BERBERIS BALUCHISTANICA: ASSESSMENT OF NATURAL ANTIOXIDANTS TO REPRIEVE FROM OXIDATIVE STRESS

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ABSTRACT

The present investigation was aimed to assess the antioxidant potential of various fractions of *Berberis baluchistanica* Ahrendt. The organic fractions were obtained by adding distilled water in crude methanolic extract of the plant and subsequently partitioning with organic solvents namely, *n*-hexane, chloroform, ethyl acetate and *n*-butanol, respectively. Phytochemical screening showed presence of useful antioxidants in more amounts in polar fractions of this plant. The antioxidant activity of all these fractions and remaining aqueous fraction was evaluated by using five methods: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, ferric thiocyanate assay by Linoleic acid, total phenolic contents (TPC) and total antioxidant activity by phosphomolybdenum method. Ethyl acetate soluble fraction possessed 87.98±1.08% inhibition of DPPH radical at a concentration of 60 µg/mL as compared to other fractions. The IC₅₀ of this fraction was 15.96 ± 1.50 µg/mL, relative to butylated hydroxytoluene (BHT, a reference standard), having IC₅₀ of 12.33±0.87 µg/mL. It also have strong degree of total antioxidant activity i.e. 0.98 ± 0.07 (absorbance at 695 nm) as well as highest FRAP value (360 ± 1.4 TEµM/mL), highest total phenolic contents (143.51 ± 1.68 GAE mg/g) and highest value of inhibition of lipid peroxidation (52.496 ± 1.16% at concentration of 500 µg/mL) as compared to the other studied fractions. *n*-Butanol fraction also showed good results.

Key words: *Berberis baluchistanica* Ahrendt, phytochemical screening, DPPH assay, total antioxidant activity, FRAP value, total phenolics, inhibition of lipid peroxidation.

INTRODUCTION

Free radicals are main cause of large number of diseases for example cancer, diabetes, heart failure, hypertension, neurodegenerative disorder and atherosclerosis¹. One or more unpaired electrons are present in free radicals due to which they are more unstable and are source to damage other molecules by removing electrons from them in order to get stability. During different physiological and biochemical processes, environmental pollutants, chemicals, toxins and radiations, living cells produce free radicals and other reactive oxygen species (ROS) which cause oxidative stress²⁻⁴. So the diseases associated with oxidative stress can be reprieved by the intake of antioxidants. Similarly, our food can also be preserved by antioxidants which stop discoloration, deterioration or rancidity caused by auto-oxidation⁵. Due to possible carcinogenicity of synthetic antioxidants, there has been a renewed interest about natural antioxidants in recent years. Several studies have shown that medicinal plants contain different nutrients and non-nutrients molecules and some molecules show antioxidant and antimicrobial activities can be useful for protection of human body against both oxidation and micro-organisms^{6,7}. So in continuation of our previous studies on medicinal plants⁸⁻¹⁰, here we have screened the phytoconstituents of *Berberis baluchistanica* Ahrendt to explore its possible use as a natural source of antioxidants. This plant belongs to family Berberidaceae and some species of this genus e.g. *Berberis aristata* is used to treat bleeding piles in the combination of other herbal formulation¹¹. *Berberis* species have been reported to treat insomnia¹², bronchial diseases, GIT discomfort, hepatic diseases and urinary discomforts¹³. These are also used as antirheumatic¹⁴, antipyretic¹⁵, antifungal¹³, and antibacterial sources¹⁶. According to our knowledge, there has been no study on *Berberis baluchistanica* as antioxidant source, therefore, in the present investigation the

antioxidant potential of various fractions of this plant was assessed by recognized methods.

MATERIALS AND METHODS

Plant Material

The plant *Berberis baluchistanica* Ahrendt was collected from Kirbi Kuch, Ziarat in August 2011, and identified by Dr. Rasool Bakhsh Tareen (Taxonomist), Department of Botany, University of Baluchistan, Quetta. A Voucher specimen (BB-RBT-08) has been deposited in the herbarium of the Botany Department of the same University.

Extraction and Fractionation of Antioxidants

The shade-dried ground whole plant (0.5 kg) was exhaustively extracted with methanol (5 L) on the soxhlet apparatus. The extract was evaporated in rotary evaporator (Laborota 4000-efficient Heidolph) at 40 °C under vacuum to yield the residue (148 g), which was dissolved in distilled water (1 L) and partitioned with *n*-hexane (1L × 5), chloroform (1 L × 4), ethyl acetate (1 L × 4) and *n*-butanol (1 L × 4) respectively. These four organic fractions and remaining water fraction were concentrated separately on rotary evaporator (*n*-hexane at 38 °C, chloroform at 40 °C, ethyl acetate at 45 °C, *n*-butanol 50 °C and water at 60 °C under vacuum) and the residues thus obtained were used to evaluate their *in vitro* antioxidant potential.

Chemicals and Standards

DPPH[•] (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin Ciocalteu's phenol reagent and BHT (butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride, ferrous chloride, ceric sulphate, hydrochloric acid, copper sulphate, aluminium chloride, lead

acetate, acetic acid, linoleic acid, tween 20 and ammonia from Merck (Pvt.) Ltd. (Germany).

Phytochemical Screening

Phytochemical screening of all the five crude extracts i.e. *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fractions was carried out to identify the phytochemical constituents (alkaloids, terpenoids, saponins, tannins, sugars, phenolics, flavonoids, cardiac glycosides) using the standard procedures⁷⁻¹⁰.

Antioxidant Assays

Following antioxidant assays were performed on all the studied fractions.

DPPH Radical Scavenging Activity

The DPPH radical scavenging activities of each crude extract of plant were examined by comparison with that of known antioxidant, (BHT) using the reported method¹⁷. Briefly, various amounts of the samples (1000 µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 60µg/mL, 30 µg/mL, and 15µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for one hour. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer (CECIL Instruments CE 7200 Cambridge England). Lower absorbance of reaction mixture indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity (\%)} = \frac{A(\text{control}) - A(\text{test})}{A(\text{control})} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total Antioxidant Activity by Phosphomolybdenum Method

The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method¹⁸. Briefly, 500 µg/mL of each crude extract was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95°C for 90 minutes. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of BHT. All determinations were assayed in triplicate and mean values were calculated.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was done according to Benzie and Strain¹⁹ with some modifications. The stock solutions included 300 mM acetate buffer (pH 3.6), 10mM TPTZ solution in 40 mM hydrochloric acid, and 20 mM ferric chloride hexahydrate solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL ferric chloride hexahydrate solution and then warmed at 37°C before using. The solutions of plant samples and that of trolox were prepared in methanol (500µM/mL). 10 µL of

each of crude extract was taken in separate test tubes and 2990 µL of FRAP solution was added in each to make total volume up to 3 mL. The plant crude extracts were allowed to react with FRAP solution in the dark for 30 minutes. Absorbance of the coloured product [ferrous tripyridyltriazine complex] was checked at 593 nm. The FRAP values were expressed as micromoles of trolox equivalents (TE) per mL of the sample solution using the standard curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

Total Phenolic Content

Total phenolics of various fractions of plant were determined by the reported method²⁰. An aliquot of 0.1 mL of each crude extract (0.5 mg/mL) was combined with 2.8 mL of 10% sodium carbonate and 0.1 mL of 2N Folin-Ciocalteu reagent. After 40 minutes absorbance at 725 nm was checked by UV-visible spectrophotometer. Total phenolics were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample using the standard calibration curve constructed for different concentrations of gallic acid. Results were expressed in GAE mg/g.

Ferric thiocyanate (FTC) Assay

The antioxidant activities of various fractions of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method²¹. Each sample (0.1 ml, 0.5 mg/ mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40°C. The mixture without extract was used as control. The mixture 0.1 mL was taken and mixed with 5.0 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid and allowed to stand at room temperature. Precisely 3 minutes after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as follows:

$$\text{Inhibition of lipid peroxidation (\%)} = \frac{1 - A(\text{sample})}{A(\text{control})} \times 100$$

The antioxidant activity of BHT as reference standard was assayed for comparison.

Statistical Analysis

All the measurements were done in triplicate and statistical analysis was performed by Statistical software. All the data were expressed as ± S.E.M. Statistical analysis were determined using one way analysis of variance (ANNOVA) followed by post-hoc Tukey's test.

RESULTS AND DISCUSSION

The phytochemical screening was done on all the studied fractions of *Berberis baluchistanica* Ahrendt and results are shown in table 1. According to the findings, the ethyl acetate soluble fraction contains phenolics, terpenoids, tannins, flavonoids and alkaloids in excess and saponins, sugar, steroids and cardiac glycosides in minute quantity. So this fraction contains excess quantity of antioxidative constituents as compared to all other fractions. *n*-Butanol soluble fractions enclosed alkaloids, sugar and cardiac glycosides excess in

quantity as compared to other fractions. It also contained phenolics, saponins, flavonoids and terpenoids in reasonable quantity but it possesses tannins in very minute quantity.

Table 1: Phytochemical constituents of various fractions of *Berberis baluchistanica*

Test	<i>n</i> -Hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate soluble fraction	<i>n</i> -Butanol soluble fraction	Remaining Aqueous fraction
Alkaloids	-	+++	++	+++	+
Terpenoids	+	++	+++	++	-
Saponins	-	-	+	++	+
Tannins	-	-	+++	+	+
Sugars	-	-	+	+++	+++
Phenolics	-	++	+++	++	+
Flavonoids	+	++	++	++	+
Cardiac glycosides	-	-	+	+++	+
Steroids	-	-	+	+	+

("+" represents presence and "-" represents absence).

Table 2: 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity of the various fractions of *Berberis baluchistanica*

Sample	Concentration in assay (µg/mL)	% Scavenging of DPPH ±S.E.M ^{a)}
<i>n</i> -Hexane soluble fraction	1000	67.87±1.43*
	500	45.63±0.48
	250	22.43±1.02
Chloroform soluble fraction	60	75.068±0.67*
	30	54.89±1.45*
	15	43.01±1.52
Ethyl acetate soluble fraction	60	87.98±0.08*
	30	67.89±0.19*
	15	45.65±0.66
<i>n</i> -Butanol soluble fraction	60	86.98±0.89*
	30	66.98±1.32*
	15	43.98±0.65
Remaining Aqueous fraction	1000	76.82±0.35*
	500	61.46±1.88*
	250	44.73±0.49
BHT ^{b)}	60	91.35 ± 0.14
	30	75.46 ± 0.08
	15	42.57 ± 0.05

^{a)} All results are presented as mean ± standard mean error of three assays. ^{b)} Standard antioxidant. *p < 0.05 when compared with negative control i.e. blank/solvent (p < 0.05 is taken as significant)

Table 3: IC₅₀, total phenolics, total antioxidant activity, FRAP values and lipid peroxidation inhibition values of various fractions of *Berberis baluchistanica*

Sr. No.	Sample	DPPH-radical scavenging activity (IC ₅₀ µg/mL)	Total antioxidant activity (abs. at 695 nm)	FRAP value (TE µM/mL)	Total phenolics (GAE mg/g)	Inhibition of lipid peroxidation (%) ^c
1	<i>n</i> -Hexane soluble fraction	667.06±1.2	0.47±0.06	150.3±1.55	38.5±1.56	10.890±0.48
2	Chloroform soluble fraction	24.1926±1.02**	0.77±0.05**	228.95±1.65*	108.66±1.06*	25.323±1.43
3	Ethyl acetate soluble fraction	15.96±1.50**	0.98±0.07**	360±1.4*	143.51±1.68*	52.496±1.16**
4	<i>n</i> -Butanol soluble fraction	17.52±0.55**	0.89±0.08**	280.85±1.33*	126.83±1.27*	47.31±0.65**
5	Remaining Aqueous fraction	315.85±1.06	0.72±0.08**	151.45±1.20	61.16±0.59	30.42±0.65
6	BHT ^{a)}	12.33±0.87	0.96±0.06	-	-	62.48±1.07
7	Blank ^{b)}	-	-	-	16.49	-

All results are presented as mean ± standard mean error of three assays.

^{a)} expressed relative to BHT, ^{b)} expressed relative to blank. ^{c)} tested concentration at 500 µg/mL *p < 0.05 when compared with negative control i.e. blank/solvent (p < 0.05 is taken as significant). **p < 0.05 when compared with reference standard (BHT).

Chloroform soluble fraction has alkaloids in excess quantity and phenolics, flavonoids and terpenoids in a reasonable quantity. *n*-Hexane soluble fraction encompassed only terpenoids and flavonoids. The remaining aqueous fraction was rich in sugars and contained saponins, tannins, flavonoids, phenolics, alkaloids and steroids in very small quantity.

DPPH radical scavenging activity is widely used to evaluate antioxidant activity in a relatively short time. It is thought that phenolic compound have hydrogen donating ability so it effects on DPPH radical scavenging activity. Phenolic compound donates hydrogen, DPPH has ability to accept electron or hydrogen radical so in this way stable free radical (DPPH) convert into stable diamagnetic molecule by accepting hydrogen from phenolic compounds²². It has been

noticed that after reaction of a phenolic compound with DPPH radical, the absorbance at 517 nm of DPPH radical decreases due to change of colour from purple to yellow²³. This assay was done on all fractions of plant and percentage scavenging of DPPH was measured which is shown in table 2. When we increased the concentration activity also increased. Results revealed that different concentrations of ethyl acetate fraction gave better percentage inhibition of DPPH radical relative to other fractions. At concentration of 60 µg/mL, percentage inhibition of DPPH radical by this fraction was 87.98±0.08. At 30 µg/mL, percentage inhibition was more than 50% i.e. 67.89±0.19. Different concentrations of *n*-butanol soluble fraction also rendered more than 50% percentage inhibition of DPPH radical, i.e. 86.98±0.89 and 66.98±1.32 at concentrations 60 µg/mL and 30 µg/mL,

respectively. A reasonable percentage inhibition was also observed by other fractions of this plant.

The IC₅₀ values for different fractions were calculated and are shown in table 3. This value is basically concentration of substrate which inhibits 50% of DPPH radical. Lower the IC₅₀ value, higher is the percentage inhibition of DPPH radicals. It was discerned that ethyl acetate soluble fraction exhibited lowest IC₅₀ value (15.96±1.5 µg/mL) as compared to those of other fractions. So we can conclude that ethyl acetate soluble fraction of this plant has high percentage inhibition of DPPH radical. *n*-Butanol soluble fraction with IC₅₀ value of 17.52±0.55 µg/mL also showed promising results relative to *n*-hexane, chloroform soluble fractions as well as remaining aqueous fraction. Formation of phosphomolybdenum complex is a method which is used to measure total antioxidant activity. In this method, plant fraction reduces Mo(VI) to Mo (V) and we can detect green phosphate Mo (V) at 695 nm by using UV-visible spectrophotometer. In this assay basically electron transfer is done and this transfer depends upon structure of antioxidant¹. Antioxidants such as phenolics, tocopherols, carotenoids and ascorbic acid are detected by phosphomolybdenum method. When total antioxidant activities of all the fractions of this plant were measured relative to standard butylated hydroxyl toluene (BHT), the following order was observed; ethyl acetate soluble fraction > *n*-butanol soluble fraction > chloroform soluble fraction > remaining aqueous fraction > *n*-hexane soluble fraction (table 3). Comparing these results with BHT, it was rationalized that ethyl acetate, *n*-butanol, chloroform and water soluble fractions were significant ($p < 0.05$) and only *n*-hexane soluble fraction was non-significant ($p > 0.05$). FRAP test is a simple method to measure antioxidant contents. FRAP test was used to measure antioxidant capacity of plasma, so we can also use this method to measure antioxidant activity of plants²⁴. The reducing capacity of any compound is the significant indicator of antioxidant activity, so in FRAP test reducing ability of different fractions of plant extract is assayed to measure antioxidant activity against reactive oxygen species. Antioxidants have ability to donate electrons so serve as reductants and by using these, oxidants can be inactivated via a typical redox reaction. So in this test, the ability of an antioxidant to reduce Fe³⁺ into Fe²⁺ is measured. The complex of Fe²⁺ and tripyridyltriazine gives an intense blue colour with maximum absorbance at wavelength of 593 nm²⁴. In the present study, it was revealed that the ethyl acetate soluble fraction exhibited highest FRAP value (360±1.4 TE µM/mL) followed by *n*-butanol soluble fraction having 280.85±1.33 TE µM/mL. Chloroform soluble fraction also showed good results (228.95±1.65) however; remaining aqueous and *n*-hexane soluble fractions depicted poor FRAP values i.e. 151.45±1.2 and 150.3±1.55 TE µM/mL, respectively. Phenolic and flavonoids have rendered good antioxidant action in human being as they act as suitable scavenging agents or chelating agents^{25,26}. The total phenolic contents of different fractions of this plant are shown in table 3. The ethyl acetate soluble fraction showed superior results with 143.51±1.68 gallic acid equivalents per gram (GAEs). The *n*-butanol soluble fraction also showed promising results as compared to other fractions with 126.83±1.27 GAEs. Lipids contain double bonds and oxygen (O₂) reacts with these double bonds and lipid hydroperoxides are produced. This type of reaction occurs *in vivo* and *in vitro* due to which reactive and cytotoxic products are produced. These cytotoxic products affect human cells and their functions.

They also affect DNA and can damage it. Lipid peroxidation can also cause cancer²⁴. Antioxidants have ability to donate hydrogen so they can react with radicals of lipid peroxyl and stop the production of new radicals. In FTC method we measure the quantity of peroxide at the start of lipid peroxidation. Here, ferrous chloride reacts with peroxide and produces ferric ions. The ferric ions then combine with ammonium thiocyanate and ferric thiocyanate is produced which shows reddish pigment⁹. This study revealed that polar fractions displayed good percentage inhibition of lipid peroxidation (table 3) as compared to medium and less polar fractions of this plant.

CONCLUSION

It was generally concluded that ethyl acetate soluble fraction of this plant is rich in valuable antioxidative phyto-constituents and can play its role to protect human body cells from damaging due to oxidative stress. It might also be utilized to preserve food products.

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