Dicliptera bupleuroides: an Imperative Source for Protection from Oxidative Stress

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Summary: *Dicliptera bupleuroides* Nees. has long been used in folkloric medicines for the treatment of various ailments. In this study, the methanolic extract of this plant was dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol sequentially. These organic fractions and the remaining aqueous fraction were tested for their phyto-constituents and antioxidant potential by four methods: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate assay along with the determination of their total phenolic contents. The results revealed that among these fractions the chloroform soluble fraction and ethyl acetate soluble fraction showed good antioxidant potential. Their IC_{50} values were found to be 108.82 ± 1.14 and 110.58 ± 0.60 µg/mL respectively. Ethyl acetate soluble fraction showed highest total antioxidant activity i.e. 1.149 ± 0.21. Chloroform and ethyl acetate soluble fractions also showed good FRAP values (227.56 ± 1.27 and 213.43 ± 1.16 TE µM/mL respectively) as well as considerable amounts of total phenolic compounds i.e. 123.17 ± 1.33 and 115.16 ± 1.09 GAE µg/g) and good values of percentage of inhibition of lipid peroxidation i.e. 42.17 ± 0.79% and 41.14 ± 1.46% respectively almost equal to each other. So, these fractions are useful potentially against the oxidative stress as compared to other studied fractions.

Key words: *Dicliptera bupleuroides* Nees., DPPH assay, total antioxidant activity, FRAP value, total phenolics, Inhibition of lipid peroxidation (%).

Introduction

Numerous studies have shown that aromatic and medicinal plants are sources of diverse nutrient and non nutrient molecules, many of which display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus it is important to characterize different types of medicinal plants for their antioxidant potential. Spices and herbs have been used for thousands of centuries by many cultures to enhance the flavor and aroma of foods. Scientific experiments since the late 19th century have documented the antioxidant properties of some spices, herbs, and their components [1]. Medicinal plants constitute the major constituents of most indigenous medicines and a large number of Western medical preparations contain one or more ingredients of plant origin [2]. There is an increasing interest in the measurement and use of plant antioxidant for scientific research as well as industrial (dietary, pharmaceutical and cosmetics) purposes. This is mainly due to their strong biological activity, excluding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis. Therefore, the need exists for safe, economic, powerful and natural antioxidants to replace these synthetic ones. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts [3].

Many antioxidant compounds, naturally occurring in plant sources, have been identified as a free radical or active oxygen scavengers [4]. A number of plants have been investigated for their biological activities and antioxidant principles [5,6]. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants [7]. In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical scavenging in the last few decades [8].Some antioxidant compounds are extracted from easy sources, such as agricultural and horticultural crops, or medicinal plants. Among them the medicinal plants and herbs are taking the main role for providing a large number of pure antioxidants. *Dicliptera bupleuroides* Nees. is a traditional medicinal plant which is commonly known as Kuthi, Kalu Ghu, Pateev, Somni and Kirch. It belongs to the family Acanthaceae. It is highly branched herb having hairy twigs. Its length is about 90 cm. It is widely distributed in Pakistan, Nepal, Afghanistan, Bangla Desh, India to Indo-China, Bhutan and West China. The flowering period of *D. bupleuroides* starts from June and ends in October [9]. It is traditionally used as a medicinal plant as tonic debility as well as for the treatment of various

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eye diseases. Its crushed fresh leaves are applied gently on the effected portion of the body thrice a day for one week in eczema [10-12]. To the best of our knowledge no antioxidant work is done on the various fractions of the *D. bupleuroides*, therefore, in the present investigation, we described the comparative *in vitro* antioxidant potential of aqueous and organic fractions of this species by four methods: 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, total antioxidant activity by phosphomolybdenum complex (PC) method, Ferric Reducing Antioxidant Power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolic contents relative to conventionally used standards. Variety of antioxidants are present in plant extracts therefore different antioxidant assays are required to access their antioxidant potential as one or two antioxidant assays cannot evaluate all types of antioxidants present in plant extracts [13].

Results And Discussion

Phytochemical Screening

The phytochemical screening was done on all the studied fractions and results have been shown in Table-1. From the results it was revealed that chloroform, ethyl acetate and *n*-butanol soluble fractions contain phenolics, flavonoides, alkaloides and cardiac glycosides while *n*- hexane fraction and remaining aqueous fraction showed absence of all these compounds. Terpenes were found to be present in all the fractions except the aqueous fraction. Tannins and sugars were present in ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction but not detected in *n*-hexane and chloroform soluble fractions. Saponins were present in ethyl acetate and *n*-butanol soluble fractions while absent in other studied fractions.

Table-1: Phytochemical constituents of various fractions of *Dicliptera bupleuroides* Nees.

Test	*n*-hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate Soluble fraction	*n*-Butanol soluble fraction	Remaining Aqueous fraction
Alkaloides	–	++	++	++	–
Terpenoides	+	++	+++	+++	–
Saponins	–	–	+	+	–
Tannins	–	–	+	+	+
Sugars	–	–	+	+	+
Phenolics	–	++	++	+	–
Flavonoides	–	+	+	+	–
Cardiac Glycosides	–	++	++	+	–

Note: '+' represents presence and '–' represents absence.

DPPH Radical Scavenging Activity

The free radical scavenging activity of the studied fractions was examined *in vitro* using DPPH radical. This assay is based on the measurement of the scavenging ability of antioxidant test substances towards the stable radical [14]. The addition of plant extract to DPPH solution causes a rapid decrease in the optical density at 517nm. The degree of discoloration indicates the scavenging capacity of the extract. Free radicals cause auto-oxidation of unsaturated lipids in food. The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. Antioxidants cease the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl group. Therefore a stable end product is formed which thus terminates the reaction [15]. The values of percent scavenging of DPPH radical have been shown in Table-2.

Table 2: 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity of the various fractions of *Dicliptera bupleuroides* Nees.

Sr.No.	Sample	Concentrationin assay (µg/ml)	% scavenging of DPPH ± S.E.M[a)]
1	*n*-Hexane soluble fraction	500	53.57 ± 1.63
		250	39.28 ± 1.36
		125	31.42 ± 0.92
2	Chloroform soluble fraction	250	81.28 ± 1.78*
		125	65.14 ± 1.33*
		60	39.28 ± 1.91
		30	22.11 ± 1.86
3	Ethyl acetate soluble fraction	500	80.42 ± 1.49*
		250	67.85 ± 1.66*
		125	54.28 ± 1.75
		60	39.87 ± 1.69
4	*n*-Butanol soluble fraction	250	70.02 ± 1.98*
		125	63.57 ± 0.71*
		60	15.09 ± 1.27
5	Aqueous fraction	1000	51.42 ± 1.58
		500	35.71 ± 0.74
		250	26.42 ± 0.57
6	BHT[b)]	60	92.46 ± 0.25*
		30	74.57 ±0.39*
		15	49.61 ± 0.55
		8	28.33 ± 0.83

[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).

It was observed that activity was increased by increasing the concentration of the samples in the assay. The various concentrations of Chloroform soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 81.28 ± 1.78 % inhibition of DPPH radical at a concentration of 250 µg/ml. Ethyl acetate fraction also showed good scavenging activity close to the chloroform fraction. However, the radical-scavenging activity decreased little as the concentration increased further. The reason is that the interference substance(s) cannot donate more protons at critical higher concentrations. The various concentrations of the fractions which showed percent inhibition greater

than 60% were found to be significant ($p < 0.05$). IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity [2]. It was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds and results have been shown in Table-3. IC_{50} value is inversely related to the activity. The lower the IC_{50} value, higher will be the scavenging potential. Chloroform soluble fraction and ethyl acetate soluble fraction showed good IC_{50} values very near to each other i.e. 108.82 ± 1.14 and 110.58 ± 0.60 respectively. *n*-butanol soluble fraction showed moderate IC_{50} value i.e. 170.93 ± 1.79. The IC_{50} values of *n*-hexane soluble fraction and remaining aqueous fraction were not found good. The results were expressed relative to butylated hydroxytoluene (BHT), a reference standard, having IC_{50} of 12.63 ± 0.97 µg/ml. The IC_{50} values of chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT, a reference standard.

Total Antioxidant Activity by Phosphomolybdenum Complex Method

The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids. This method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidants and the subsequent formation of a green phosphate Mo (V) complex at acidic pH. Electron transfer occurs in this assay which depends upon the structure of the antioxidant [16]. The total antioxidant activities of the studied fractions were meared and compared with the standard antioxidant BHT and the results have been shown in Table-3. It was revealed from the results that ethyl acetate fraction showed highest total antioxidant activity i.e. 1.149 ± 0.21 as compared to other fractions. The chloroform fraction also showed good value i.e. 0.934 ± 0.65. *n*-butanol fraction showed moderate value (0.776 ± 0.41) while *n*-hexane fraction and remaining aqueous fraction showed very less values (0.352 ± 0.09 and 0.295 ± 0.28 respectively). The results were compared with BHT, a reference standard having total antioxidant activity 1.22 ± 0.53. The total antioxidant activity shown by chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction was found to be significant ($p < 0.05$) while that of *n*-hexane and aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Ferric Reducing Antioxidant Power (FRAP) Assay

This assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. It utilizes the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (Fe(III)-TPTZ) complex and produce an intense blue colored ferrous tripyridyltriazine (Fe(II)-TPTZ) complex with an absorbance maximum at 593 nm. Increasing absorbance indicates an increase in reductive ability. This assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions [15,17]. The FRAP values of the studied fractions were calculated and results have been shown in Table-3. Among all the fractions the chloroform fraction and ethyl acetate fraction showed good FRAP values very near to each other (227.56 ± 1.27 and 213.43 ± 1.16 TE µM/mL respectively). *n*-butanol fraction showed moderate FRAP value (170.36 ± 0.91 TE µM/mL) while remaining aqueous fraction and *n*-hexane fraction showed poor FRAP values i.e. 34.12 ± 0.59 and 45.51 ± 0.78 TE µM/mL High FRAP values may be ascribed partially to the presence of phenolic and flavonoid contents. The results were compared with the blank having value 22.73. The FRAP values of chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank.

Total Phenolic Contents

In recent years, many publications have reported evidence of beneficial health effects attributed to phenolic contents such as flavonoids including antiallergenic, antiinflammatory, cardioprotective, vasodilatory, anticarcenogenic and antioxidant properties. Several epidemiological studies have also associated the consumption of flavonoids with a reduction of the risk of chronic diseases including, cancer, diabetes and coronary heart problems [18]. The phenolic compounds may contribute directly to antioxidative action. The antioxidative activities observed can be ascribed both the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. The antioxidants have different functional properties, such as reactive oxygen species scavenging e.g. quercetin and catechin, inhibition of the generation of free radicals and chain-breaking activity, e.g. *p*-coumaric acids and metal chelation.

These compounds are normally phenolic compounds, which are effective proton donors, and include tocopherols, flavonoids, and other organic acid [19]. Table-3 shows the phenolic concentration in the studied fractions, expressed as micrograms of gallic acid equivalents (GAEs) per gram of fraction. Among them, the chloroform fraction and ethyl acetate fraction showed considerable amounts of total phenolic compounds i.e. 123.17 ± 1.33 and 115.16 ± 1.09 GAE µg/g). *n*-butanol fraction showed moderate amount of total phenolics (68.34 ± 0.96 GAE µg/g) while that of *n*-hexane soluble fraction and remaining aqueous fraction were found to be very less (33.67 ± 0.91, and 43.27 ± 0.82 GAE µg/g respectively). The results for total phenolic contents of chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank(14.96).

Ferric Thiocyanate (FTC) Assay

This assay measures the amount of peroxide generated at the initial stage of linoleic acid emulsion during incubation. Oxygen reacts with unsaturated double bond on the lipid which results in generation of free radicals and lipid hydroperoxides. Peroxidation of lipids occurs both *in vivo* and *in vitro* and gives rise to cytotoxic and reactive products. Here, peroxide reacts with ferrous chloride to form ferric chloride, which in turn reacts with ammonium thiocyanate to produce ferric thiocyanate, a reddish pigment. Low absorbance values measured via the FTC method indicate high antioxidant activity [20]. The specific mode of inhibition of lipid peroxidation by the polyphenols is not clear. They may act by chelating copper ions via the *ortho*-dihydroxy phenolic structure or by acting as hydrogen-donating radical scavengers for example by scavenging lipid alkoxyl or peroxy radical [21]. The various fractions of plant were tested by this assay and results have been shown in Table-3. chloroform fraction and ethyl acetate fraction showed good values of percentage of inhibition of lipid peroxidation nearly equal to each other i.e. 42.17 ± 0.79% and 41.14 ± 1.46% respectively, while *n*-hexane fraction, *n*-butanol fraction and aqueous fractions exhibited very low values of percent inhibition of lipid peroxidation i.e. 10.51 ± 0.98%, 21.52 ± 0.56% and 15.18 ± 0.87% respectively. The inhibition of lipid peroxidation by BHT (standard) was found to be 62.96 ± 1.18%. The results for percent inhibition of lipid peroxidation by chloroform soluble fraction and ethyl acetate soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Experimental

Plant Material

The plant *Dicliptera bupleuroides* Nees. was collected from district Kotli, Azad Kashmir in October 2010, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, Government College University, Lahore. A Voucher specimen (G.C.Bot.Herb.968) 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 (0.725 g) was exhaustively extracted with methanol on the soxchlet apparatus. The extract was evaporated in rotary evaporator (Laborta 4000-efficent Heidolph) at 45^0C under vaccum to yield the residue (51 g), which was dissolved in distilled water (600 mL) and partitioned with *n*-hexane (500 mL × 4), chloroform (500 mL × 4), ethyl acetate (500 mL × 4) and *n*-butanol (300 mL × 4) respectively. These organic fractions and remaining water fraction were concentrated separately on rotary evaporator (*n*-hexane at 37 ^{o}C, chloroform at 40 ^{o}C , ethyl acetate at 48 ^{o}C , *n*-butanol 50 ^{o}C and water at 60 ^{o}C under vaccum) and the residues thus obtained were used to evaluate their Phytochemical constituents and *in vitro* antioxidant potential.

Table-3: IC_{50}, total phenolics, total antioxidant activity, FRAP values and lipid peroxidation inhibition values of different fractions of *Dicliptera bupleuroides* Nees.

Sr No.	Sample	DPPH-radical scavenging activity(IC_{50},µg/mL)	Total antioxidant activity	FRAP value (TE µM/mL)	Total phenolics (GAE µg /g)	Inhibition of lipid peroxidation (%)
1	*n*-Hexane soluble fraction	443.62 ± 1.96	0.352 ± 0.09	45.51 ± 0.78	33.67 ± 0.91	10.51 ± 0.98
2	Chloroform Soluble fraction	108.82 ± 1.14**	0.934 ± 0.65**	227.56 ± 1.27*	123.17 ± 1.33*	42.17 ± 0.79**
3	Ethyl acetate soluble fraction	110.58 ± 0.60**	1.149 ± 0.21**	213.43 ± 1.16*	115.16 ± 1.09*	41.14 ± 1.46**
4	*n*-Butanol soluble fraction	170.93 ± 1.79**	0.776 ± 0.41**	170.36 ± 0.91*	68.34 ± 0.96*	21.52 ± 0.56
5	Aqueous fraction	952.72 ± 1.91	0.295 ± 0.28	34.12 ± 0.59	43.27 ± 0.82	15.18 ± 0.87
6	BHT [a)]	12.63 ± 0.97	1.22 ± 0.53			62.96 ± 1.18
7	Blank [b)]			22.73	14.96	

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, hydrocloric acid, copper sulphate, aluminium chloride, lead acetate, acetic acid and ammonia from Merck (Pvt.) Ltd. (Germany).

Phytochemical Screening

Phytochemical screening of the crude methanolic extract, *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fractions were carried out to identify the phytochemical constituents (alkaloides, terpenoides, saponins, tannins, sugars, phenolics, flavonoides and cardiac glycosides using standard procedures [22-24].

Antioxidant Assays

Following antioxidant assays were performed on all the studied fractions.

DPPH Radical Scavenging Activity

The DPPH radical scavenging activities of various fractions of plant were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the reported method [25]. Briefly, various concentrations of the samples (1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 60 μg/mL, 30 μg/mL, 15 μg/mL, 8 μg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1mM). The mixture was shaken vigorously and allowed to stand at room temperature for one an hour.

Then absorbance was measured at 517 nm against methanol as a blank in the UV-visible spectrophotometer (CECIL Instruments CE 7200 Cambridge England). Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula:
Antiradical activity = $A_{control}$ - A_{sample}/ $A_{control}$ ×100
Each sample was assayed in triplicate and mean values were calculated.

Total Antioxidant Activity by Phosphomolybdenum Complex Method

The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method [16]. Briefly, 500 μg/mL of each sample 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 ^{o}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 Strain [17] with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa.3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2,4,6-Tripyridyl-s-triazine) 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 $FeCl_3.6H_2O$ solution and then warmed at 37 ^{o}C before using. The solutions of plant samples and that of trolox were formed in methanol (250 μg/mL). 10 μL of each of sample solution and BHT solution were 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 samples were allowed to react with FRAP solution in the dark for 30 minutes. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm by UV-visible spectrophotometer. The FRAP values were determined as micromoles of trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE μM/mL.

Total Phenolic Contents

Total phenolics of various fractions of plant were determined reported method [26]. The 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10% Sodium carbonate and 0.1 mL of 2N Folin-Ciocalteu's phenol reagent. After 40 minutes absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolic contents were

expressed as micrograms of gallic acid equivalents(GAE) per gram of sample using the standard calibration curve constructed for different concentrations of gallic acid. The curve was linear between 50 μg/mL to 500 μg/mL of gallic acid. Results were expressed in GAE μg/mL.

Ferric Thiocyanate (FTC) Assay

The antioxidant activities of various fractions of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method [27-29]. The 0.1 mL of each of sample solution (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 ^{o}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% HCl 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 percentage inhibition of peroxidation (IP%) [IP% = {1-(abs. of sample) /(abs. of control)} × 100]. The antioxidant activity of BHT was assayed for comparison as reference standard.

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.

Conclusion

It was observed from the results that chloroform fraction and ethyl acetate fraction contain considerable amounts of phenolics, flavonoides, alkaloides and cardiac glycosides while *n*-butanol fraction contains moderate amounts of such compounds whereas *n*- hexane fraction and remaining aqueous fraction showed absence of all these compounds. Because of presence of considerable amounts of such compounds chloroform fraction and ethyl acetate fraction showed good antioxidant activity, *n*-butanol fraction showed moderate activity due to fewer amounts of such compounds while *n*- hexane fraction and aqueous fraction showed no activity due to absence of all these compounds. Chloroform soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 81.28 ± 1.78 % inhibition of DPPH radical at a concentration of 250 μg/ml. Ethyl acetate fraction also showed good scavenging activity close to the chloroform fraction. Their IC_{50} values were found very near to each other i.e. 108.82 ± 1.14 and 110.58 ± 0.60 respectively, relative to butylated hydroxytoluene (BHT), a reference standard, having IC_{50} of 12.63 ± 0.97 μg/ml.

Ethyl acetate fraction showed highest total antioxidant activity i.e. 1.149 ± 0.21. Chloroform fraction and ethyl acetate fraction showed good FRAP values (227.56 ± 1.27 and 213.43 ± 1.16 TE μM/mL respectively) as well as considerable amounts of total phenolic compounds i.e. 123.17 ± 1.33 and 115.16 ± 1.09 GAE μg/g) and good values of percentage of inhibition of lipid peroxidation i.e. 42.17 ± 0.79% and 41.14 ± 1.46% respectively. All these values were found nearly equal to each other. So it was concluded that chloroform fraction and ethyl acetate fraction and are rich in strong antioxidants and are potentially valuable sources of natural antioxidants and bioactive materials, which would be expected to increase shelf life of foods and fortify against peroxidative damage in living systems in relation to aging and carcinogenesis.

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References

1. M. Sengul, H. Yildiz, N. Gungor, B. Cetin, Z. Eser and S. Ercilsi, *Pakistan Journal of Pharmaceutical Sciences,* **22** 2009).
2. G. Nahak and R.K. Sahu, *Journal of American Sciences,* **6**, 123 (2010).
3. J. McClements and E. A. Decker, *Journal of Food Sciences,* **65**, 1270 (2000).
4. W. Zheng and S. Y. Wang, *Journal of Agricultural and Food Chemistry,* **49**, 5165 (2001).
5. O. Baris, M. Golloce, R. Sahin and H. Ozer, *Turkish Journal of Biology,* **30**, 65 (2006).
6. A. Saleem, M. Ahotupa and K. Pihlaja, *Zeitschrift fur Natureforschung,* **56**, 973 (2001).
7. N. Ito, S. Fukushima, A. Hasegawa, M. Shibata and T. Ogiso, *Journal of the National Cancer Institute,* **70**, 343 (1983).

8. R. P. Singh, K. N. C. Murthy and G. K. Jayaprakasha, *Journal of Agricultural and Food Chemistry,* **50**, 81 (2002).
9. E. Nasir and S. I. Ali, Flora of West Pakistan. Vol 188, Acanthaceae. Department of Botany, University of Karachi, Karachi, 23-25 (1988).
10. K. R. Arya and S. C. Agarwal, *Indian Journal of Traditional Knowledge,* **7**, 443 (2008).
11. P. C. Pande, L. Tiwari and H. C. Pande, *Indian Journal of Traditional Knowledge*, **6**, 444 (2007).
12. M. Khan, S. Kumar and I. A. Hamal, *Ethnobotanical Leaflets,* **13**, 1113 (2009).
13. E. Souri, G. Amin, H. Farsam, H. Jalalizadeh and S. Barezi, *Iranian Journal of Pharmaceutical Reasearch*, **7,** 149 (2007).
14. R. Ilavarasan**,** M. Mallika and S. Venkataraman, *African Journal of Traditional CAM,* **2**, 70 (2005).
15. T. Riaz, M. A. Abbasi, M. I. Umer, A. Rehman, T. Shahzadi, K. M. Khan and V. U. Ahmad., *Journal of Chemical Society of Pakistan,* **33**, 200 (2011).
16. P. Prieto, M. Pineda and M. Aguilar, *Analytical Biochemistry,* **269**, 337 (1999).
17. I.F.F. Benzie and J.J. Strain, *Analytical Biochemistry,* **239**, 70 (1996).
18. J. Santas, M. P Almajan and R. Carbo, *International Journal of Food Science and Technology*, **45**, 403 (2010).
19. M. A. Abbasi, A. Zafar, T. Riaz, Aziz-ur-Rehman, S. Arshad, D. Shahwar, M. Jahangir, S. Z. Siddiqui, T. Shahzadi and M. Ajaib, *Journal of Medicinal Plants Res*earch, **4**, 1883 (2010).
20. J. S. J. Kim and M. J. Kim, *Journal of Medicinal Plants Research*, **4**, 674 (2010).
21. N. Salah, N. J. Miller, G. Paganga, L. Tigburg, G. P. Bolwell, C. Rice-Evans, *Archives of Biochemistry and Biophysics,* **332**, 339 (1995).
22. A. Sofowora, Medicinal plants and Traditional Medicine in Africa. Spectrum Books, Ibadan, 150 (1993).
23. G. E. Trease and W. C. Evans, *Pharmacog*nosy 13th edition. Bailliere Tindall, London, 176, 180 (1989).
24. G. A. Ayoola, H. A. B. Coker, S. A. Adesegun, A. A. Adepoju-Bello, K. Obaweya, E. C. Ezennia and T. O. Atangbayila, *Tropical Journal of Pharmaceutical Research*, **7**, 1019 (2008).
25. K. Lee and T. Shibamoto, *Food Chemistry,* **74**, 443 (2001).
26. H. P. S. Makkar, M. Bluemmel, N. K. Borowy and K. Becker, *Journal of the Science of Food and Agriculture,* **61**, 161 (1993).
27. P. Valentao, E. Fernandes, F. Carvalho, P. B. Andrade, R. M. Seabra and M. L. Bastos, *Journal of Agricultural and Food Chemistry,* **50**, 4989 (2002).
28. A. Sharif , E. Ahmed , M. M. Ali, S. Jabeen, M. U. A. Khan, R. Begum and A. Farrukh, *Journal of the Chemical Society of Pakistan,* **33,** 578 (2011).
29. J. Zhang and R. Liu, , *Journal of the Chemical Society of Pakistan,* **33,** 356 (2011).