Journal of Medicinal Plants Research Vol. 5(30), pp. 6597-6603, 16 December, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals
DOI: 10.5897/JMPR10.528

Full Length Research Paper

In vitro assessment of fortification from oxidative stress by various fractions of *Rhynchosia pseudo-cajan*

Tauheeda Riaz[1], Muhammad Athar Abbasi[1]*, Aziz-ur-Rehman[1], Tayyaba Shahzadi[1], Sabahat Zahra Siddiqui[1], Hira Khalid[1] and Muhammad Ajaib[2]

[1]Department of Chemistry, Government College University, Lahore-54000, Pakistan.
[2]Department of Botany, Government College University, Lahore-54000, Pakistan.

Accepted 22 October, 2010

The methanolic extract of *Rhynchosia pseudo-cajan* Cambess. 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: 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. All the fractions showed noteworthy antioxidant potential. The results revealed that ethyl acetate soluble fraction showed highest value of % inhibition of DPPH (92.45% ± 0.98) at concentration of 125 µg/ml. The IC_{50} of this fraction was 22.75 ± 0.27 µg/ml, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/ml. It also showed highest total antioxidant activity, that is, 1.946 ± 0.22 and total phenolic contents (129.49 ± 1.2 mg of gallic acid equivalents) as compared to other fractions. However, the crude methanolic extract displayed highest FRAP value (639.66 ± 1.9 µg of trolox equivalents) as well as highest value of inhibition of lipid peroxidation (54.48% ± 1.18) as compared to the studied fractions.

Key words: *Rhynchosia pseudo-cajan* Cambess., DPPH assay, total antioxidant activity, FRAP value, total phenolics, inhibition of lipid peroxidation (%).

INTRODUCTION

Free radicals are constantly generated *in vivo* for physiological purposes. They can be over produced in pathological conditions, causing oxidative stress. A large number of civilization-associated diseases such as autoimmune diseases, inflammation, cardiovascular-neurological diseases, cancer and aging are attributed to oxidative stress. An adequate intake of natural antioxidants could protect macromolecules against oxidative damage in cells. The term antioxidant refers to free radical scavengers, inhibitors of lipid per oxidation and chelating agents. Phenolic compounds possess a wide spectrum of biological effects including antioxidant and free radical scavenging (Khanavi et al., 2009). The most effective way to eliminate free radicals which cause the oxidative stress is with the help of antioxidants. Antioxidants, both exogenous and endogenous, whether synthetic or natural, can be effective in preventing free radical formation by scavenging them or promoting their decomposition and suppressing many disorders (Souri et al., 2008). Number of synthetic antioxidants such as BHA, BHT and TBHQ, have been added to foodstuffs. Although these synthetic antioxidants are efficient and relatively cheap, there are some disadvantages, because they are suspected of having some toxic properties. Therefore search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones (Khan et al., 2010).

Currently, there is growing interest towards natural antioxidants of herbal 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 (Souri et al., 2008). *Rhynchosia pseudo-cajan* Cambess.

*Corresponding author. E-mail: atrabbasi@yahoo.com. Tel: (+92)-42-111000010 ext 264.

(papilionaceae) is a common weed which is medicinally important. It is an erect shrub having whitish leaflets on lower side. Its common name is Lahrr. It is distributed in Azad Jammu and Kashmir, Pakistan. Its leaves are used as tonic and stomach disorders, leaves, and bark powder is useful for peptic ulcer and other digestive problems (Ajaib et al., 2010). The crude drugs of *Rhynchosia* are used in the formation of fermented liquor having anti-oxidative, anti-tumor, skin whitening and arthritis preventing and treating effects (Lee and Soon, 2006). So far no phytochemical studies have been carried out on this species, many important compounds have been discovered from other species of the genus *Rhynchosia*. Tirumalin, a prenylated dihydroflavanol, was isolated from *Rhynchosia cyanosperma* (Dama et al., 1980). Rhynchosin, a new 5-deoxyflavonol, was isolated from leaves of *Rhynchosia beddomei* (Dama et al., 1980). The leaves of *Rhynchosia rufescenes* contain methylated flavonols such as kaempferol, kaempferol 3-methyl ether and quercetin 3-methyl ether which are of chemotaxonomic importance (Dama and Posupulati, 1984). Many flavonoides have been discovered from various *Rhynchosia* species (Dama et al., 1985). Many glycosides have also been isolated from *Rhynchosia rothii* (Rao and Gunasikar, 1987). To the best of our knowledge, no detailed antioxidant studies have been carried out on *R. pseudo-cajan* Cambess.; 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, FRAP assay and ferric thiocyanate assay along with determination of their total phenolic contents relative to conventionally used standards.

MATERIALS AND METHODS

Plant material

The plant *R. pseudo-cajan* Cambess. was collected from Kotli, Azad Kashmir in June 2009, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A voucher specimen (GC.Herb.Bot. 623) has been deposited in the herbarium of the same university.

Extraction and fractionation of antioxidants

The shade-dried ground whole plant (15 kg) was exhaustively extracted with methanol (20 L × 4) at room temperature. The extract was evaporated to yield the residue (950 g), which was dissolved in distilled water (2 L) and partitioned with *n*-hexane (1.5 L × 4), chloroform (1.5 L × 4), ethyl acetate (1.5 L × 4) and *n*-butanol (1.5 L × 4), respectively. These organic fractions and remaining water fraction were concentrated separately on rotary evaporator. The yield of *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction was obtained as 203, 226 198, 185 and 138 g, respectively. All these fractions were used to evaluate their *in vitro* antioxidant potential. As no phytochemical studies have been done on *R. pseudo-cajan* Cambess. as yet; therefore, we are unable to predict how complex these fractions are.

Chemicals and standards

DPPH$^{\cdot}$ (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin Ciocalteu 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 and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

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 method of Lee and Shibamoto (2001). Briefly, various amounts of the samples (1000, 500, 250, 125, 60, 30, 15, 8 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 one hour. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity. The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

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 (Prieto et al., 1999). 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℃ for 90 min. 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 butylated hydroxytoluene (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 (1996) 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 HCl, and 20 mM $FeCl_3.6H_2O$ 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℃ before use. 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 min. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. 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 by the method of Makkar et al. (1993). The 0.1 ml (0.5 mg/ml) of sample was combined with 2.8 ml of 10% Na_2CO_3 and 0.1 ml of 2N Folin-Ciocalteu reagent. After 40 min, absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. The standard curve was linear between 50 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 (Valentao et al., 2002). 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°C. The mixture without extract was used as control. The 0.1 ml of the mixture 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 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]. 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 was determined using one way analysis of variance (ANOVA) followed by post-hoc Turkey's test.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples. The method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus, reduces it to become stable diamagnetic molecule. DPPH radical decolourizes in the presence of antioxidants. It contains an odd electron which is responsible for visible deep purple colour (Lee and Shibamoto, 2001). The proton transfer from antioxidant to DPPH radical depends upon the structure of the antioxidant. The colour changes from purple to yellow after reduction and its absorbance at wavelength 517 nm decreases (Jalili et al., 2010). In the present study, the aqueous and organic fractions of *R. pseudo-cajan* Cambess. were evaluated for their free radical scavenging activity using the DPPH radical assay. The compounds which contain hydroxyl groups may donate hydrogen to free radical to reduce the DPPH radical. So many hydroxyl containing compounds such as flavonoides, glycosides, etc have been isolated from Rhynchosia species (Dama et al., 1985; Rao and Gunasikar, 1987), so these compounds may also be present in *R. pseudo-cajan* Cambess. due to the fact that it showed very good DPPH Radical Scavenging Activity. Reduction of DPPH radical was observed by the decrease in absorbance at 517 nm. These fractions reduced DPPH radicals significantly. The values of percent scavenging of DPPH radical are shown in Table 1. It was observed that activity was increased by increasing the concentration of the samples. The various concentrations of ethyl acetate soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 92.45% ± 0.98 inhibition of DPPH radical at a concentration of 125 µg/ml. The various concentrations of the fractions which showed percent inhibition greater than 60% were found to be significant ($p < 0.05$). The IC_{50} value for each fraction, defined as the concentration of sample causing 50 percent inhibition of free radicals, was determined and tabulated (Table 2). Since IC_{50} is a measure of inhibitory concentration, a lower value would reflect greater antioxidant activity of the fraction. Ethyl acetate soluble fraction of this plant revealed lowest IC_{50} value (22.75 ± 0.27 µg/ml) as compared to other fractions so it had highest antioxidant activity, followed by *n*-butanol soluble fraction (IC_{50} 55.86 ± 1.69 µg/ml), methanolic extract (IC_{50} 60.08 ± 2.06 µg/ml), aqueous fraction (IC_{50} 122.94 ± 0.95 µg/ml), chloroform soluble fraction (IC_{50} 124.76 ± 2.85 µg/ml) and *n*-hexane soluble fraction (IC_{50} 167.32 ± 14.0 µg/ml) relative to butylated hydroxytoluene (BHT), a reference standard, having IC_{50} of 12.1 ± 0.92 µg/ml. The IC_{50} values of chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction, aqueous fraction and methanolic extract 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.

Total antioxidant activity by phosphomolybdenum complex method

Total antioxidant activity of the studied fractions was determined by phosphomolybdenum method. This

Table 1. Free radical scavenging activity of various fractions of *Rhynchosia pseudo-cajan* Cambess. using 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH).

S/N	Sample	Concentration in assay (µg/4 ml)	% Age scavenging of DPPH radical ± S.E.M[a)]
1	Crude methanolic extract	500	89.32 ± 0.98*
		250	66.85 ± 1.30*
		125	51.62 ± 1.88
		60	40.38 ± 0.99
2	*n*-Hexane soluble fraction	1000	87.76 ± 0.84*
		500	74.51 ± 0.14*
		250	57.85 ± 0.17
		125	47.40 ± 0.29
		60	38.16 ± 1.73
3	Chloroform soluble fraction	500	87.60 ± 0.73*
		250	66.83 ± 0.81*
		60	41.23 ± 1.02
4	Ethyl acetate soluble fraction	125	92.45 ± 0.98*
		60	75.63 ± 0.15*
		30	59.28 ± 0.68
		15	43.28 ± 0.63
		8	39.63 ± 1.8
5	*n*-Butanol soluble fraction	250	91.52 ± 0.68*
		125	72.54± 0.91*
		60	52.80 ± 0.29
		30	43.15 ± 0.54
		15	38.07 ± 2.0
6	Aqueous fraction	500	89.32 ± 1.20*
		250	66.85 ± 1.6*
		125	51.62 ± 0.2
		60	40.38 ± 0.79
7	BHT[b)]	60	91.25 ± 0.13*
		30	75.56 ± 0.07*
		15	42.67 ± 0.04
		8	23.57 ± 0.31

[a)] Standard mean error of three assays; [b)] Standard antioxidant; *p < 0.05 when compared with negative control, that is, blank/solvent (p<0.05 is taken as significant).

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 (Prieto et al., 1999). The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids. As most species of Rhynchosia contain flavonoides which also contain phenolic groups, so *R. pseudo-cajan* Cambess. may also contain flavonoides due to the fact that it showed good total antioxidant activity (Dama et al., 1985; Rao and Gunasikar, 1987). The total antioxidant activities of these fractions were compared with the standard antioxidant BHT and the results are shown in Table 2. The results indicated that ethyl acetate soluble fraction had highest total antioxidant activity (1.946 ± 0.22). The antioxidant activity of the various plant fractions was decreased

Table 2. IC_{50}, total antioxidant activity, FRAP values, total phenolics and lipid peroxidation inhibition values of different fractions of *Rhynchosia pseudo-cajan* Cambess.

S/N	Sample	IC_{50} of DPPH assay (µg/mL) ± S.E.M [a)]	Total antioxidant activity ± S.E.M [a)]	FRAP value TE (µg/ml) ± S.E.M [a)]	Total phenolics (GAE mg/g of sample) ± S.E.M [a)]	Inhibition of lipid peroxidation (%) ± S.E.M [a)]
1	Methanolic extract	60.08 ± 2.06**	1.220 ± 0.14**	639.66 ± 1.9*	108.95 ± 1.90*	54.48 ± 1.18**
2	*n*-Hexane soluble fraction	167.32 ±14.0	1.223 ± 0.07**	49.29 ± 1.7	76.00 ± 1.4	38.63 ± 0.62
3	Chloroform soluble fraction	124.76 ± 2.85**	1.419 ± 0.03**	119.6 ± 1.3*	102.82 ± 1.2*	51.27± 0.70**
4	Ethyl acetate soluble fraction	22.75 ± 0.27**	1.946 ± 0.22**	348.24 ± 1.8*	129.49 ± 1.2*	50.16 ± 1.2**
5	*n*-Butanol soluble fraction	55.86 ± 1.69**	1.470 ± 0.15**	108.87 ± 2.0*	92.36 ± 1.27*	53.16 ± 0.34**
6	Aqueous fraction	122.94 ± 0.95**	1.148 ± 0.10	125.67 ± 1.5*	85.69 ± 0.68*	42.32 ± 0.55
7	BHT [b)]	12.1 ± 0.92	1.2186 ± 0.09	-	-	62.91 ± 0.60

[a)] Standard mean error of three assays; [b)] Standard antioxidant; *p< 0.05 when compared with negative control, that is, blank/solvent (p<0.05 is taken as significant); **p < 0.05 when compared with reference standard (BHT).

in the following order: ethyl acetate soluble fraction > *n*-butanol soluble fraction (1.470 ± 0.15) > chloroform soluble fraction (1.419 ± 0.03) > *n*-hexane soluble fraction (1.223 ± 0.07) > methanolic extract (1.220 ± 0.14) > aqueous fraction (1.148 ± 0.10). BHT showed total antioxidant activity 1.2186 ± 0.09. Thus, it was obvious from the results that ethyl acetate soluble fraction displayed highest phenolic content while aqueous fraction had lowest phenolic content. The total antioxidant activity shown by *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and methanolic extract were found to be significant ($p < 0.05$) while that of aqueous fraction was found to be non significant ($p > 0.05$) when compared with BHT, a reference standard.

Ferric reducing antioxidant power (FRAP) assay

FRAP is a simple direct test for measuring antioxidative contents. This method was initially developed to assay plasma antioxidant capacity, but can be used with plant extracts too (Gourine et al., 2010). The ferric reducing antioxidant power (FRAP) 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. This assay is based on the 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 (Benzie and Strain, 1996). Increasing absorbance indicates an increase in reductive ability. In the current study, *n*-hexane soluble, chloroform soluble, ethyl acetate soluble, *n*-butanol soluble, methanolic extract and aqueous fractions of this plant species exhibited promising antioxidant power with FRAP values 49.29 ± 1.7, 119.6 ± 1.3, 348.24 ± 1.8, 108.87 ± 2.0, 639.66 ± 1.9 and

125.67 ± 1.5 (µg of trolox equivalent) respectively. The results are shown in Table 2. The value of blank was 20.54 ± 1.7. The crude methanolic extract exhibited a highest FRAP value showing a synergic effect of antioxidant constituents while *n*-hexane soluble fraction expressed lowest FRAP value. Higher FRAP values were obtained for samples extracted in more polar solvents. High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents. The FRAP values of chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction, aqueous fraction and methanolic extract 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

Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Saha et al., 2008). Their antioxidative activities observed can be ascribed both to the different mechanisms exerted by different phenolic compounds and to the synergistic effects of different compounds. These antioxidants have different functional properties, such as reactive oxygen species scavenging, for example, quercetin and catechin, inhibition of the generation of free radicals and chain-breaking activity, for example, *p*-coumaric acids and metal chelation. These compounds like tocopherols, flavonoids, and other organic acids are also effective proton donors (Huang et al., 2005). It has been suggested that the phenolic content of plant material is correlated with their antioxidant activity (Juan and Chou, 2010). Most commonly encountered flavonoides were flavonols, quercetin, flavanols and anthocyanins. Rhynchosia species contain nearly all these compounds so *R. pseudo-cajan* Cambess. showed high antioxidant power due to phenolic contents. Table 2 shows the phenolic concentration in the different fractions, expressed as milligram of gallic acid equivalents (GAEs) per gram of fraction. Among these six fractions, the ethyl acetate soluble fraction showed the highest amount of total phenolic compounds (129.49 ± 1.2 mg/g) followed by the methanolic extract (108.95 ± 1.90 mg/g), chloroform soluble fraction (102.82 ± 1.2 mg/g), *n*-butanol soluble fraction (92.36 ± 1.27 mg/g), aqueous fraction (85.69 ± 0.68 mg/g) and *n*-hexane soluble fraction (76.00 ± 1.4 mg/g) respectively. The value of blank was 19.98 ± 0.9. The results for total phenolic contents of chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction, aqueous fraction and methanolic extract 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 (Aqil et al., 2006). The various fractions of plant were tested by this assay and results are shown in Table 2. None of the fractions showed absorbance value greater than the negative controls (without plant extracts) at the end point of the method, indicating the presence of antioxidant activity. Significantly lower absorbances as compared to control were observed which indicate that these fractions have greater antioxidant activities. The fractions which showed greater values of percent inhibition of lipid peroxidation might contain primary antioxidant compounds, which are able to react aggressively with free radicals, particularly hydroxyl radicals, thereby terminating the radical-chained reaction and retarding the formation of hydroperoxides (Ismail et al., 2010). Highest percentage of inhibition of lipid peroxidation was exhibited by methanolic extract (54.48% ± 1.18), while *n*-hexane soluble fraction had lowest percentage of inhibition of lipid peroxidation (38.63% ± 0.62). Chloroform, ethyl acetate and *n*-butanol soluble fractions exhibited percent inhibition of lipid peroxidation nearly close to each other, that is, 51.27% ± 0.70, 50.16% ± 1.2 and 53.16% ± 0.34 respectively while that of aqueous fraction was 42.32% ± 0.55. The inhibition of lipid peroxidation by BHT (standard) was 62.91% ± 0.60. The results for percent inhibition of lipid peroxidation of chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and methanolic extract 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.

Conclusion

From the results, it is evident that ethyl acetate fraction showed the highest value of percent inhibition of DPPH (92.45% ± 0.98) at concentration of 125 µg/ml. It also showed the highest total antioxidant activity, that is, 1.946 ± 0.22 as well as the highest total phenolic contents (129.49 ± 1.2 mg of gallic acid equivalents) as compared

to other studied fractions. Its IC_{50} was 22.75 ± 0.27 µg/ml, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/ml. However, the crude methanolic extracts displayed highest FRAP value (639.66 ± 1.9 µg of trolox equivalents) and highest value of inhibition of lipid peroxidation (54.48% ± 1.18) as compared to other fractions. So it was concluded that although the polar fractions are rich in strong antioxidants but it is also promising to say that all the fractions of this plant 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. The overall antioxidant activity results for the chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and methanolic extract were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and aqueous fraction were mostly found to be non significant ($p > 0.05$) when compared with BHT/blank.

ACKNOWLEDGEMENT

The authors are thankful to Higher Education Commission of Pakistan for financial support.

REFERENCES

Ajaib M, Khan Z, Khan N, Wahab M (2010). Ethnobotanical studies on useful shrubs of district kotli, azad jammu and Kashmir, Pakistan. Pak. J. Bot., 42: 1407-1415.

Aqil F, Ahmad I, Mehmood Z (2006). Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk. J. Biol., 30: 177-183.

Benzie IEF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Anal. Biochem., 239: 70-76.

Dama A, Duvvuru G, Pasupulati R, Otto S, Hildebebert S (1980). Tirumalin- a new prenylated dihydroflavanol from *Rhynchosia cyanosperma.* Phytochemistry, 19: 478-480.

Dama A, Duvvuru G, Pasupulati R, Otto S, Hildebebert S (1980). Rhynchosin- a new 5-deoxyflavanol from *Rhynchosia beddomei.* Phytochemistry, 19: 483-484.

Dama A, Pasupulati R (1984). Methylated flavonols of Rhynchosia rufescenes. Pharmazie, 39: 714-715.

Dama A, Pasupulati R, Rao KN (1985). Flavonoides from the leaves of seven *Rhynchosia* species. Experientia, 41: 251-252.

Gourine N, Yousfi M, Bombarda , Nadjem B, Stocker P, Gaydou EM (2010). Antioxidant activities and chemical composition of essential oil of *Pistacia atlantica* from Algeria. Ind. Crops Prod., 31: 203-208.

Huang DJ, Chen HJ, Lin CD, Lin YH (2005). Antioxidant and antiproliferative activities of water spinach (*Ipomea aquatica* Forsk) constituents. Bot. Bull. Acad. Sin., 46: 99-106.

Ismail HI, Chan KW, Mariod AA, Ismail M (2010). Phenolic content and antioxidant activity of cantaloupe (cucumis melo) methanolic extracts. Food Chem., 119: 643-647.

Jelili BA, Oyeronke OA, Efere OM, Oyeboade OO (2010). Phytochemicals and *in vitro* antioxidant potentials of defatted methanolic extract of *Holarrhena floribunda* leaves. Afr. J. Biotechnol., 9: 340-346.

Juan MY, Chou CC (2010). Enhancement of antioxidant activity, total phenolic and flavonoid content of black soybeans by solid state fermentation with *Bacillus subtilis*. Food Microbiol., 27: 586-591.

Khan MA, Shahwar D, Ahmad N, Khan Z, Ajaib M (2010). Chemical constituents of *Carissa opaca* extracts and their evaluation as antioxidants and preservatives in edible oils. Asian J. Chem., 22: 379-388.

Khanavi M, Aajimahmoodi M, Niromand MC, Karger Z, Ajani Y, Hadjiakhoondi A, Oveisi MR (2009). Comparison of the antioxidant activity and total phenolic contents in some *Stachys* species. Afr. J. Biotechnol., 8: 1143-1147.

Lee EJ, Soon LK (2006). Republic Korean, Kongkae Taeho Konjbo KR2006 65, 250, 14 June (2006), Appl. 104,049.

Lee K, Shibamoto T (2001). Antioxidant property of aroma extract isolated from clove bud [Syzygiun aromaticum (L.) Merr. et Perry]. Food Chem., 74: 443-448.

Makkar HPS, Bluemmel M, Borowy NK, Becker K (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric., 61: 161-165.

Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric Quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.

Rao CV, Gunasikar D (1987). C-Glycosides from Rhynchosia rothii. Acta Cienc. Indica. Chem., 13(3): 187-189.

Saha MR, Hasan MSR, Akter R, Hossain MM, Alam MS, Alam MA, Mazumder MEH (2008). *In vitro* free radical scavenging activity of methanol extract of the leaves of *Mimusops elengi linn*. Bangl. J. Vet. Med., 6: 197-202.

Souri E, Amin G, Farsam H, Jalalizadeh H, Barezi S (2008). Screening of thirteen medicinal plant extracts for antioxidant activity. Iran. J. Pharm. Res., 7: 149-154.

Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML (2002). Antioxidative properties of cardoon (*Cynara cardunculus* L.) infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. J. Agric. Food Chem., 50: 4989-4993.