Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2014; 6(3): 598-605

ISSN: 0975-4873

Research Article

Antioxidant Activity of *Boerhavia Diffusa* Extract

Patel M.[1], *Verma r.[1], Srivastav P.[2]

[1]*Department of Zoology, University School of Sciences, Gujarat University, Ahmedabad - 380009, India*
[2]*Department of Chemistry, University School of Sciences, Gujarat University, Ahmedabad - 380009, India*

Available Online: 1st September 2014

ABSTRACT
In vitro antioxidant activity of hydro-alcoholic extract of *Boerhavia diffusa* (Nyctaginaceae) was evaluated by studying superoxide radical scavenging activity, hydroxyl radical scavenging activity, nitrous oxide radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing ability and Fe^{+2} chelating ability using standard procedure. Identification and quantification of the boerhavinone B, one of the active constituents of the *Boerhavia diffusa* plant extract, was carried out by HPLC analysis. Result of the present study indicates that the *Boerhavia diffusa* extract shows high amount of phenolics, flavonoids, tannins and ascorbic acid contents. Hydro-alcoholic extract of *Boerhavia diffusa* effectively scavenged free radicals at all different concentrations and showed potent antioxidant potency and effects were in a dose-dependent manner. *Boerhavia diffusa* extract possess potent antioxidative properties.

Key words: *Boerhavia diffusa*, Antioxidants, Free radical scavenging, 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

INTRODUCTION

Medicines derived from plants have played a pivotal role in the health care of many cultures, both ancient and modern. Scientific evaluation of plants has often shown that active principles in these are responsible for therapeutic success. Medicinal plants may serve as a vital source of potentially useful new compounds for the development of effective therapy to combat a variety of ailments. Plant derived natural products such as flavonoids, terpenoids, steroids, etc. have diverse pharmacological properties including antioxidant activity. Antioxidants are compounds that help to inhibit many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl and hydroxyl radicals etc., thereby preventing or delaying damage to the cells and tissues. Antioxidant compounds have potential to mitigate the effect of free radicals and play an important role as a health protecting factor[1].

Boerhavia diffusa (Nyctaginaceae), commonly known as 'Punarnava' in the Indian system of medicine is a perennial creeping herb. *Boerhavia diffusa* has a long history of uses by the tribal people and in Ayurvedic and Unani medicines. It is widely distributed throughout India and flourishes during rainy seasons. The aerial parts then disappear but revive or sprout again next year[2]. It is a diffusely branched pubescent or glabrous, prostrate herbs, abundantly occurring as a weed throughout India, up to an altitude of 2000 m in the Himalayas. Pharmacological studies have demonstrated that *Boerhavia diffusa* known to possess anticonvulsant, diuretic, anti-inflammatory, antifibrinolytic, antibacterial, antihelminthic, antileprosy, antiasthmatic, antiurethritis, antilymphoproliferative, antimetastatic, antidiabetic, immune-modulation, anti-nociceptive, nephroprotective and antiurolithiatic[3].

The main aim of the present study was to evaluate antioxidative properties of the hydro-alcoholic extract of *Boerhavia diffusa*.

MATERIALS AND METHODS

Chemicals: The chemicals used in the entire study were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India; Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Sigma-Aldrich, St. Louis, MO, USA and was of analytical grade. HPLC grade methanol, acetonitrile and acetic acid were obtained from Merk specialities Pvt. Ltd., Mumbai, India.

Plant material: The whole plant of *Boerhavia diffusa* was collected during September-october, 2011 from the Botanical garden of the Gujarat University, Ahmedabad. Herbarium specimens were prepared and authenticated by Dr. Hitesh Solanki, Associate Professor, Department of Botany, Gujarat University, Ahmedabad.

Extract preparation: The extract was prepared according to World Health Organization protocol CG-06 (1983) with slight modifications[4]. The whole plant materials were shade dried and powdered. 5 gm of whole powdered plant material was extracted by overnight soaking method using 100 ml of 50% aqueous-ethanol (v/v). The content was filtered successively through ordinary and then Whatman filter paper No. 1. Extraction procedure was repeated. Both the fractions were pooled, dried and stored in dark bottle at 4 °C. During the experiment known amount of dried extract was redissolved in double distilled water and used. The percent yield of the extract was calculated.

**Author for correspondence: E-mail: ramtejverma2000@yahoo.com*

Table 1: Phytochemical analysis of the Boerhavia diffusa extract

Phytochemical parameters	*Boerhavia diffusa*
Extract yield (%)	10.89 ± 1.05
Qualitative analysis	
Phenolic content	+
Flavonoid content	+
Tannin content	+
Ascorbic acid content	+
Quantitative analysis	
Phenolic content	70.80 ± 0.48
Flavonoid content	35.91 ± 1.50
Tannin content	25.71 ± 1.02
Ascorbic acid content	0.67 ± 0.04

Results are expressed as mean ± SEM; n = 6.

Qualitative and quantitative analysis: The hydro-alcoholic extracts of *Boerhavia diffusa* were subjected to phytochemical analysis for the detection of the major chemical groups. Qualitative and quantitative estimation of phytochemical constituents were done as described below.

Qualitative analysis: Qualitative analysis for determining the presence of phenolics, flavonoids, tannins, and ascorbic acid in the plant extracts were carried out using standard methods.

Test for phenolics: The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride ($FeCl_3$) solution was added. A dark green colour indicated the presence of phenolic contents.

Test for flavonoids: 5 ml of dilute ammonia solution was added to plant extract, followed by addition of concentrated H_2SO_4. A yellow colouration indicated presence of flavonoids.

Test for tannins: 0.5 gm of extract was dissolved in 20 ml distilled water in a test tube and then filtered. A few drops of 0.1% $FeCl_3$ was added and observed for brownish green or blue black colour.

Test for ascorbic acid: To the extract, one drop of 2, 6 dichlorophenolindophenol (DCPIP) solution was added. Formation of blue to red colour indicates the presence of ascorbic acid.

Quantitative analysis: Total phenolic content: Total phenolic content of the extract was estimated by the method as described by Singleton *et al*. (1999)[5]. Briefly extract react with Folin-Ciocalteu reagent in the presence of sodium carbonate to form a blue coloured complex which was read at 760 nm. Various concentrations of gallic acid were used to plot standard curve. Total phenolic content of extract was expressed as mg gallic acid equivalent/gm dry wt. of extract.

Flavonoid content: The flavonoid content of the plant extract was estimated by the method of (Lamaison and Carnat, 1990) [6]. Briefly 1.0 ml of plant extract was mixed with 1.0 ml of aluminium chloride reagent and resultant colour was read at 430 nm. The flavonoid content of the extract was expressed as mg quercetin equivalent/gm dry wt. of extract.

Tannin content: Tannin content of the extracts was estimated by the method as described by (Price and Butler, 1977)[7]. Plant extract was allowed to react with $K_3Fe(CN)_6$-$FeCl_3$ reagent for five min and the intensity of colour developed was measured spectrophotometrically at 720 nm. The tannin content of the extract was expressed as mg rutin equivalent/gm dry wt. of extract.

Ascorbic acid content: Ascorbic acid, also known as vitamin C, is one of the most abundant antioxidant present in plant was quantified by the method of (Jagota and Dani, 1982)[8]. The ascorbic acid content of plant extract was expressed as µg/gm dry wt. of extract.

Free radical scavenging assays: Antioxidative potency of the plant extract of *Boerhavia diffusa* was estimated by various chemical assay systems as described below:

Superoxide radical scavenging assay: Superoxide radical scavenging activity was assessed by the method described by (Liu et al., 1997)[9]. In the Phenazine methosulphate (PMS)/ Nicotinamide adenine dinucleotide reduced disodium salt-Nitro blue Tetrazolium chloride (NADH-NBT) system, superoxide anion derived from dissolved O_2 by PMS/NADH coupling reaction reduces NBT. Addition of various concentrations of extracts resulted in decreased colour intensity which was read at 560 nm against blank to determine the quantity of the formazon generated.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity of the extracts was estimated by the method described by (Halliwell et al., 1987)[10], where radicals were generated from Fe^{+3}/ ascorbate/ EDTA/ H_2O_2 system by Fenton's reaction. Briefly different concentrations of plant extracts were made to react with 2-deoxy-2-ribose, H_2O_2, $FeCl_3$ and EDTA. The reaction was initiated by the addition of ascorbic acid. After incubation for 90 min the reaction was terminated by addition of thiobarbituric acid (TBA) and resulting colour was read at 590 nm.

Nitrous oxide radical scavenging assay: Nitrous oxide radical scavenging activity was measured using the method described by (Sreejayan and Rao, 1997)[11]. Various concentrations of plant extracts were incubated with 10 mM sodium nitroprusside for 150 min. After incubation, Griess reagent was added to the tubes and absorbance of chromophore formed was read at 590 nm.

DPPH radical scavenging assay: Ability of the plant extracts to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured by the method described by (Gyamfi et al., 1999)[12]. DPPH is a purple colour radical compound which changes to stable compound having

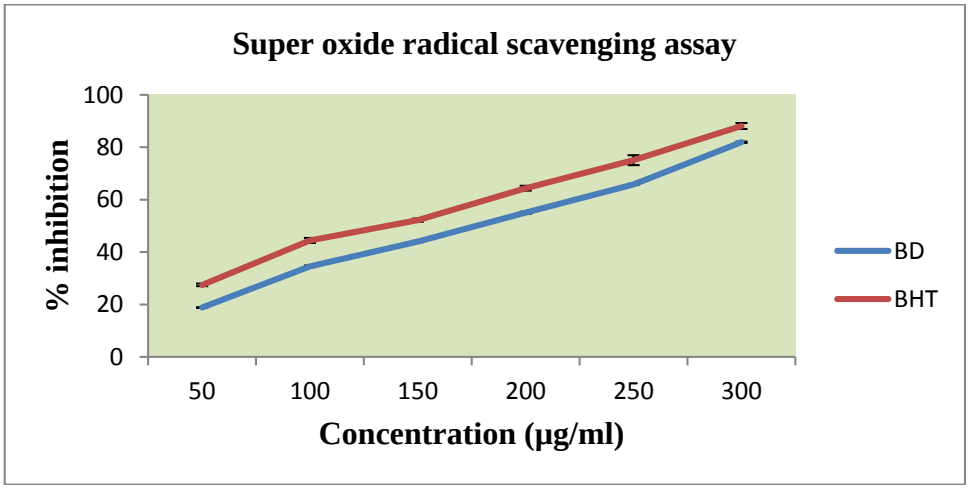

Fig. 1: Superoxide radical scavenging activity of Boerhavia diffusa extract

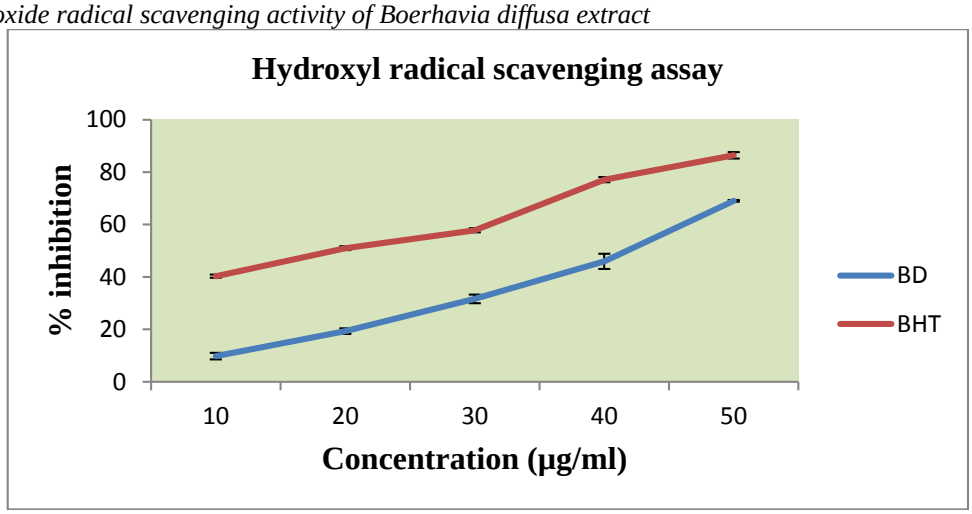

Fig: 2 Hydroxyl radical scavenging activity of Boerhavia diffusa extract

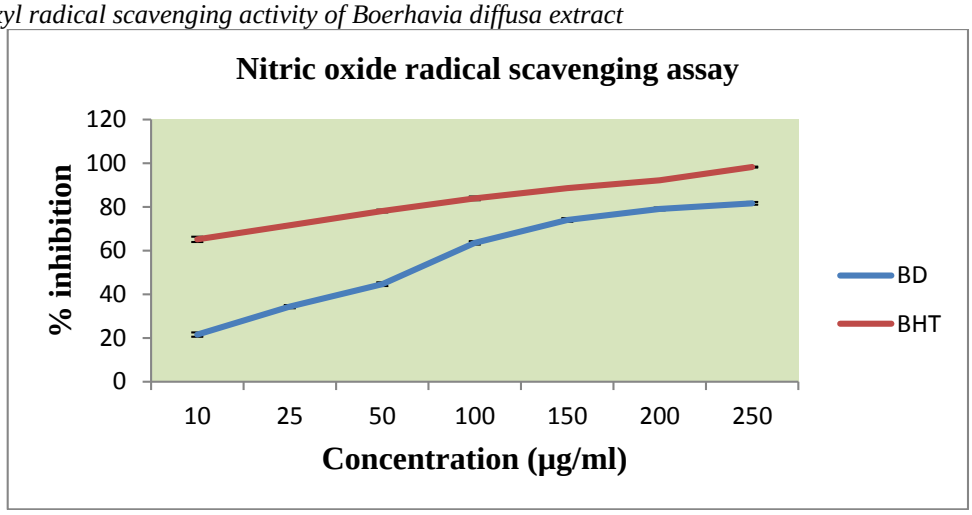

Fig: 3 Nitric oxide radical scavenging activity of Boerhavia diffusa extract

yellow colour by reacting with antioxidant compounds. Addition of 0.1 mM DPPH solution in various concentrations of extracts in presence of tris-HCl buffer (50 mM, pH-7.4) resulted in decreased absorbance which was measured at 517 nm. Percent inhibition was calculated by measuring the absorbance of plant extract treated samples against blank.

Reducing ability: Reducing ability of *Boerhavia diffusa* plant extracts was evaluated using method decribed by (Yildirim, 2000)[13]. Briefly various concentrations of both the extracts were mixed with potassium phosphate buffer and potassium hexacyanoferrate [$K_3Fe(CN)_6$] and incubated for 30 min. Reaction was terminated by addition of trichloroacetic acid (TCA) followed by addition of $FeCl_3$. Antioxidants present in the tested plant samples reduces Fe^{+3}/ferricyanide complex to the ferrous form (Fe^{+2}) resulting in formation of perl's Prussian blue colour which was read at 700 nm.

Fe^{+2} chelating ability: The Fe^{+2} chelating activity of the extract was estimated using the method described by (Dinis et al., 1994)[14]. Plant extracts were allowed to react with ferrozine (5 mM) in presence of $FeCl_3$ (2 mM). Blue coloured Fe^{+2} – ferrozine complex formed was read at 562 nm. Chelating ability of the extracts was compared with EDTA (0.01 mM).

Hi-performance liquid chromatography (HPLC): Identification and quantification of Boerhabinone B from hydro-alcoholic extracts of *Boerhavia diffusa*:

Standard stock preparation: Standard stock of boerhavinone B was prepared by dissolving 10 mg of dry extracts in 10 ml milique water.

Plant extracts preparation: Whole plant extract of *Boerhavia diffusa* was prepared by dissolving 10 mg of dry extract in 10 ml milique water.

Chromatographic conditions: A Shimadzu LC-VP HPLC system (Kyoto, Japan) consisting of LC-10ADVP pump, SIL-HTc autosampler, CTO 10 ASvp column oven and a DGU-14A degasser was used for setting the reverse-phase liquid chromatographic conditions. Inertsil ODS-C18 (150 mm length × 4.6 mm inner diameter, 5µ particle diameter) analytical column from Phenomenex Inc. (Torrance, CA, USA) was used. Column oven temperature was 30°C, UV detector was used, separation mode was isocratic, mobile phase was acetonitrile-methanol (70:30 v/v) and flow rate was 1 ml/min. Total chromatographic run time was 30 min and injection volume was 20 µl.

Plant extract and boerhavinone B standard were separately run on chromatographic column. Based on the retention period of boerhavinoe B standard and its corresponding peak in the crude extract chromatogram identification and quantification of the same was achieved. Spiking the standard boerhavinone B solution with plant extract *Boerhavia diffusa* was done to confirm presence of boerhavinone B in the extract.

RESULTS

Phytochemical analyses of the plant extract**:** Qualitative analysis: Table 1 depicts the results of phytochemical analysis of *Boerhavia diffusa* extract. The qualitative analysis of *Boerhavia diffusa* extract indicated the presence of phenolics, flavonoids, tannins and ascorbic acid contents, which were later determined quantitatively using standard methods.

Units: Phenolics – mg gallic acid equivalent/gm dry wt. of extract; Flavonoids – mg quercetin equivalent/gm dry wt. of extract; Tannins – mg rutin equivalent/gm dry wt. of extract; Ascorbic acid - µg/gm dry wt. of extract.

Quantitative analysis

Total phenolic content: Total phenolic content (TPC) of the *Boerhavia diffusa* extract was estimated using standard method. The concentration of TPC determined in hydro-alcoholic extract of *Boerhavia diffusa* was 70.80 mg gallic acid equivalent/gm dry weight calculated using equation that was obtained from standard gallic acid graph (Table 1).

Flavonoid content: Quantification of flavonoid content showed that hydro-alcoholic extract of *Boerhavia diffusa* contained 35.91 mg quercetin equivalent/gm dry weight of extract (Table 1).

Tannin content: Standard curve for tannin estimation was plotted using various concentrations of rutin. Tannin content of hydro-alcoholic extract of *Boerhavia diffusa* was found to be 25.71 mg rutin equivalent/gm dry weight (Table 1).

Ascorbic acid content: Ascorbic acid content of hydro-alcoholic extract of *Boerhavia diffusa* was found to 0.67 µg/gm dry weight of extract (Table 1).

Free radical scavenging assays: Superoxide radical scavenging assay: Superoxide radicals generated from PMS/NADH-NBT system were strongly scavenged by various concentrations of *Boerhavia diffusa* extracts. The decrease in colour intensity was observed with increasing concentration of extracts indicating consumption of the radicals in the reactions mixture (Fig. 1). Maximum scavenging effect was achieved at 300 µg/ml concentration of the extracts. Maximum scavenging effect found with *Boerhavia diffusa* extract was 81.91%. The effect was concentration-dependent (R^2 = 0.9932). Concentration required to scavenge 50% (IC_{50}) of the radicals was 200 µg/ml for *Boerhavia diffusa* extract.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging capacity of extract is directly proportional to its antioxidative potency. The percent inhibition of hydroxyl radical increased significantly ($p<0.05$) with increasing concentrations of hydro-alcoholic extracts of *Boerhavia diffusa* (Fig. 2). Maximum protection for *Boerhavia diffusa* extract was (69.06%). The protective effect was concentration-dependent (R^2 = 0.9700) and was highest at 50 µg/ml concentration (Fig. 3.2). IC_{50} value of *Boerhavia diffusa* extract was 40 µg/ml.

Nitrous oxide radical scavenging assay: Nitrous oxide radical generated from sodium nitroprusside at physiological pH was significantly ($p<0.05$) inhibited by *Boerhavia diffusa* extracts. Percent inhibition was concentration-dependent (R^2 = 0.8948) and maximum at 250 µg/ml concentration of the extract. Highest scavenging effect found with *Boerhavia diffusa* extract was 81.65%. IC_{50} value for nitrous oxide scavenging activity was 100 µg/ml for *Boerhavia diffusa* extract.

DPPH radical scavenging assay: DPPH radical scavenging activity of various concentrations of *Boerhavia diffusa* were found statistically significant ($p<0.05$). Decrease in absorbance due to antioxidative effect of soluble solids of *Boerhavia diffusa* was highest at 250 µg/ml concentration (Fig. 4). Maximum percentage inhibition achieved with *Boerhavia diffusa* extract was 80.00%. Scavenging effect of the extracts were concentration-dependent (R^2 = 0.9146). IC_{50} value for *Boerhavia diffusa* extract was 100 µg/ml.

Reducing ability: The presence of reductant (antioxidant) in the tested extracts of *Boerhavia diffusa* resulted in the reduction in Fe^{+3}/ferricyanide complex to ferrous form (Fe^{+2}). Increasing concentrations of the extracts resulted in simultaneous increase of reducing power (Fig. 5). Highest reducing ability was found with *Boerhavia diffusa* extract (72.12%), whereas it was at 250 µg/ml concentration (Fig. 5). Increase in reducing ability of the extracts was

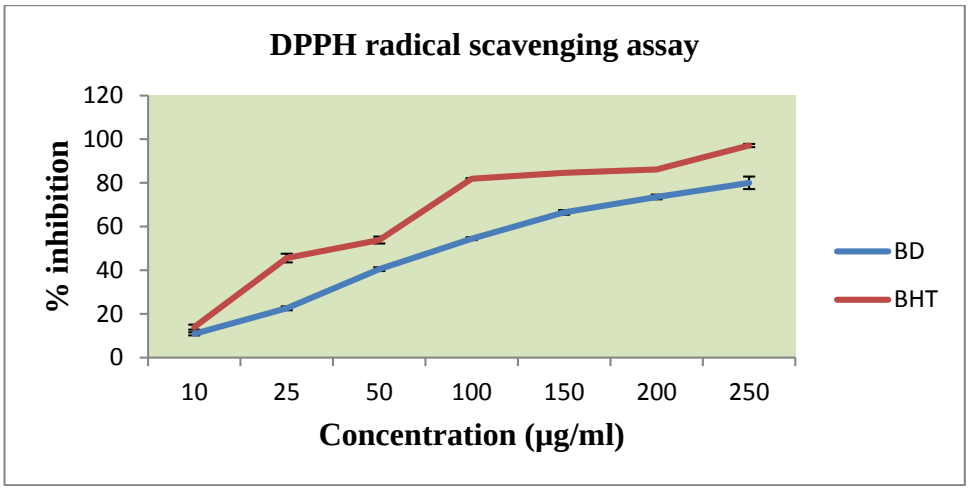

Fig: 4 DPPH radical scavenging activity of Boerhavia diffusa extract

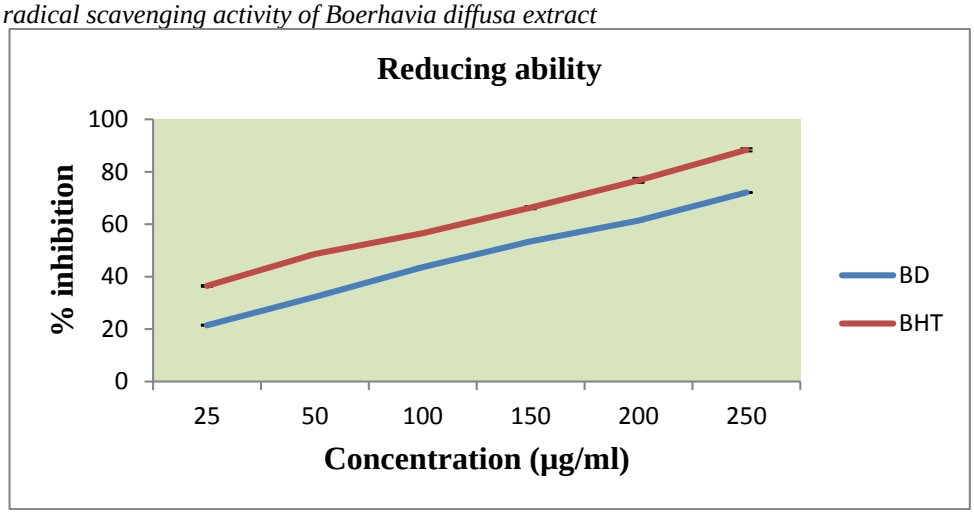

Fig: 5 Reducing ability of Boerhavia diffusa extract

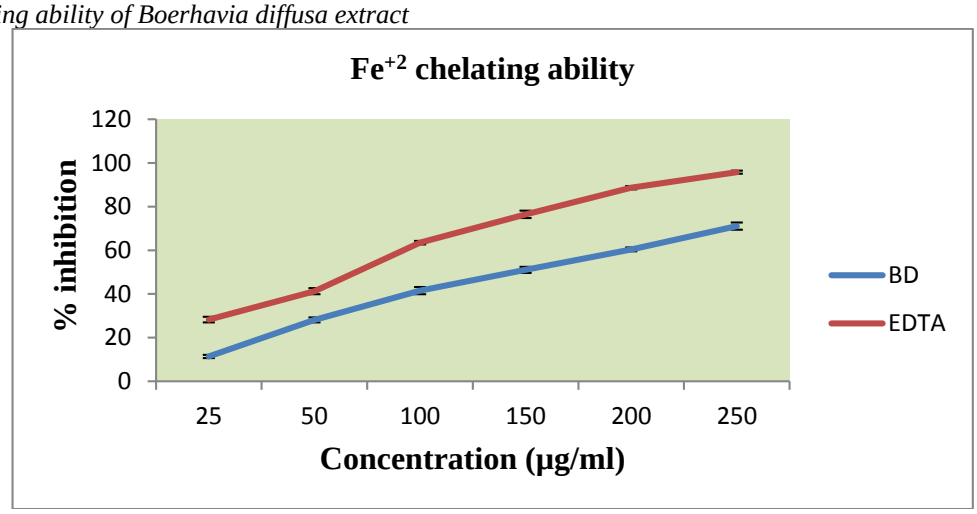

Fig: 6 Fe^{+2} chelating activity of Boerhavia diffusa extract

concentration-dependent (R^2 = 0.9842). IC_{50} value for reducing ability scavenging were 150 µg/ml for *Boerhavia diffusa* extracts.

Fe^{+2} chelating activity: Ferrozine - Fe^{+2} complex produces violet colour in the reaction mixture in presence of metal ions which was significantly reduced by addition of *Boerhavia diffusa* extracts. Formation of coloured chromaphore is interrupted in the presence of chelating agents of *Boerhavia diffusa* extracts and resulted in decreased optical density (Fig. 6). Maximum inhibition achieved with *Boerhavia diffusa* extract was 71.08%. *Boerhavia diffusa* extracts chelated metal ions in a concentration – dependent manner (R^2= 0.9862). IC_{50} values for *Boerhavia diffusa* extract was 150 µg/ml.

Identification and quantification of active components of plant extracts: Standardization of plant extracts was done by quantifying the major active components of the plant

extracts. The major active component present in *Boerhavia diffusa* extract was Boerhavinone B and was separated from other constituents by reverse phase HPLC analysis.

Under the optimized chromatographic condition the retention time for standard Boerhavinone B was 2.252 as shown Fig.7. Chromatograph of hydro-alcoholic extract of *Boerhavia diffusa* showed four peaks of 1.622, 2.252, 2.598 and 4.329 min as shown Fig. 8. The peak of 2.252 in the crude extract corresponds to Boerhavinone B, which was confirmed by spiking the sample with standard stock solution of Boerhavinone B (1000 µg/ml) as depicted in Fig. 8. Percent concentration of Boerhavinone B present in *Boerhavia diffusa* extract was 0.194 %.

DISCUSSION

In traditional societies nutrition and health care are strongly interconnected and many plants have been consumed both as food and for medicinal purposes. The consumption of non-cultivated botanicals plays a central role in the diet, but very few ethnopharmacological and phytopharmacological studies have dealt exhaustively with the potential health benefits of such diets. In the past few years, there has been growing interest in the involvement of ROS in several pathological situations.

Currently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant - derived phytochemicals from vegetables, fruits, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status[15]. Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years[16].

In the present study qualitative assessment of phytochemical constituents of *Boerhavia diffusa* plant extract showed presence of phenolics, flavonoids, tannins and ascorbic acid contents. The quantitative analysis revealed presence of phenolic content in *Boerhavia diffusa* extract. Phenolic as well as flavonoids were in high amount as compared to other phytoconstituents analysed. The presence of alkaloids, phenolic compounds, flavonoids, tannins have been associated with various degrees of antioxidant activities[21]. Phenolics are antioxidants by nature due to their redox properties and metal chelating effects[17,18].

In the present study quantitative estimation of crude polyphenols from hydro-alcoholic extract of *Boerhavia diffusa* revealed presence of significantly high amount of phytochemicals principally responsible for its protective effect (Table 1). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing

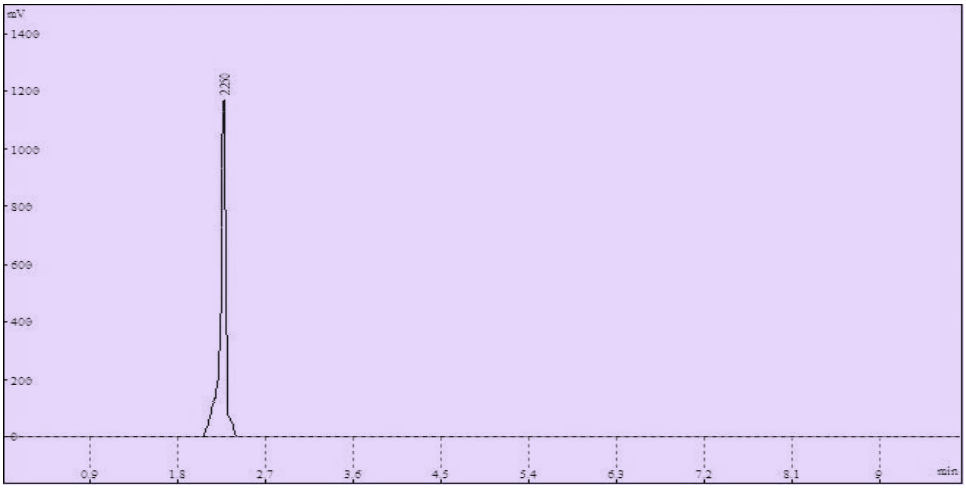

Fig. 7: Chromatogram of boerhavinone B

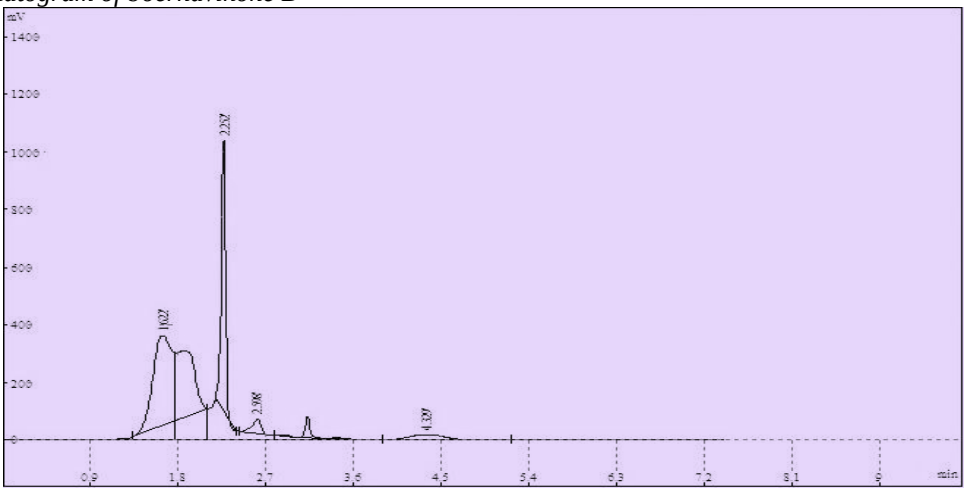

Fig. 8: Chromatogram of Boerhavia diffusa extract

free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[19].

The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular diseases[20]. Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS[21,22] , continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage[23]. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated[24]. In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity[25,26,27,28].

Reactive oxygen species produced *in vivo* include superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet-}$) can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical (•OH)[29].

In vitro assessment of antioxidative properties of various plant extracts using chemical models provides biochemical basis for the *in vivo* ethnopharmacological uses of the plants. *Boerhavia diffusa* extract was found to be potent scavenger of superoxide (Fig. 1), hydroxyl (Fig. 2), nitrous oxide (Fig. 3) and DPPH (Fig. 4) radicals. Free radical scavenging activity of *Boerhavia diffusa* extract was reported by Gopal *et al*. (2010)[30]. These scavenging properties are generally due to high reducing capacity of the polyphenols acting as primary antioxidants[31]. 1, 1-Diphenyl-2-pecryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge[32]. 1, 1-Diphenyl-2-pecryl-hydrazyl (DPPH) assay has many advantages, such as good stability, credible sensitivity, simplicity and feasibility[33]. Metal ions play central role in reactive oxygen species generation as they can change the state from reduced to oxidised causing removal of electron from various biomolecules[34]. *Boerhavia diffusa* plant extract showed good metal chelating activity as indicated in Fig. 6.

Medicinal values of the plants depend on the bioactive constituents exerting desirable physiological action in humans. Antioxidative effect of *Boerhavia diffusa* extract is principally denoted by the phytochemicals acting as reductant and free radical scavenger. An attempt was made in the present study to isolate and characterize the major active component from the indigenous medicinal plant namely *Boerhavia diffusa*. Boerhavinone B was isolated from *Boerhavia diffusa* (Fig.7,8) whole plant extract by RP-HPLC analysis. Lami et al., 1991 and Kadota et al., 1989 have isolated many rotanoids from the roots of the *Boerhavia diffusa*[35,36]. These include a series of boeravinones viz., boerhavinone A, boerhavinone B, boerhavinone C, boerhavinone D, boerhavinone E and boerhavinone F. Bhope et al., 2013 have isolated boerhavinone B by RP-HPLC method[37].

ACKNOWLEDGEMENTS

We thank the Gujarat University, Ahmedabad, India for providing laboratory facility for the study.

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