In vitro assessment of relief to oxidative stress by different fractions of *Boerhavia procumbens*

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Abstract: Methanolic extract of *Boerhavia procumbens* Bank ex Roxb. was partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol sequentially after dissolving in distilled water. Phytochemical screening showed presence of phenolics, flavonoides and cardiac glycosides in large amount in chloroform, ethyl acetate and *n*-butanol soluble fraction. The antioxidant activity of all these fractions and the remaining aqueous fraction was evaluated by four methods such as: 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, total antioxidant activity and ferric thiocyanate assay. Total phenolics were also determined. Some fractions showed noteworthy antioxidant activity. The results of the antioxidant activity revealed that the ethyl acetate soluble fraction showed the highest value of percent inhibition of DPPH (82.54 ±0.62) at the concentration of 125 µg/ml. The IC_{50} of this fraction was 37.11± 0.23 µg/ml, compared with butylated hydroxytoluene (BHT), which have IC_{50} of 12.1 ± 0.92 µg/mL. It also showed the highest FRAP value (251.08 ± 1.46 µg of trolox equivalents) as well as the highest value of lipid peroxidation inhibition (57.21 ± 52%), the highest total antioxidant activity (0.549 ± 0.08) and also the highest total phenolic contents (77.1 ± 0.6) as compared to the studied fractions. Phytochemical screening showed high percentage of phenolics, flavonoides and cardiac glycosides in this fraction.

Keywords: *Boerhavia procumbens* Bank ex Roxb., DPPH assay, total antioxidant activity, FRAP value, inhibition of lipid peroxidation (%) and total phenolics.

INTRODUCTION

Medicinal plants are in greater demand due to their increased popularity and it is being suggested by a large number of conservation groups, that wild medicinal plants should be brought into cultivation because numerous medicinal plants as well as their purified components have shown beneficial therapeutic potentials. Various herbs and other plant species are reported to show antioxidant activity. Majority of the antioxidant potential is due to the presence of flavones, flavonoids, isoflavones, anthocyanin, lignans, coumarin, catechins and isocatechins (Aqil *et al*., 2004). Antioxidant-based drug products are being used for the treatment and prevention of complicated diseases like atherosclerosis, diabetes, stroke, Alzheimer's disease, and cancer (Devasagayam *et al*., 2004). In living organisms, free radicals are produced as a result of the normal metabolic process, and also free radical chain reactions normally occur as respiratory chain reaction in the mitochondria, through xanthene oxidase activity, liver mixed function oxidases, atmospheric pollutants and from the transitional metal catalysts, xenobiotics, and drugs. In addition to this chemical mobilization of the fat stores in different conditions such as lactation, fever, exercise, infection, and even fasting, may result in enhanced radical activity, and damage. Oxidative injury or free radicals now appears as if the fundamental mechanism, causing a number of the human neurologic and many other disorders. Peroxidation of lipids can be initiated by the oxygen free radical, which in turn stimulates the glycation of protein, inactivation of some enzymes, and alteration in the function and structure of collagen basement and a few other membranes, and also play a role in chronic complication of diabetes (Ara and Nur, 2009). *Boerhavia procumbens* Bank ex Roxb. is a medicinal plant belonging to family Nyctaginaceae and is commonly known as red Hogweed, Punarnanava, spreading Hogweed, Itsit (Punjabi), Sentori (Sind), and Wasao (Blochistan). It is found in South West Asia, Pakistan and India. The plant chiefly contains punarnavine alkaloid. It causes a diuretic effect, activating the kidney gloumeruli by initiating heart functionality and resulting in the provision of a considerable amount of blood flow to that area, and increasing arterial tension. Root and whole herb are commonly used in herbal medicine. It is applied for stimulating stomach, fighting constipation, rejuvenating the system, initiating expectoration and vomiting, boosting the perspiration and urination. The root is specifically used for cleansing the bowel, reducing fevers, and for the killing of intestine helminthes. The list of diseases cured by the red hogweed includes wasting diseases, urethritis, skin diseases, snake and rat bites, rheumatism, nervous system conditions, jaundice, kidney stones, insomnia, hemorrhoids, eye diseases, heart disease, asthma, edema, and alcoholism in chronic situation even (Nasir and Rafiq, 1995; Nasir, 1997). According to our knowledge, as no detailed study on antioxidant activity and phytochemical screening of

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Boerhavia procumbens have been carried out yet, therefore, in the present investigation, we have evaluated the comparative *in vitro* antioxidant capacity of aqueous as well as organic fractions of *Boerhavia procumbens* by four different antioxidant methods such as total antioxidant activity by phosphomolybdenum complex (PC) method, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), scavenging activity, FRAP assay, and ferric thiocyanate assay alongwith determination of their total phenolic contents relative to conventionally used standards.

MATERIAL AND METHODS

Plant material

The plant *Boerhavia procumbens* Bank ex Roxb. was collected in August 2010 from Kotli, Azad Kashmir and Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore identified it. The voucher specimen no. of the plant (GCU.Herb.Bot.857) has been submitted in the Herbarium of Botany Department of the same university.

Extraction and fractionation of antioxidants

The plant was dried in shade. After grinding the whole plant (900g), the antioxidant compounds were extracted with methanol (4.5L × 4) at ambient temperature. The extract was evaporated at rotary evaporator to yield the residue (516g). Then it was dissolved in the distilled water (500ml), and partitioned with *n*-hexane (500ml × 4), chloroform (500ml × 4), ethyl acetate (500ml × 4) and *n*-butanol (400ml × 4) respectively. All these organic fractions and the remaining aqueous fraction were concentrated separately on rotary evaporator. The residues of *n*-hexane soluble fraction (107g), chloroform soluble fraction (76g) ethyl acetate soluble fraction (83g), *n*-butanol soluble fraction (62g), and remaining aqueous fraction (123g) were collected separately. The residues thus obtained were used to study their *in vitro* antioxidant capacity.

Chemicals and standards

DPPH˙ (2,2-Diphenyl-1-picrylhydrazyl radical), trolox, TPTZ (2,4,6-Tripyridyl-s-triazine), BHT (Butylated hydroxytoluene), Gallic acid, and Follin Ciocalteu reagent were obtained from the Sigma Chemical Company Ltd., USA. Organic solvents i.e. *n*-hexane, ethyl acetate, chloroform, and *n*-butanol as well as sulphuric acid, sodium phosphate, ferrous chloride, ammonium molybdate, ferric chlorid, ammonia, aluminium chloride, acetic acid, bismuth nitrate, potassium iodide, and ceric sulphate from Merck (Pvt.) Ltd., Germany.

Phytochemical screening

Phytochemical screening was performed using the standard methods (Sofowara, 1993; Trease and Evans, 1989; Ayoola *et al*., 2008) as described below.

Test for alkaloides

For the test of alkaloides the TLC card having spots of the studied samples was sprayed with Draggendorff's reagent. Appearance of orange colour indicates the presence of alkaloids.

Test for terpenoides

Two methods were adopted to test the presence of terpenoides. First, Ceric sulphate solution was sprayed on TLC card having spots of samples. TLC card was heated on TLC heater. Appearance of brown color indicated the presence of terpenoides. Second, to 0.5 g of each of the extract, 2 ml of chloroform was added .3 ml of concentrated H_2SO_4 was carefully added along the wall of test tube, thus a layer was formed. A reddish brown coloration at the interface of two layers indicated the presence of terpenoides.

Test for saponins

5 ml of distilled water was added to 0.5g of extract in a test tube. The solution was shaken vigorously and a stable persistent froth was observed. 3 drops of olive oil were mixed with the frothing and shaken vigorously, after which it was noted that an emulsion was formed.

Test for tannins

2 ml of the sample was taken in a test tube and 5 ml of *n*-butanol-HCl solution was added. Mixture was warmed for 1 hour at 95^{o}C in a water bath. Appearance of red colour indicated the presence of tannins.

Test for sugars

Fehling's solution (A and B) was added to the sample solutions (0.5 g in 5ml water), in a test tube, and boiled at water bath for half an hour. 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 presence of phenolics.

Test for flavonoides

Four methods were adopted to test for the presence of flavonoides. Firstly, 5ml dilute ammonia was added, to a portion of sample solution in water. Then about 1ml of conc. sulphuric acid was added. A yellow colouration that disappears on standing indicated the presence of flavonoides. Secondly, 3 to 4 drops of 1% aluminium chloride solution were added to sample solution. A yellow colouration indicated the presence of flavonoides. Thirdly, the TLC card having spots of samples was sprayed with Benidict's reagent. Green fluorescence in UV light indicated the presence of flavonoides. Fourth, the TLC card having spots of samples was sprayed with lead acetate solution. Green fluorescence in UV light indicated the presence of flavonoides.

Test for cardiac glycosides (Keller-Killiyani test)

The 0.5g of each sample was diluted up to 5ml in water and 2ml of the glacial acetic acid having one drop of $FeCl_2$ solution was added. It was underlayed by 1 ml of conc. sulphuric acid. Formation of brown ring at the junction of two solutions showed the presence of deoxysugar which is the characteristic of cardenolides. A violet ring appeared below the brown ring, while a greenish ring appeared just above the brown ring, in the acetic acid layer which slowly spread throughout this layer.

Antioxidant assays

Following antioxidant assays were performed on all studied fractions of *Boerhavia procumbens.*

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 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 60 μg/mL, 30 μg/mL, 15 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 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:

$$\text{Antiradical activity} = \frac{\text{Acontrol - Asample}}{\text{Acontrol}} \times 100$$

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°C for 90 minutes. The absorbance of mixture was measured at 695 nm against blank when the contents of the test tubes were cooled to room temperature. 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°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 upto 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. 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 minutes 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 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 (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 at 40 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 Microsoft Excel 2007. Results are presented as average ± S.E.M.

RESULTS

The screening of various phytoconstituents, namely alkaloids, flavonoids, saponins, tannins, cardiac glycosides, terpenoids, and other phenolics of *Boerhavia procumbens* was carried out by reported methods (Sofowara, 1993; Trease and Evans, 1989; Ayoola *et al*., 2008) and results are shown in table 1. The antioxidant potential of *n*-hexane, chloroform, ethylacetate, *n*-butanol soluble fractions and remaining aqueous fraction was evaluated by DPPH, ferric reducing antioxidant power assay, total antioxidant activity by phosphomolybdenum complex method, and ferricthiocyanate assay along with total phenolics. Results obtained are shown in table 2 and table 3.

DISCUSSION

Phytochemical screening

The tests for phytochemical screening were performed on all the studied fractions and results are shown in table 1. Cardiac glycosides were present in more amount in ethyl acetate soluble fraction and *n*-butanol soluble fraction as compared to chloroform fraction while absent in remaining aqueous fraction and *n*-hexane soluble fraction. Ethyl acetate fraction, chloroform fraction and *n*-butanol fraction contained large number of phenolics and flavonoides as well as alkaloids while *n*-hexane soluble fraction and remaining aqueous fraction showed absence of all these compounds. Terpeniods showed very good results in all the fractions except remaining aqueous fraction. Tannins were also present in all fractions except *n*-hexane and chloroform soluble fraction. Saponins were present in ethyl acetate soluble fraction, *n*-butanol soluble fraction, *n*-hexane and chloroform soluble fraction but found absent in remaining aqueous fraction. Sugars were absent in *n*-hexane and chloroform soluble fraction while present in all remaining studied fractions.

DPPH radical scavenging activity

Antioxidants are being placed in the most important category particularly those which are intended to avert the presumed carcenogenic effects of free radicals within the human body, and to avoid the deterioration of fats, and other components of foodstuffs. In both cases, there is a preference that antioxidants should be from natural rather than, from synthetic sources. There is therefore, a growing interest in the utilization of methods for the efficiency estimation of antioxidant substances. One of the methods that is more popular, is based upon the usage of a stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). DPPH is considered to be a stable free radical due to the delocalization of a spare electron at the whole molecule, so that the molecules could not dimerise, as is the case with most of other free radicals. The delocalization causes to develop a deep violet color, characterized by an absorption band, in ethanolic solution at 520nm. A substance donates a hydrogen atom, when DPPH solution is mixed with it, thus giving the reduced form, with loss of violet color (Molyneux, 2004). It is anticipated that free-radical scavenging capacity of tannins, flavonoids, and many other phenols is mainly because of their aromatic hydroxyl groups, which give great stability to the phenolic radical when it forms, after donating one hydrogen radical to DPPH (Zocoler *et al*., 2009). Reduction of DPPH radical was observed by a decrease in absorbance at 517 nm whereas colour change takes place from purple to yellow. The various fractions of *Boerhavia procumbens* reduced DPPH radicals significantly. The results of percent scavenging of DPPH radical are shown in table 2. It was observed that antioxidant potential increases with increase in the concentration of samples in the assay. The various concentrations of ethyl acetate soluble fraction exhibited the highest percent inhibition of DPPH radical, as compared to other four fractions. It showed 82.54±0.62% inhibition of DPPH radical at a concentration of 125µg/ml. "IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds" (Nahak and Sahu, 2010). IC_{50} values of all the fractions were also calculated and results have been shown in table 3. The lower the IC_{50} value, higher will be scavenging power. Ethyl acetate soluble fraction showed the lowest IC_{50} value i.e. 37.11± 0.10 as compared to remaining four fractions. The IC_{50} values of *n*-hexane soluble fraction, ethyl acetate soluble fraction, chloroform soluble fraction, and that of *n*-butanol soluble fraction were found to be 227.88 ± 1.82, 37.11 ± 0.10, 115.44 ± 1.85,58.61 ± 0.23 respectively, relative to BHT, while remaining aqueous fraction showed no reasonable activity because its all %age inhibition values were lower than 50%.

Total antioxidant activity by phosphomolybdenum complex method

The phosphomolybdenum method is based upon the reduction of Mo (VI) to that of Mo (V) by the antioxidant substance, with a maximal absorption at 695 nm, which is due to the formation of a greenish phosphate/Mo (V) complex. This assay is successfully employed to quantify vitamin E in seeds and, being a simple and independent of many other antioxidant measurements techniques

commonly used, it was decided to pull-out the application of this method to plant extracts. As the antioxidant potential is the expression of the number of equivalents, of ascorbic acid, hence, it is considered as a quantitative one. The study shows that the antioxidant potential of the extract increases with the increase in concentration of a plant extract (Saha *et al*., 2008). Hydroxyl group of the 6-hydroxychroman ring, which is shared by all flavan-3-ols (condensed tannins) and flavonoids, is considered to be, the base of the phosphomolybdenum complex method. Thus, this method employs the reduction of Mo(VI) to that of Mo(V) by the sample (Zocoler *et al*., 2009). It is a quick, cheap and reproducible test, gives the measurement of the antioxidant potential of an analyte. In the presence of a reducing agent, reduction of phosphormolybdate acid to phosphomolybdate blue (Mo^{+6} _ Mo^{+5}) took place. The UV/visible spectrum of this compound has a characteristic maximum at 695 nm. This method has been used successfully to determine the antioxidant potentials of plant extracts and isolated compounds for example,

Table 1: Phytochemical screening results of various fractions of *Boerhavia procumbens* Bank ex Roxb.

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

+: Present, **_:** Absent

Table 2: Free radical scavenging activity of various fractions of *Boerhavia procumbens* Bank ex Roxb. using 1,1-Diphenyl-2-picrylhydrazyl radical(DPPH)

Sr. No.	Sample	Concentration in assay (μg/ml)	%age scavenging of DPPH radical ± S.E.M[a)]
1	*n*-Hexane soluble fraction	500	68.16 ± 0. 85
		252	55.85 ± 0.54
		125	44.17 ± 0.51
		60	10.12 ± 0.83
2	Chloroform soluble fraction	250	79.14 ± 0.14
		125	69.87 ± 0.71
		60	35.01 ± 0.14
		30	26.78 ± 0.73
3	Ethyl acetate soluble fraction	125	82.54 ± 0.62
		60	55.85 ± 1.06
		30	42.19 ± 0.71
		15	16.9 9 ± 0.89
4	*n*-Butanol soluble fraction	500	91.40 ± 0.54
		250	77.30 ± 0.72
		125	61.89 ± 0.99
		60	52.93 ± 0.77
		30	41.31 ± 0.86
5	Remaining aqueous fraction	1000	45.00 ±1.01
		500	34.36 ± 0.66
		250	27.93 ± 0.97
		125	24.46 ± 0.46
6	BHT[b)]	60	91.25 ± 0.13
		30	75.56 ± 0.07
		15	42.67 ± 0.04
		8	23.56 ± 0.31

[a)] Standard mean error of three assays. [b)] Standard antioxidant

Table 3: IC_{50} total antioxidant activity, FRAP values, total phenolics and lipid peroxidation inhibition values of different fractions of *Boerharvia procumbens* Bank ex Roxb.

Sr. No.	Sample	IC_{50} of DPPH assay (ug/ml) ± S.E.M[a)]	Total anti-oxidant activity ± S.E.M[a)]	FRAP value TE (uM/ml) ± S.E.M[a)]	Total phenolics (GAE)mg/g of sample) ± S.E.M[a)]	Inhibition of lipid peroxi-dation (%) ± S.E.M[a)]
1	*n*-Hexane soluble fraction	227.88±1.82	0.322 ± 0.009	34.16 ± 1.04	29.61± 1.38	37.15 ± 1.23
2	Chlorofom soluble fraction	115.44 ± 1.85	0.540 ± 0.003	76.60 ± 1.15	74.06 ± 1.07	51.52 ± 0.76
3	Ethyl acetate soluble fraction	37.11± 0.10	0.549 ± 0.026	251.0 ± 1.00	77.19 ± 0.60	57.2 ± 0.64
4	*n*-Butanol soluble fraction	58.61± 0.23	0.219± 0.01	33.00 ± 0.86	39.79 ± 0.88	45.56 ± 0.36
5	Aqueous fraction	*	0.064± 0.003	14.2 ± 0.34	31.11± 0.33	21.27 ±0.59
6	BHT[b]	12.1 ± 0.09	0.818 ± 0.09			62.91 ± 0.60

[a)] Standard mean error of three assays, [b)] Standard antioxidant, *) Not detected

flavidin (Biskup and Lojkowska, 2009). The total antioxidant activities of these fractions were compared with that of standard antioxidant BHT and the results have been shown in table 3. It was revealed from the results that ethyl acetate soluble fraction showed highest total antioxidant activity i.e. 0.549±0.026 while chloroform soluble fraction showed activity slightly less than this. The total antioxidant capacity of ethyl acetate soluble fraction was found to be 0.615 ± 0.009 while that of *n*-butanol soluble fraction and remaining aqueous fraction was 0.417 ± 0.02 and 0.59 ± 0.04. The *n*-hexane soluble fraction exhibited the lowest antioxidant activity (0.217 ± 0.01). The results were compared with BHT, a reference standard, having total antioxidant potential 0.818 ± 0.09.

Ferric reducing antioxidant power (FRAP) assay

Antioxidants can reduce the oxidative stress when administered additionally. Antioxidants are closely related to the prevention of degenerative diseases, such as cardiovascular, cancer, oxidative stress dysfunctions, and neurological diseases. Foods from plant origin not only give us important antioxidant vitamins such as vitamin C, provitamin A or vitamin E, but also provide a complex mixture of many other natural substances which exhibit antioxidant capacity. It is possible to determine all of the antioxidant constituents in a sample separately, but this is a costly and time-consuming process. A lot of methods are known to determine the total antioxidant potential of biological samples, we tried the FRAP assay however, which is dependent upon the reduction of (Fe(III)-TPTZ) i.e. ferrictripyridyltriazine complex to form the ferrous tripyridyltriazine (Fe(II)-TPTZ), by a reductant at a lower pH. Fe(II)-TPTZ has a bluish color and can be monitored, at 593 nm. Although this method was developed for human plasma, yet we wanted to get an answer to the question "Is this assay applicable for investigation of fresh plant samples and herbs?" (Szôllôsi and Varga, 2002). Reducing ability of a compound usually depends upon the presence of reductants, which exhibit antioxidative capacity by donating a hydrogen atom, cosequently breaking the free radical chain. The presence of reductants (i.e. antioxidants) in *Boerhavia procumbens* extracts causes the reduction of Fe^{3+} (ferricyanide complex), to the ferrous form. Therefore, Fe^{2+} is monitored by measuring the development of Perl's Prussian blue, at 700 nm (Ara and Nur, 2009). Reductive ability increases with increasing absorbance. The FRAP values of the studied fractions were calculated and results have been shown in table 3. Among all the fractions the ethyl acetate soluble fraction showed the highest FRAP value (251.00±1.00 TE µM/mL). Chloroform also showed a pretty good activity as 71.60±1.15 TE µM/mL. *n*-butanol fraction and *n*-hexane fraction showed almost equal FRAP values i.e. 33.00±0.86 T µM/mL and 34.16±1.04 TE µM/mL respectively while that of aqueous fraction was not good enough i.e. 14.20±0.34. High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents.

Total phenolic contents

It is a well-known fact that phenolic compounds contribute pretty well to quality and nutritional value, in terms of the modification of color, aroma, taste, and flavor, and also contributing to the beneficial effects for health. Phenolic compounds also serve in defense mechanisms of plants to combate reactive oxygen species (ROS), in order to preserve total antioxidant activity (Vaya *et al*., 1997). Polyphenolic compounds have an important role in the lipid-oxidation, and are closely associated with antioxidant activity. The phenolic compounds are considered to contribute directly to oxidative capacity. The oxidative activities observed may be ascribed both the different mechanism exerted by various phenolic compounds and to the different

synergistic effects of different substances. The antioxidants posses different functional properties, such as: reactive oxygen scavenging for example quercitin and catichin, inhibition of free radicals generation, and chain breaking activity, such as p-coumaric acid and metal chelation. These compounds are usually phenolic compounds, which are potential protein donors and include tochopherols, flavonoids, and many other organic acids (Huang *et al*., 2005). Table 3 shows the total phenolic concentrations in different fractions, expressed as micrograms of the gallic acid equivalents (GAEs), per gram of any fraction. Among these five fractions, ethylacetate soluble fraction showed the highest amount of the total phenolic compounds 77.19 ± 0.06 GAE mg/g while chloroform soluble fraction showed activity (74.06 ± 1.07), a little less than that of ethyl acetate. The total phenolic contents of *n*-hexane soluble fraction were 29.61 ± 1.38, while *n*-butanol soluble fraction, and aqueous fraction were found to be 39.79 ± 0.88 and 31.11 ± 0.33 GAE µg/g respectively.

Ferric thiocyanate (FTC) assay

The ferric thiocyanate assay measures at the initial stage of linoleic acid emultion, the amount of peroxide generated, during incubation. Here peroxide reacts with $FeCl_2$, which produces ferric thiocyanate, a reddish pigment, after reacting with ammonium thiocyanate. Low absorbance values which are measured via FTC method exhibit high antioxidant activity (Kim and Kim, 2010). Different fractions of plant were assessed by this assay and results have been shown in table 3. Significantly lower absorbance as compared to control was observed for chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction which indicated that these fractions have greater antioxidant activities. The fractions which showed greater values of percent inhibition of lipid peroxidation might have primary antioxidant compounds, having the ability to react aggressively with any free radical, particularly hydroxyl radicals, thus resulting in termination of a radical-chained reaction and slowing down the formation of hydroperoxides (Ismail *et al*., 2010). Ethyl acetate fraction exhibited the highest %age of lipid peroxidation inhibition value (57.21 ± 0.64%), while aqueous fraction had lowest %age of lipid peroxidation inhibition value (21.27 ± 0.59%). Chloroform, *n*-butanol and that of *n*-hexane soluble fractions exhibited percent inhibition of lipid peroxidation 51.52 ± 0.76%, 45.56 ± 0.36% and 37.15 ± 1.23% respectively. Lipid peroxidation inhibition by BHT (standard) was 62.91 ± 0.60%.

CONCLUSION

Phytochemical screening showed that cardiac glycosides were present in more amounts in ethyl acetate fraction and *n*-butanol soluble fraction. Ethyl acetate, chloroform, and *n*-butanol soluble fractions contain large number of phenolics and flavonoides as well as alkaloids. Due to presence of these compounds these fractions showed noteworthy antioxidant potential. The results showed that ethyl acetate soluble fraction showed the highest value of percent inhibition of DPPH (82.54±0.62) at concentration of 125 µg/ml. The IC_{50} of this fraction was 37.11± 0.23µg/ml, with reference to butylated hydroxytoluene (BHT), which have IC_{50} of 12.1 ± 0.92 µg/ml. It also showed the highest FRAP value (251.08 ± 1.46 µg of trolox equivalents), as well as the highest lipid peroxidation inhibition value (57.21 ± 52%), the highest total antioxidant activity (0.549 ± 0.08), and also the highest total phenolic contents (77.1 ± 0.6) as compared to other studied fractions. So it was concluded that ethyl acetate, chloroform, and *n*-butanol soluble fractions are rich in strong antioxidants. These fractions are sources of potentially valuable natural antioxidants and many other bioactive materials, which are expected to enhance the shelf-life of food stuffs and also fortify against peroxidative damage within the living systems and retarding the process of aging and carcinogenesis.

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