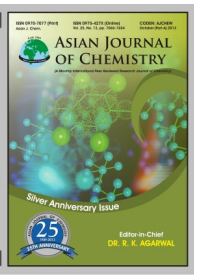
Asian Journal of Chemistry; Vol. 25, No. 14 (2013), 7921-7926

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.14689



Bergia ammannioides: Phytochemical Screening, Antioxidant Activity and Radical Scavenging Effects of its Various Fractions

AZIZ-UR-REHMAN[1,*], ASIA SIDDIQA[1], MUHAMMAD ATHAR ABBASI[1], TAUHEEDA RIAZ[1], GHULAM HUSSAIN[1], SABAHAT ZAHRA SIDDIQUI[1], MISBAH IRSHAD[1], MUHAMMAD AJAIB[2] and IRSHAD AHMAD[3]

[1]Department of Chemistry, Government College University, Lahore-54000, Pakistan
[2]Department of Botany, Government College University, Lahore-54000, Pakistan
[3]Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur-63100, Pakistan

*Corresponding author: Tel: +92 42 111000010; Ext. 449; E-mail: azizryk@yahoo.com

(*Received*: 16 October 2012; *Accepted*: 25 July 2013) AJC-13841

The methanolic extract of *Bergia ammannioides* (Henye ex Roth) was dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol sequentially. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated by four methods *e.g.*, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, total antioxidant activity, ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolics. Polar fractions showed noteworthy antioxidant potential. The results revealed that ethyl acetate soluble fraction showed highest value of % inhibition of DPPH radical (81.16 ± 0.87 %) at a concentration of 120 µg/mL. The IC_{50} of this fraction was 26.65 ± 1.8 µg/mL, relative to ascorbic acid, a reference standard, having IC_{50} 58.8 ± 0.89 µg/mL. It also showed highest total antioxidant activity *i.e.*, 1.142 ± 0.08, highest FRAP value (378.5 ± 0.4 TE µM/mL) as well as highest total phenolic contents *i.e.*, 226.109 ± 0.3 GAE mg/g and highest value of per cent inhibition of lipid peroxidation (54.23 ± 0.57 %) as compared to the studied fractions.

Key Words: *Bergia ammannioides*, DPPH assay, Total antioxidant activity, FRAP value, Total phenolics.

INTRODUCTION

Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite[1]. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Several authors demonstrated that antioxidant intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks[2,3]. Antioxidants that are mainly supplied as dietary consumptions can also impede carcinogenesis by scavenging oxygen radicals or interfering with the binding of carcinogens to DNA which includes vitamin C, vitamin E (α-tocopherol, γ-tocopherol), carotenoids (β-carotene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin, lycopene) and several polyphenolic compounds including flavonoids (catechins, flavonols, flavones, isoflavonoids)[4]. In particular, phenolic compounds have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals[5]. DNA is probably the most biologically significant target of oxidative attack and it is widely thought that continuous oxidative damage to DNA is a significant contributor to the age-related development of the major cancers[6]. Plant products are widely used in testing because of their low toxicity and great medicinal value. Much research has concentrated on different plant extracts, abilities to induce antioxidant effects[7]. Ascorbic acid also has antioxidant activity and is essential for the maintenance of normal function of living cells[8]. It has been reported that the majority of drugs come from natural resources and that approximately 60-80 % and of the world's population still believe in folk/traditional medicine[9]. Medicinal plants are therefore the main source of new pharmaceutical and health care products[10]. Currently, there is growing interest towards natural antioxidants of plant resources. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems. The world is gradually turning to herbal formulations which are known to be effective against a large repertoire of diseases and ailments[11]. *Bergia ammannioides* (Henye ex Roth) commonly known as Rohwan in Pakistan, belongs to a family of Elatinaceae. Its flowering period is from November to June[12].

Elatinaceae is a small aquatic and semi-aquatic family comprising of 40 species. The whole plant infusion of *Bergia ammannioides* is applied to cure scabies, wounds and cuts[13]. Other plants of the genus Bergia are also show medicinal properties, *e.g*. *Bergia suffruticosa* act as antioxidant and knownto exhibit free-radical scavenging activity[14]. The *Bergia suffruticosa* is traditionally used in Burkina Faso to treat childhood malaria and it is also used as a tonic[15]. It is also used traditionally to repair bones and applied as a poultice on sores. Ethnomedically, it is used as an antidote to scorpion sting and for stomach troubles. The plant has been reported to show antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*[14]. Four compounds *viz*., gallicin, gallicacid, lupeol and β-sitosterol have been reported from *B. suffruticosa*[16].

To the best of our knowledge, no detailed antioxidant studies have been carried out yet on *Bergia ammannioides*. Therefore, in continuation of our current interests to explore safe antioxidants from plant origin[17,18], in the present investigation, we described the comparative *in vitro* antioxidant potential of aqueous and organic fractions of this species by four methods *i.e.*, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging, total antioxidant activity, FRAP assay and ferric thiocyanate assay along with determination of their total phenolic contents relative to conventionally used standards.

EXPERIMENTAL

DPPH$^{\bullet}$ (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox, gallic acid, Follin Ciocalteu's phenol reagent and BHT (butylated hydroxy toluene) 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, lenoleic acid, Tween-20 and ammonia from Merck (Pvt.) Ltd. (Germany).

The plant *Bergia ammannioides*, was collected from Azad Kashmir (Pakistan) in September 2011 and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen (GC. Herb. Bot. 985), 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 (733 g) was exhaustively extracted with methanol on soxhlet apparatus. The extract was evaporated on rotary evaporator (laborta 4000-efficient Heidopph) at 42 ºC under vacuum to yield the residue (83 g), which was dissolved in distilled water (500 mL) and partitioned with *n*-hexane (300 mL × 4), chloroform (300 mL × 4), ethyl acetate (300 mL × 4) and *n*-butanol (300 mL × 4), respectively. These organic fractions and remaining water fraction were concentrated separately on rotary evaporator. The yields of *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were 20, 12, 19, 18 and 13 g, respectively. All these fractions were used to screen the contained phytochemicals followed by evaluation of their *in vitro* antioxidant potential.

Phytochemical screening: The phytochemical screening was performed using the standard methods[19-21] as described as under.

Test for alkaloids: For the test of alkaloids, the TLC card having spots of the studied samples was sprayed with Draggen dorff's reagent. Appearance of orange colour indicates the presence of alkaloids.

Test for terpenoids: Two methods were used to test the presence of terpenoids. First, ceric sulphate solution was sprayed on TLC card having spots of samples. TLC card was heated on TLC heater. Appearance of brown colour indicates the presence of terpenoids. Second, to 0.5 g of each of the extract was added 2 mL of chloroform. 3 mL of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

Test for saponins: To 0.5 g of extract was added 5 mL of distilled water in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for tannins: 2 mL of sample was taken in test tube and 5 mL of *n*-butanol-HCl solution was added. Mixture was warmed for 1 h at 95 ºC in a water bath. Appearance of red colour indicated the presence of tannins.

Test for sugars: Sample solutions (0.5 g in 5 mL water) were added to boiling Fehling's solution (A and B) in a test tube. Formation of red precipitates indicated the presence of sugars.

Test for phenolics: Neutral ferric chloride was added to each fraction. Appearance of bluish green colour indicated the presence of phenolics.

Test for flavonoids: Four methods were used to test for flavonoids. First, dilute ammonia (5 mL) was added to a portion of sample solution in water. Concentrated sulphuric acid (1 mL) was added. A yellow colouration that disappears on standing indicated the presence of flavonoids. Second, a few drops of 1 % aluminium chloride solution were added to sample solution. A yellow colouration indicated the presence of flavonoids. Third, the TLC card having spots of samples was sprayed with Benedict's reagent. Green fluorescence in UV light indicated the presence of flavonoids. Fourth, the TLC card having spots of samples was sprayed with lead acetate solution. Green fluorescence in UV light indicated the presence of flavonoids.

Test for cardiac glycosides (Keller-Killiyani test): To 0.5 g of each sample diluted to 5 mL in water was added 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Antioxidant assays: The 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,

ascorbic acid using the reported method[17]. Briefly, various concentrations of the samples (1000, 500, 250, 125, 60, 30 and 15 µg/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 1 h. 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 decolouration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

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

Total antioxidant activity: The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method[22]. 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 ºC for 90 min. After the samples were 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[23] with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa \cdot 3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl 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 $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 ºC before using. The solutions of plant samples (500 µg/mL) and that of Trolox were formed in methanol.10 µL of each of the sample solution and BHT solution were taken in separate test tubes and 2990 µL of FRAP solution was added in each to make the total volume up to 3 mL. The plant samples were allowed to react with FRAP solution in the dark for 0.5 h. 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 using the method of Makkar *et al.*[24]. 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 min, absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolic contents 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 method[25]. 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) and 2 mL of phosphate buffer (0.02 M, pH 7). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40 ºC. The mixture without extract was used as control. The mixture (0.1 mL) was taken and mixed with 5 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 min 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]. 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 ± SEM. Statistical analysis were determined using one way analysis of variance (ANNOVA) followed by post-hoc Tukey's test.

RESULTS AND DISCUSSION

Phytochemical screening: The phytochemical screening was done on all the studied fractions and results have been shown in Table-1. It was observed from the results that chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction contained phenolics and flavonoids, while all these were absent in *n*-hexane soluble fraction. The remaining aqueous fraction showed the presence of saponins, tannins, sugars and phenolics. Cardiac glycosides were only present in ethyl acetate soluble fraction and *n*-butanol soluble fraction. Terpenes were detected in all the fractions except remaining aqueous fraction. Tannins, saponins and sugars were present in *n*-butanol soluble fraction and remaining aqueous fraction. Alkaloids were only present in ethyl acetate fraction.

DPPH radical scavenging capacity: The effects of phenolic compounds on DPPH radical scavenging are thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[26]. It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discolouration from purple to yellow[27]. The greater the discolouration of the methanolic solution of DPPH radical, the lower will be the absorbance of the reaction mixture, thereby indicating significant free radical scavenging capacity. The various fractions of *Bergia ammannioides* were tested by this assay and the values of per cent scavenging of DPPH radical have been shown in Table-2. It was observed that activity was increased by increasing the concentration of the samples in the assay mixture. The various concentrations of ethyl acetate soluble fraction exhibited highest per cent inhibition of DPPH radical as compared to other fractions. It showed 81.16 ± 0.87 %

TABLE-1
PHYTOCHEMICAL CONSTITUENTS OF VARIOUS FRACTIONS OF *Bergia ammannioides* (HENYE EX ROTH)

Test	*n*-Hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate soluble fraction	*n*-Butanol soluble fraction	Remaining aqueous fraction
Alkaloids	–	–	+	–	–
Terpenoids	++	++	+++	+	–
Saponins	–	–	–	+	+
Tannins	–	–	–	+	+
Sugars	–	–	–	++	+++
Phenolics	–	++	+++	++	–
Flavonoids	–	++	+++	++	+
Cardiac glycosides	–	–	+	+	–

'+' Represents presence and '–' represents absence.

inhibition of DPPH radical at a concentration of 120 µg/mL. Chloroform soluble fraction also showed good scavenging activity. 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 per cent inhibition greater than 60 % were found to be significant ($p < 0.05$). IC_{50} values (concentration of sample required to scavenge 50 % free radical) were also calculated and results have been shown in Table-3. IC_{50} value is inversely related to the activity. In the present study, ethyl acetate soluble fraction showed lowest IC_{50} value (26.65 ± 1.8 µg/mL) while chloroform fraction showed IC_{50} value 72.83 ± 1.02 µg/mL relative to ascorbic acid, a reference standard having IC_{50} 58.8 ± 0.89 µg/mL. Remaining aqueous fraction and *n*-butanol soluble fraction showed moderate activity (124.63 ± 0.7 and124.51 ± 1.04 µg/mL, respectively) while *n*-hexane soluble fraction showed no significant activity. The IC_{50} values of chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction was found to be non-significant ($p > 0.05$) when compared with ascorbic acid, a reference standard.

Total antioxidant activity: Total antioxidant activity of the studied fractions was determined by phosphomolybdenum complex formation method. 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[22]. The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids. From results (Table-3), it was observed that ethyl acetate soluble fraction showed highest total antioxidant activity *i.e.*, 1.142 ± 0.08 as compared to other fractions. Chloroform soluble fraction also showed good total antioxidant activity (1.007 ± 0.06). Remaining aqueous fraction (0.605 ± 0.04) and *n*-butanol soluble fraction (0.654 ± 0.03) showed moderate activity while *n*-hexane soluble fraction showed no significant activity. The results were compared with BHT whose total antioxidant activity was found to be 1.22 ± 0.08. The total antioxidant activity shown by chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and

TABLE-2
DPPH RADICAL SCAVENGING ACTIVITY OF *Bergia ammannioides* (HENYE EX ROTH)

S. No.	Sample	Conc.(µg/mL)	Scavening (%) of DPPH radical ± SEM[a]
1	*n*-Hexane soluble fraction	1000, 500, 250, 120	59.23±1.48*, 38.43±0.44, 32.72±0.22, 27.38±0.34
2	Chloroform soluble fraction	1000, 500, 250, 120	89.09±1.2*, 75.21±1.05*, 56.87±0.79*, 49.58±1.01
3	Ethyl acetate soluble fraction	120, 60, 30, 15	81.16±0.87*, 66.17±0.34*, 41.75±0.23, 53.14±0.76
4	*n*-Butanol soluble fraction	250, 120, 60, 30, 15	77.19±0.89*, 58.83 ±1.09*, 32.71±1.4, 28.74 ± 0.87, 56.58±0.56
5	Remaining aqueous fraction	250, 120, 60, 30	79.30±0.98*, 56.87±1.12*, 31.84±0.8, 22.18±0.34
6	Ascorbic acid[b]	125, 60, 30	79.14± 0.93, 58.09 ±0.86, 29.97± 0.55

[a]Standard mean error of three assays. [b]A reference standard antioxidant.

TABLE-3
IC_{50}, TOTAL ANTIOXIDANT ACTIVITY, FRAP VALUES, TOTALPHENOLICS AND LIPID PEROXIDATION OF DIFFERENT FRACTIONS OF *Bergia ammannioides* (HENYE EX ROTH)

S. No.	Sample	IC_{50} (µg/mL)	Total antioxidant activity	FRAP value TE (µM/mL)	Total phenolics (GAE mg/g)	Inhibition of Lipid peroxidation (%)
1	*n*-Hexane soluble fraction	777.5 ± 1.5	0.25 ± 0.07	42.1 ± 0.8	15.83 ± 1.23	12.09 ± 0.8
2	Chloroform soluble fraction	72.83 ± 1.02**	1.007 ± 0.06**	137.8 ± 0.2*	103.99 ± 1.4*	41.31 ± 0.53**
3	Ethyl acetate soluble fraction	26.65 ± 1.8**	1.142 ± 0.08**	378.4 ± 0.4*	226.109 ± 0.3*	54.23 ± 0.57**
4	*n*-Butanol soluble fraction	124.63 ± 0.7**	0.654 ± 0.03**	105.5 ± 0.3*	78.38 ± 1.21*	30.15 ± 0.9
5	Remaining aqueous fraction	124.51 ± 1.04**	0.605 ± 0.04**	92.5 ± 0.9*	73.05 ± 0.9*	25.01 ± 0.96
6	Ascorbic acid[a]	58.8 ± 0.89	–	–	–	–
6	BHT[a]	–	1.22 ± 0.08	–	–	62.48 ± 1.07
7	Blank	–		24.11 ± 0.95	14.96 ± 0.56	–

All results are presented as mean ± standard mean error of three assays. [a]Standard antioxidant. *$p < 0.05$ when compared with negative control *i.e.*, blank/solvent ($p < 0.05$ is taken as significant). **$p < 0.05$ when compared with reference standards (BHT/Ascorbic acid).

remaining aqueous fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction was found to be non-significant ($p > 0.05$) when compared with BHT, a reference standard.

Ferric reducing antioxidant power (FRAP) assay: The ferric reducing antioxidant power (FRAP) assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating antioxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based onthe ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine [TPTZ] forming an intense blue Fe^{2+}-TPTZ complex with an absorbance maximum at 593 nm[23]. Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied fractions were calculated and results are shown in Table-3. Among all the fractions, the ethyl acetate fraction showed highest FRAP value (378.5 ± 0.4 TE µM/mL). FRAP values of the *n*-hexane soluble fraction, chloroform soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were 42.1 ± 0.8, 137.8 ± 0.2, 105.5 ± 0.3 and 92.5 ± 0.9 TE µM/mL, respectively. High FRAP values may be ascribed partially to the presence of phenolic and flavonoid contents. The results were compared with the blank having value 24.11 ± 0.95. The FRAP values shown by chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction was found to be non-significant ($p > 0.05$) when compared with blank.

Total phenolic contents: The antioxidative potential of phenolic compounds can be attributed to their strong capability to transfer electrons to ROS/free radicals, chelating metal ions, activate antioxidant enzymes and inhibitory oxidases[28]. Phenolic compounds and flavonoids are important plant secondary metabolites. These compounds have numerous defense functions in plants and thus several environmental factors, such as light, temperature, humidity and internal factors, including genetic differences, nutrients, hormones *etc*., contribute to their synthesis[29]. Similarly, other factors, such as germination, degree of ripening, variety, processing and storage, also influence the content of plant phenolics[30]. It was reported that the phenolics are responsible for the variation in the antioxidant activity of the plant. They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals or chelate metal ions and protect against pathogens and predators[31]. Most commonly encountered flavonoids are flavonols, quercetin, flavanols and anthocyanins. Table-3 shows the phenolic concentration in the different fractions, expressed as milligram of gallic acid equivalents (GAEs) per gram of fraction. Among these five fractions, the ethyl acetate soluble fraction showed the highest amount of total phenolic contents (226.109 ± 0.3 GAE mg/g). The total phenolic contents of *n*-hexane soluble fraction, chloroform soluble fraction, *n*-butanol soluble fraction and aqueous fraction were found to be15.83 ± 1.23, 103.99 ± 1.4, 78.38 ± 1.21 and 73.05 ± 0.9 GAE mg/g, respectively. The values of total phenolic contents shown by chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction was found to be non-significant ($p > 0.05$) when compared with blank.

Ferric thiocyanate (FTC) assay: The antioxidant activity of these fractions was also tested using the ferric thiocyanate (FTC) method. Peroxidation of lipids occurs both *in vivo* and *in vitro* and gives rise to cytotoxic and reactive products. These products disturb the normal functioning of the cell and can give rise to damaged or modified DNA. Oxygen reacts with unsaturated double bond on the lipid which results in generation of free radicals and lipid hydroperoxides. Hydrogen donating antioxidants can react with lipid peroxyl radicals and break the cycle of generation of new radicals. The FTC method is used to measure the amount of peroxide at the beginning of lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce ferric thiocyanate, a reddish pigment[32]. The results revealed that the ethyl acetate soluble fraction showed the highest inhibition of lipid peroxidation (54.23 ± 0.57 %) and the *n*-hexane soluble fraction showed the lowest value (12.09 ± 0.8 %) at a concentration of 500 µg/mL. Values exhibited by chloroform soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were found to be 41.31 ± 0.53, 30.15 ± 0.9 and 25.01 ± 0.96 %, respectively. The inhibition of lipid peroxidation shown by BHT was 62.48 ± 1.07 % (Table-3). The values shown 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 was found to be non-significant ($p > 0.05$) when compared with BHT.

Conclusion

It was observed from the results that ethyl acetate fraction was enriched with more phenolics and flavonoids. Due to presence of such compounds, ethyl acetate soluble fraction showed highest antioxidant activity as compared to other studied fractions. It exhibited highest value of percentage inhibition of DPPH (81.16 ± 0.87) at concentration of 120 µg/mL. The IC_{50} of this fraction was 26.65 ± 1.8 µg/mL, relative to ascorbic acid, having IC_{50} 58.8 ± 0.89 µg/mL. It also showed highest total antioxidant activity *i.e*., 1.142 ± 0.08, highest FRAP value (378.5 ± 0.4 TE µM/mL) as well as highest total phenolic contents *i.e*., 226.109 ± 0.3 GAE mg/g and highest value of percent inhibition of lipid peroxidation (54.23 ± 0.57 %) as compared to the studied fractions. Chloroform soluble fraction also showed good results. So generally, it was concluded that chloroform soluble fraction and ethyl acetate fraction are rich in strong antioxidants. These fractions are potentially valuable sources of natural antioxidants and bioactive materials, which would be expected to increase shelf life of foods and buttress against peroxidative damage in living systems in relation to aging, carcinogenesis, neurodegenerative diseases and coronary heart diseases *etc*.

REFERENCES

1. J.M. David, A.L.B.S. Barreisors and J.P. David, *Pharm. Biol*., **42**, 36 (2004).
2. M.G. Hertog, E.J. Feskens, P.C. Hollman, M.B. Katan and D. Kromhout, *Lancet*, **342**, 1007 (1993).
3. J.V. Anderson, R.D. Palombo and R. Earl, *J. Am. Diet. Assoc*., **98**, 205 (1998).

4. N.M. Elsayed and A. Bendich, *Nutr. Res*., **21**, 551 (2001).
5. C.A. Rice-Evans, N.J. Muller, P.G. Bolwell, P.M. BramLey and J.B. Pridham, *Free Radic. Res*., **22**, 375 (1995).
6. J.R. Totter, *Proc. Nat. Acad. Sci*., **77**, 1763 (1980).
7. D.J. Newman, G.M. Cragg and K.M. Snader, *Nat. Prod. Rep*., **17**, 215 (2000).
8. N. Peckenpaugh, Nutrition Essentials and Diet Therapy, Saunders Elsevier, St. Louis, USA, pp. 106-108 (2010).
9. S. Kumar, P.K. Suresh, M.R. Vijayababu, A. Arunkumar and J. Arunakaran, *J. Ethnopharmacol*., **105**, 246 (2006).
10. S. Tewtrakul, S. Subhadhirasakul, C. Karalai, C. Ponglimanont and S. Cheenpracha, *Food Chem*., **115**, 534 (2009).
11. Aziz-ur-Rehman, S. Gulzar, M.A. Abbasi, T. Shahzadi, T. Riaz, S.Z. Siddiqui and M. Ajaib, *J. Serb. Chem. Soc*., **76**, 1379 (2011).
12. A. Ghafoor, In eds.: E. Nasir and S.I. Ali, Flora of West Pakistan. Elatinaceae, Department Botany, University of Karachi, 19: pp. 1-4 (1972).
13. L.R. Dangwal, A. Sharma, K. Naveen, C.S. Rana and S. Upvan, *Researcher*, **2**, 49 (2010).
14. S. Anandjiwala, H. Srinivasa, J. Kalola and M. Rajani, *J. Nat. Med*., **61**, 59 (2007).
15. J. Olivia, L. Angenot, M. Tits, J.P. Nicolas, P.D. Mol, J.B. Nikiéma and M. Frederich, *J. Ethnopharmacol*., **130**, 143 (2010).
16. S. Anandjiwala, H. Srinivasa and M. Rajani, *Chromatographia*, **66**, 725 (2007).
17. Aziz-ur-Rehman, A. Abbas, T. Riaz, S. Ahmad, Shahzaman, M.A. Abbasi, S.Z. Siddiqui and M. Ajaib, *Asian J. Pharm. Biol. Res*., **1**, 534 (2011).
18. I. Hafeez, Aziz-ur-Rehman, I. Mariam, T. Riaz, M.A. Abbasi, S.Z. Siddiqui, M. Ajaib and I. Ahmad, *Asian J. Chem*., **24**, 4436 (2012).
19. A. Sofowara, Medicinal Plants and Traditional Medicine in Africa. Spectrum books, Ibadan, p. 150 (1993).
20. G.E. Trease and W.C. Evans, Pharmacognosy, Bailliere Tindall, edn. 13, pp. 176-180 (1989).
21. G.A. Ayoola, H.A.B. Coker, S.A. Adesegun, B.A.A. Adepoju, K. Obaweya, E.C. Ezennia and T.O. Atangbayila, *Trop. J. Phar. Res*., **7**, 1019 (2008).
22. P. Prieto, M. Pineda and M. Aguilar, *Anal. Biochem*., **269**, 337 (1999).
23. I.E.F. Benzie and J.J. Strain, *Anal. Biochem*., **239**, 70 (1996).
24. H.P.S. Makkar, M. Bluemmel, N.K. Borowy and K. Becker, *J. Sci. Food Agric*., **61**, 161 (1993).
25. P. Valentao, E. Fernandes, F. Carvalho, P.B. Andrade, R.M. Seabra and M.L. Bastos, *J. Agric. Food Chem*., **50**, 4989 (2002).
26. M.N. Siddaraju and S.M. Dharmesh, *J. Agric. Food Chem*., **55**, 7377 (2007).
27. S.J. Meir, B. Kanner and S.P.H. Akiri, *J. Agric. Food Chem*., **43**, 1813 (1995).
28. P. Cos, L. Ying, M. Calomme, J.P. Hu, K. Cimanga, B. van Poel, L. Pieter, A.J. Vlietinck and D.V. Berghe, *J. Nat. Prod*., **61**, 71 (1998).
29. D. Strack, In eds.: P.M. Dey and J.B. Harborne, Phenolic Metabolism, In Plant Biochemistry, Academic Press, San Diego, pp. 387-416 (1997).
30. L. Bravo, *Nutr. Rev*., **56**, 317 (1998).
31. T. Riaz, M.A. Abbasi, T. Shahzadi, A. Rehman, S.Z. Siddiqui and M. Ajaib, *J. Med. Plants. Res*., **5**, 4180 (2011).
32. F. Aqil, I. Ahmad and Z. Mehmood, *Turk. J. Biol*., **30**, 177 (2006).