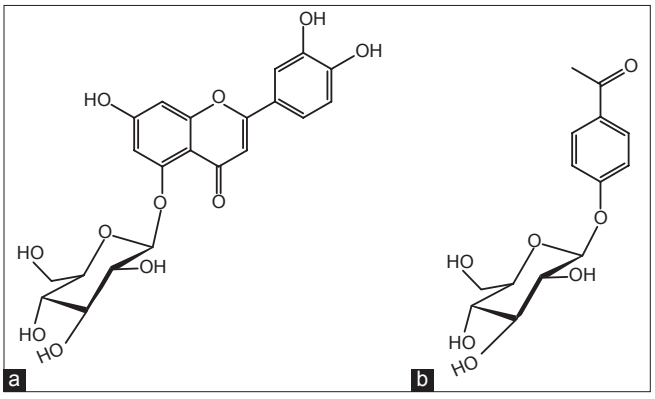

Fig. 1: Structures of isolated compounds.
Structures of isolated (a) compound 1(luteolin-5-O-glucoside) and (b) compound 2 (picein).

HPLC analysis:

For HPLC analysis, isolated compound (2), picein has been used as a standard. One milligram extracts and picein standard were dissolved in 5 ml of acetonitrile:water (10:90%, v/v) and filtered through 0.45 µm (millipore) filters prior to injection into HPLC. HPLC analysis was performed with a Waters HPLC system equipped with 600 quaternary gradient pump, (7725i Rheodyne injector) Waters 717 plus autosampler, 996 PDA detector, and Empower 2 software (version-4.01). The temperature of the column was set at 30±1°. Elution of standards and samples (20 µl) was performed. The mobile phase was acetonitrile:water (10:90%, v/v). The flow rate was 1 ml/min, the run time 20 min, and the detection wavelength was set at 254 nm. Identification of compounds was performed on the basis of the retention time, coinjections and spectral matching with standard. A series of standard solutions of picein were prepared to obtain solutions with final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 µg/ml. Calibration curve for picein was constructed. Triplicate injections were made for each concentration. The linearity of standard curve was confirmed by plotting the peak area versus the concentration. The correlation coefficient and the regression equation are shown in Table 1.

Evaluation of antioxidant activity:

Radical scavenging activity of extract, fractions and purified compounds against stable DPPH was determined by DPPH assay. The effects of antioxidants in the DPPH radical scavenging test reflect the hydrogen donating capacity of a compound. When the radical form of DPPH is scavenged by an antioxidant through the donation of hydrogen to form a stable DPPH molecule, this leads to a color change from purple to yellow, and a decrease in absorbance was measured at 517 nm[19]. The radical scavenging activity of extracts was measured by method with slight modifications[16]. Stock solution (1 mg/ml) of the fractions (ethanol, ethyl acetate, and butanol), isolated compounds (1 and 2) and the standard ascorbic acid were prepared in methanol (for DPPH assay) and ethanol (for ABTS assay), respectively. An amount of 2.5, 5.0, 7.5, and 10.0 µg/ml ascorbic acid and isolated compounds (1 and 2) respective and 40, 80, 120, 160, 200, and 240 µg/ml butanol, ethyl acetate fractions and ethanol extract respective aliquots were taken in test tubes and measuring 300 µl (0.75, 1.5, 2.25, and 3.0 µg standard, isolated compounds (1 and 2) and 12, 24, 36, 48, 60, and 72 µg extract and fractions, respectively) were added to 2 ml of the 0.100 *m*M DPPH solution prepared in methanol. The mixture was shaken vigorously, allowed to stand at 25° in the dark for 30 min and decrease in absorbance of the resulting solution was monitored at 517 nm (Shimadzu 2450, Japan) against a blank consisted of 300 µl of methanol and 2 ml of DPPH solution.

All measurements were done in triplicate. Inhibition of free radical DPPH in percent *I* (%) was calculated using the formula, $I\%=(A_b-A_s/A_b)\times100$, where, A_b is the absorbance of the control reaction and A_s is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample.

$ABTS^{\bullet+}$ scavenging activity was carried out with a slight modification from earlier reported method[20]. The radical cation was prepared by reacting 7 mM aqueous ABTS with 2.45 mM potassium persulfate and the mixture was allow to stand in the dark at room temperature for 16 h before use, by which the ABTS turned blue green. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700±0.020 at 734 nm. An amount of 40, 60, 80, and 100 µg/ml (standard and the isolated compounds 1 and 2) and 200, 400, 600, 800, and 1000 µg/ml of ethanol extract, its different fractions aliquots were taken in test tubes. Measuring 50 µl each (2.0, 3.0, 4.0, and 5.0 µg standard and compounds and 10, 20, 30, 40 and 50 µg extract and fractions) were added to 2.0 ml of ethanol diluted $ABTS^{\bullet+}$ solution. The absorbance was recorded

TABLE 1: SUMMARY OF HPLC METHOD FOR PICEIN

Compound regression equation	Correlation coefficient (R^2)	LOD (µg/ml)	LOQ (µg/ml)
Picein y=306436x-57760	0.9999	2.34	7.81

LOD=Limit of detection, LOQ=Limit of quantitation.

TABLE 2: IC_{50} VALUES OF STANDARD, COMPOUNDS (1 AND 2) AND DIFFERENT FRACTIONS OF *P. KURROA* LEAVES IN DPPH AND ABTS ASSAYS

Picrorhiza kurroa (leaves fractions)	IC_{50} value (µg) (DPPH)	IC_{50} value (µg) (ABTS)
Ethanol	67.48	48.36
Ethyl Acetate	39.58	33.24
Butanol	37.12	29.48
Luteolin-5-O-glucopyranoside (1)	1.04	4.02
Picein (2)	NA*	NA*
Ascorbic acid	0.81	2.59

*Not active up to the concentration of 3.0 µg for DPPH activity and 5.0 µg for ABTS activity, DPPH=2,2-diphenyl-1-picrylhydrazyl radical, ABTS=2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

after 4 min. The IC_{50} and percentage inhibition of absorbance at 734 nm calculated. All measurements were done in triplicate. Inhibition of $ABTS^{\bullet+}$ in percent I (%) was calculated as per the above equation.

RESULTS

The structures of the isolated compounds were determined as luteolin-5-O-glucoside (compound 1, fig 1) and picein (compound 2, fig 1) with the help of NMR data, LC-MS/MS analysis and also get compared with spectroscopic data of literature[21,22]. The content of picein in the different extracts of *P. kurroa* was quantified by HPLC method. The optimum HPLC separation of picein was achieved using acetonitrile:water (10:90%, v/v). The HPLC chromatogram obtained is shown in fig 2. The retention time was 6.5 min for picein. Linearity was confirmed by construction of a calibration curve. For this curve, standard solutions were prepared at eight concentrations, and chromatograms were recorded. The correlation coefficient obtained for picein was 0.9999 (Table 1). The linear range was 1.56-200 µg/ml for picein. The limits of detection and limits of quantification were 2.34 and 7.81 µg/ml, respectively. The picein content was determined to be 20.09, 10.68 and 10.63% in butanol, ethyl acetate and ethanol extract, respectively. The total antioxidant activity of *P. kurroa* leaves extract was evaluated by two tests, DPPH and $ABTS^{\bullet+}$ free radical. Their ability to scavenge those free radicals at different concentrations was analyzed. The percentage inhibition of absorbance was calculated and plotted as a function of concentration of the extract and of standard. The results of IC_{50} of DPPH and ABTS radical scavenging activity assays are shown in Table 2. The antioxidant capacity was expressed as IC_{50}, which is the concentration of an antioxidant needed to trap 50% of DPPH and ABTS absorbance. Consequently, a low IC_{50} value indicates a high antioxidant capacity. The antioxidant activity of parent extract, different fractions and isolated compounds of *P. kurroa* was determined by comparing the IC_{50} values evaluated by DPPH and $ABTS^{\bullet+}$ assays. The IC_{50} values of ascorbic acid, compound 1, butanol, ethyl acetate fractions and ethanol extract determined by DPPH assay were 0.81, 1.04, 37.12, 39.58, and 67.48 µg for 2.0 ml of 0.100 mM DPPH solution, respectively. The results demonstrated that fractions showed less antioxidant activity as compare to the standard (ascorbic acid) and isolated compound 1. Butanol fraction of *P. kurroa* showed maximum activity against DPPH and $ABTS^{\bullet+}$ free radical followed by ethyl acetate and ethanol fractions. The IC_{50} values of ascorbic acid, compound 1, butanol, ethyl acetate fractions and ethanol extract determined

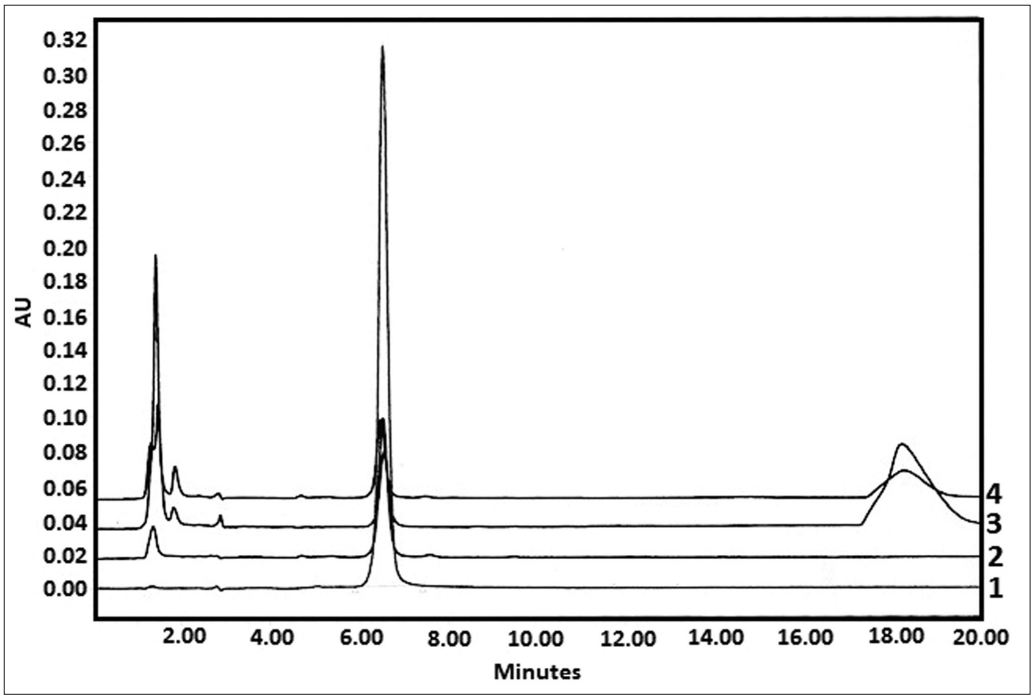

Fig. 2: HPLC chromatogram of different extracts of *P. kurroa* leaves.
The chromatogram tracks represent, 1. picein standard, 2. butanol extract, 3. ethyl acetate extract and 4. ethanol extract.

by ABTS assay were 2.59, 4.02, 29.48, 33.24 and 48.36 µg for diluted ABTS (A=0.700±0.020) solution, respectively. However, isolated compound 2 (picein) was lacking with significant antioxidant activity in both assays. Both DPPH and ABTS assays showed nearly identical antioxidant potential of fractions (figs 3 and 4).

DISCUSSION

Natural antioxidants have gained lot of interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with reduction of cardiac and other disorders with no side effects. A number of studies are going on throughout the world to identify pharmacologically potent source of antioxidant compounds. It is well known that *P. kurroa* is an important herb and largely used in Ayurveda for skin disorders, fever, burning sensation, respiratory diseases, hepatitis and anorexia[23]. The present study employed two different (DPPH and ABTS assays) antioxidant testing systems to confirm the antioxidant potentials of the leaves fractions of *P. kurroa*.

The $ABTS^{\bullet+}$ and the DPPH assays are widely used for formation of stable chromogen compounds which depicts the promising antioxidant activity. The isolated compound 1 (luteolin-5-O-glucoside), butanol, ethyl acetate fractions, and ethanol extract were able to scavenge DPPH and $ABTS^{\bullet+}$ free radicals. The ethanol extract, however, was less efficient in the scavenging of the radicals as compare to ethyl acetate and butanol fractions. The IC_{50} values of isolated compound 1, butanol, ethyl acetate fractions, and ethanol extract showed that isolated compound 1 and butanol fraction were better scavenger of $^{\bullet}OH$ radical than ethyl acetate fraction and ethanol extract. Although the chemical assays are commonly used as they are technically simple and give accurate and repeatable results. However, there are limitations on antioxidant activity assessed by chemical assays as few compounds may interfere with free radical scavenger's activity by causing alteration in some parameters such as thermodynamic, absorption spectra and steric accessibility[24]. This study in general conclude the preliminary antioxidant activity of leaves of *P. kurroa*. Currently, a lot off reports on the antioxidant activity of *P. kurroa* rhizomes has been available in the literature. Some paper reported the antioxidant activity (IC_{50}) of methanol extract and ethyl acetate extract of *P. kurroa* rhizomes assessed by DPPH method as 47.4±0.75 µg/ml and 44.5±0.52 µg/ml, respectively[25]. Another report

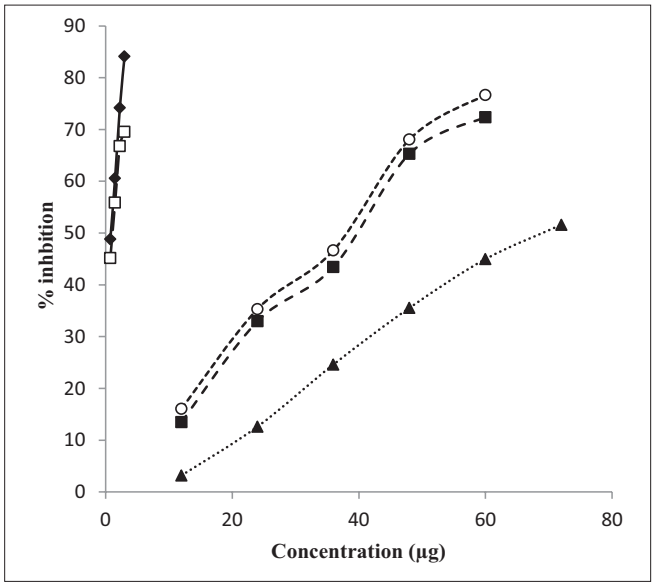

Fig. 3: DPPH assay.
Comparative scavenging of DPPH radical by ascorbic acid, luteolin-5-O-glucoside (compound 1) and different fractions (parent ethanol, ethyl acetate and butanol) of *P. kurroa* leaves.
—◆— % inhibition ascorbic acid, —□— % inhibition luteolin-5-O-glucoside, ····▲···· % inhibition ethanol extract, – –■– – % inhibition ethyl acetate extract, – –○– – % inhibition butanol extract.

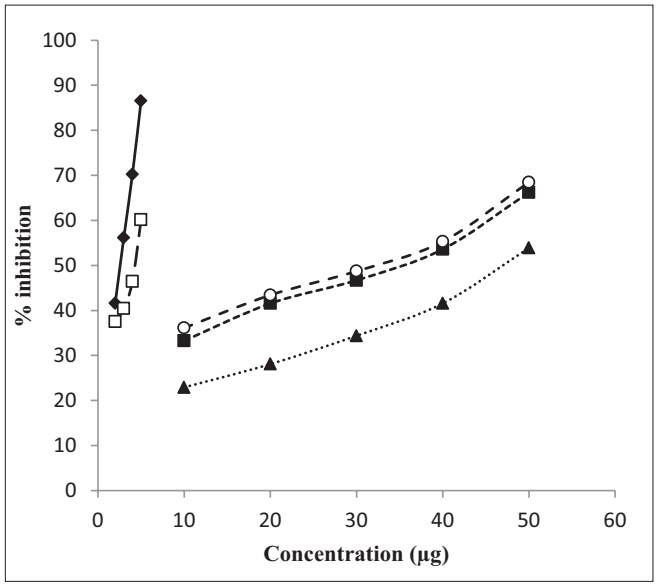

Fig. 4: ABTS assay.
Comparative scavenging of ABTS radical cation by ascorbic acid, luteolin-5-O-glucoside (compound 1), and different fractions (parent ethanol, ethyl acetate and butanol) of *P. kurroa* leaves.
—◆— % inhibition ascorbic acid, —□— % inhibition luteolin-5-O-glucoside, ····▲···· % inhibition ethanol extract, – –■– – % inhibition ethyl acetate extract, – –○– – % inhibition butanol extract.

on antioxidant activity (IC_{50}) of ethanol extract of *P. kurroa* rhizomes assessed by nitric oxide scavenging method as 206.69 µg/ml[26]. Comparison of the IC_{50} values for DPPH assay for ethanol extract of leaves (33.74 µg/ml) and ethyl acetate fraction (19.79 µg/ml) with reported literature values for *P. kurroa* rhizome's extract[25] showed that observed antioxidant activity for leaves is better than *P. kurroa* rhizomes antioxidant activity.

Antioxidant and radical-scavenging activity of parent extract, fractions, and isolated compound of *P. kurroa* leaves indicate its role toward various oxidative stress related diseases, as a food supplement and source of natural antioxidants. This study discloses that the isolated compound 1, butanol, and ethyl acetate fractions found to be promising with antioxidant potential as compared to parent ethanol extract. Thus, isolated molecule and fractions can be considered for further detailed pharmacological studies to develop a new natural product for the treatment of oxidative stress-related diseases.

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