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Comparative Evaluation of Phytochemical and *in vitro* Antioxidant Activities of two Endangered Plant Species of Western Himalayas

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ABSTRACT: Present study deals with the quantitative evaluation of phytochemical and antioxidant activities of two endangered plant species, i.e. *Rauwolfia serpentina* (RS) and *Picrorhiza kurroa* (PC) of Western Himalayas. The leaf samples of both the plants were collected from wild or natural (W) and field grown (FG) habitats. The phytochemical study showed the presence of different phytochemicals such as total phenols, terpenoids, flavanoids and reducing sugars, whereas alkaloids, steroids and saponins were absent in both plant extracts taken from wild and field grown habitats. Tannins were only present in field grown and wild variety of *Picrorhiza kurroa*. The total phenolic content was found to be low in field grown variety of RS i.e. 102±0.42 GAE mg/g and highest in the wild variety of PC i.e. 181±0.52 GAE mg/g. Similarly, the total flavanoid content was found to be low in field grown variety of RS i.e. 12.6±0.34 RU mg/g and highest in the wild variety of PC i.e. 65±0.31 RU mg/g. In DPPH assay, the IC50 value was found to be high in acetone leaf extract of PC (W) i.e. 56.8µg/ml. In Nitric oxide radical scavenging assay, the IC50 value was found to be high in acetone leaf extract of PC (W) 50.1µg/ml. The extracts showed good FRAP activity with the increase in concentration. The highest values of FRAP were observed in the leaf extract of PC (W) i.e. 0.536 ± 0.045 respectively. Results revealed that the acetone leaf extract of *Picrorhiza kurroa* possesses better antioxidant activity than *Rauwolfia serpentina* and wild varieties of both plants exhibited good phytochemical as well as antioxidant properties than field grown varieties.

Keywords: *Rauwolfia serpentina, Picrorhiza kurroa*, IC50 value, DPPH assay, FRAP assay

INTRODUCTION

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. This knowledge is accessible from thousands of medical texts and manuscripts (Pratibha *et al*., 2013). These medicinal plants are an important source of phytochemicals that offer traditional medicinal treatment of various ailments (Maobe *et al*., 2012). Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties (Ahmed *et al*., 2010). Plants produce these chemicals to protect itself, but research demonstrates that many phytochemicals can protect humans against diseases (Kubmarawa *et al*., 2008). There are many phytochemicals in herbs and each works differently. These phytochemicals have various health benefits such as antioxidant, anti-microbial, anti-inflammatory, cancer preventive, anti-diabetic and antihypertensive properties (Rupasinghe *et al*., 2003). Rather than phytochemicals, much attention has been devoted to the natural antioxidant (Arnous *et al.,* 2001). There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body and to prevent the deterioration of fats and other constituents of foodstuffs. Moreover, it was also concluded in the few years that medicinal plant species are increasingly under threat day by day. At present, it is feared that 15.20 percent of the total vascular flora of India (over 3,000 species) may fall under one of the IUCN categories of threatened, rare or endangered. While a comprehensive analysis of the status of medicinal plant species has never been carried out, it is estimated that approximately one third of the plant species listed in the Red Data Book of India, may have medicinal properties. Together with increasing populations, increased demands for crude drugs and lack of a comprehensive knowledge base, the continued availability of plant material from the wild cannot be scientifically or practically assured (Mazid *et al*., 2012).

Rauwolfia serpentine is an important medicinal plant, commonly known as Sarapgandha or it is also known as Indian snake root due to its high antivenom activity. It is a medicinally famous herb in Western and ayurvedic system. Moreover, the International union of conservation of nature and natural resources (IUCN) has assigned endangered status to *Rauwolfia serpentina* (Deshmukh *et al*., 2012). In India, it is widely distributed in the sub-Himalayan tract from Punjab to Nepal, Sikkim and Bhutan and Himalayan range. The generic name of *Picrorhiza kurroa* is derived from the bitter root, which is used in native medicine. The specific name is derived from "Karu", the Punjabi name of the plant, which means bitter (Coventry, 1927). It is listed as an 'endangered' herb due to reckless collection from its natural habitat (Dhar *et al*., 1983). It is found in the North-Western Himalayan region from Kashmir to Kumaun and Garhwal regions in India and Nepal (Sharma *et al.,* 2012).

MATERIAL AND METHODS

In the present study leaf samples of field grown *Rauwolfia serpentina* and *Picrorhiza kurroa* were collected from Dr. Y.S. Parmar University of Horticulture and Forestry, Solan, HP, India and wild variety of *Rauwolfia serpentine* leaves was collected from the forest area of Paonta Sahib, HP, whereas the wild variety of *Picrorhiza kurroa* leaves were collected from the upper area of Choodhar, Solan, HP, India. Acetone extracts of these plant samples were prepared by using soxhlet extraction.

A. Qualitative estimation of phytochemicals

The plant extracts were screened for the presence of reducing sugars, alkaloids, saponnin, tannins, flavonoids, anthraquinones, phlobatannin, steroids, terpenoids and cardiac glycosides (Ayoola *et al*., 2008).

Total phenolic content. Total phenolic content of each plant extract was determined by the Folin–Ciocalteu method (Rashid *et al*., 2010).

Total flavonoid content. The total flavonoid content of each plant extract was determined by the method of Zou *et al*. (2004).

B. Determination of antioxidant activity

DPPH (2, 2-diphenyl-l-picryl hydrazyl) radical scavenging assay (Sutharsingh *et al*., 2011). DPPH solution (0.004% w/v) was prepared in 95% ethanol. A stock solution of acetone extract and standard ascorbic acid were prepared in the concentration of 10mg/100ml (100µg/ml). From stock solution 2ml, 4ml, 6ml, 8ml & 10ml of this solution were taken in five test tubes respectively. 2 ml of freshly prepared DPPH solution (0.004% w/v) was added in each of these test tubes. The reaction mixture was incubated in the dark for 15 min and there after the optical density was recorded at 523 nm against the blank. For the control, 2 ml of DPPH solution in ethanol was mixed with 10ml of ethanol and the optical density of the solution was recorded after 15 min. The assay was carried out in triplicate. The decrease in optical density of DPPH on addition to test samples in relation to the control was used to calculate the antioxidant activity, as percentage inhibition (%IP) of DPPH radical. The capability to scavenge the DPPH free radical was calculated using the following formula:

$$\% \text{ of radical scavenging activity} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100\%$$

Where,

Abs control = Absorbance of DPPH solution

Abs sample = Absorbance of extracts and ascorbic acid solutions

IC50 values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

Nitric oxide scavenging activity assay (Garrat, 1964). 2 ml of 10 M sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of both the extracts at various concentrations and the mixture incubated at 25 °C for 2hrs. From the mixture 0.5 ml was and 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min followed by addition of 1ml naphthylethylenediamine dihydrochloride (0.1% w/v) and incubated at room temperature for 30 min. The nitric oxide radicals scavenging activity was calculated using following formula:

$$\% \text{ of radical scavenging activity} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100\%$$

Absorbance was taken at 540 nm.

Ferric reducing antioxidant power (Oyaizu, 1986). In ferric reducing antioxidant power assay 1 ml of test sample of extracts in different concentration were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated at a temperature-controlled water bath at 50°C for 20 min, followed by the addition of 1 ml of 10% trichloroacetic acid. The mixtures were then centrifuged for 10 min at room temperature. The supernatant obtained (1 ml) was added with 1 ml of deionised water and 200 µl of 0.1% $FeCl_3$. The blank was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by distilled water. The absorbance of the reaction mixture was measured at 700 nm. Reducing the power was expressed as an increase in absorbance after blank subtraction.

RESULTS AND DISCUSSION

A. Qualitative estimation of phytochemicals

The preliminary phytochemical screening of leaf extracts of *Rauwolfia serpentina* and *Picrorhiza kurroa* showed the presence of reducing sugar, terpenoids and flavanoid whereas alkaloid, saponins, steroids and cardiac glycoside. Tannins were present in wild (W) and field grown (FG) variety of *Picrorhiza kurroa* (PC) acetone extracts while absent in wild (W) and field grown (FG) variety of *Rauwolfia serpentina* (RS) acetone extracts. The previous study conducted by Panda *et al*., (2012) with methanolic extract of *Picrorhiza kurrooa* showed the presence of alkaloids, glycoside, steroids, reducing sugars, flavanoids, tannin in leaf and stem extract where as in root extract glycoside, flavanoids and saponins were absent.

Total phenolic content. The total phenolic content in plant extracts was examined by using Folin-ciocalteu reagent which was expressed in terms of gallic acid equivalent and was determined by standard curve having equation: $y= 0.003x + 0.027$, $R^2 = 0.990$. The total phenolic content in the examined extracts was between 102±0.42 to 181±0.52 mg/g of gallic acid (Table 1).

Table 1: Total phenolic content in the plant extracts expressed in terms of gallic acid equivalent.

Acetone extracts	
Extracts	**mg of GAE/g of extract**
Field grown *Rauwolfia serpentina*	102±0.42
Wild *Rauwolfia serpentina*	122±0.67
Field grown *Picrorhiza kurroa*	169±0.21
Wild *Picrorhiza kurroa*	181±0.52

The lowest total phenolic content was obtained in field grown *Rauwolfia serpentine* acetone extract i.e. 102±0.42mg/g and highest total phenolic content was obtained in wild variety of *Picrorhiza kurroa* acetone extract that was 181±0.52mg/g of gallic acid. High solubility of phenol in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for extraction. According to Kalivani *et al*., (2010), total phenolic content present in the *picrorhiza kurroa* dichloromethane was found to be 1845.14±0.32 mg/g and 100.14±0.54 in petroleum ether extract mg/g of gallic acid than that of the ethanol, chloroform and water rhizome extracts. According to Sinha *et al*., (2011), with the concentration of 20mg/l the phenolic content was found to be 42±1.3, 10 mg/l was 29.4 ±2.3 and 0.5mg/l was depicted to be 11±0.4 of ethanolic extract of *Rauwolfia* plant. Harisranraj *et al*., (2009) reported that the total phenolic content on leaf acetone (80%) extract of *Rauwolfia serpentina* was found to be 352.5 mg/l.

Total flavonoid content. The total flavanoid content was expressed in terms of rutin equivalent by standard curve equation $y=0.002x+0.09$, $R^2=0.966$**.** The concentration of flavanoids in the plant extracts depends upon the polarity of solvents used in the extract preparation.

Table 2: Total flavanoid content in the plant extracts expressed in terms of rutin equivalent.

Acetone extracts	
Extracts	**mg of RU/g of extract**
Field grown *Rauwolfia serpentina*	12.6±0.34
Wild *Rauwolfia serpentina*	43±0.007
Field grown *Picrorhiza kurroa*	59±0.63
Wild *Picrorhiza kurroa*	65±0.031

The total flavanoid content in the examined extracts ranges from 12.6±0.34 to 65±0.31 mg/g of Rutin (Table 2). The lowest total flavanoid content was obtained in field grown *Rauwolfia serpentina* extract i.e. 12.6±0.34 and highest total flavanoid content was obtained in wild variety of *Picrorhiza kurroa* extract.i.e.65±0.031 mg/g of Rutin. In the study of Kalivani *et al*., (2010), the highest total flavanoid content was found to be 21.56±20.4 mg/g in *Picrorhiza kurroa* ethanolic extract.

B. Antioxidant activity analysis

DPPH assay. The antioxidant activity of *Rauwolfia serpentine* and *Picrorhiza kurroo* acetone extracts was determined using ethanol solution of DPPH reagent. The extract which has shown highest %inhibition was found to having low inhibitory concentration that is called as minimum inhibitory concentration or IC50 value expressed in µg/ml**.** The results were found to be statistically significant.

The lowest % inhibition was of field grown *Rauwolfia serpentine* acetone extract i.e. 69.1± 0.43 and highest was of wild *Picrorhiza kurroa* acetone extract *i.e.* 83.5±0.18.

Ascorbic acid was taken as standard having % inhibition of 85.3±0.76**.** Wild *Picrorhiza kurroa* acetone extract exhibited greater IC50 value which was found to be 56.8µg/ml (Table 3).

Table 3: DPPH scavenging activity of different plant extracts.

Sample	Conc. (µg/ml)	%inhibition			
		FG.RS (Ac)	W.RS (Ac)	FG.PC (Ac)	W.PC (Ac)
TI	20	12.3±0.23	13.6±0.43	14±0.11	19.7±0.34
T2	40	22.1±0.40	25.1±0.42	28.2±0.024	31.1±0.21
T3	60	39.4±0.21	42.1±0.22	47.1±0.03	53.2±0.21
T4	80	54±0.23	58.5±0.11	65.4±0.13	76.1±0.11
T5	**100**	**69.1±0.43**	**72.1±0.33**	**80.2±0.42**	**83.5±0.18**
IC50		76.2±0.23	70.1±0.42	62.4±0.22	56.8±0.21

Govindarajan *et al*., (2003) studied the free radical scavenging activity with root ethanolic extract of *Picrorhiza kurroa* and the scavenging activity of this extract was found to be 67.81% and IC50 value was 50.2±1.92 µg /ml. Similarly, Kant *et al*. (2013) evaluated the antioxidant activity of *Picrorhiza kurroa* extract. They studied plant ethanol, ethylacetate and butanol extracts and IC50 was found to be 67.48, 39.58, 37.12 µg/ml at the concentration of 20-250 µg/ml. Bhandari *et al*. (2009) reported that the DPPH scavenging activity with methanolic and ethylacetate extract was found to be 32.8% and 29.3% at 100 µg/ml and IC50 value was found to be 47.4±0.75 and 44.5±0.52 µg /ml in ethanolic extracts.

According to Fazal *et al*. (2011) the DPPH scavenging activity of root ethanolic extract of *Rauwolfia serpentine* was found to be 61.8%.

Nitric oxide (NO) scavenging activity. The results of NO scavenging activity of the selected plant extracts were shown as percent of NO scavenging. The lowest % inhibition was of field grown *Rauwolfia serpentina* extract i.e. 70.4±0.005 and highest was of wild *Picrorhiza kurroa* extract i.e. 82.7±0.005. Ascorbic acid was taken as standard having % inhibition of 85±0.47. Wild *Picrorhiza kurroa* acetone extract exhibited greater IC50 value which was found to be 50.1µg/ml (Table 4).

Table 4: Nitric oxide scavenging activity of different plant extracts.

Sample	Conc. (µg/ml)	%inhibition			
		FG.RS (Ac)	W.RS (Ac)	FG.PC (Ac)	W.PC (Ac)
TI	20	14.6±0.001	15.0±0.05	17.4±0.034	20.1±0.056
T2	40	27.2±0.23	30.4±0.002	39.4±0.003	40.3±0.004
T3	60	39.3±0.34	40.1±0.006	49.3±0.45	50.6±0.008
T4	80	56.7±0.012	59.3±0.134	65.2±0.023	76.2±0.26
T5	**100**	**70.4±0.005**	**77.2±0.45**	**81.2±0.006**	**82.7±0.005**
IC50		70.1±0.002	68.5±0.001	60.1±0.21	50.1±0.22

Govindranrajan *et al*. (2003) reported that the scavenging of nitric oxide by root alcoholic *Picrorhiza kurroa* extract was concentration dependant and IC50 value was found to be 23±3.93 µg/ml.

Ferric reducing antioxidant power. The higher the FRAP value the greater is the antioxidant activity. The highest absorbance for FRAP assay was observed in wild *Picrorhiza kurroa* acetone extract i.e. 0.536 ± 0.045 and that the lowest was found in Field grown *Rauwolfia serpentina* acetone extract i.e.0.290 ± 0.002 (Table 5). Root ethanolic extract of *Rauwolfia serpentine* showed higher ferric reducing activity with the absorbance from 0.12 to 0.80 at the concentration of 10-500 µg/ml (Kaliavani *et al*., (2010).

Table 5: FRAP assay for different plant extracts.

Sample	Conc. µg/ml	FG.*RS* (Ac)	W.*RS* (Ac)	FG.*PC* (Ac)	W.*PC* (Ac)
TI	20	0.111±0.004	0.145±0.010	0.234±0.071	0.282±0.13
T2	40	0.122±0.005	0.170±0.002	0.299±0.023	0.362±0.002
T3	60	0.154±0.006	0.181±0.008	0.378±0.031	0.461±0.001
T4	80	0.181±0.002	0.196±0.12	0.456±0.002	0.514±0.024
T5	**100**	**0.290±0.002**	**0.250±0.18**	**0.480±0.005**	**0.536±0.045**

CONCLUSION

In the present study, the wild *Picrorhiza kurroa* acetone extract exhibited the highest total phenolic content as well as high flavanoid content and its greater radical scavenging and reducing capacity may be due to its higher content of phenolic compounds and flavanoid content. Thus, the therapeutic properties of wild *Picrorhiza kurroa* leaves may be possibly attributed to the phenolic and flavanoid compounds present. Hence it has been concluded that leaf parts of these plants can also be used as an alternative to other plant parts and acetone can also be used as an alternative to other solvent systems for the detection of phytochemicals.

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