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Research Article

Natural Antioxidant Activity of *Boerhavia diffusa* L.

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ABSTRACT

Objective: To evaluate antioxidant properties of *Boerhavia diffusa* ethanolic, chloroform and petroleum ether extracts. Methods: *In vitro* antioxidant potential as well as total phenolic and flavanoid content of *B.diffusa* have been investigated by different assays including scavenging activity and reducing power assays. Results: All the three extracts of *B.diffusa* showed significant antioxidant activities compared to the standard antioxidants in a dose dependent manner. IC_{50} values indicated that ethanolic extract of the plant has more scavenging activities and reducing power than others. Ethanolic extract was found to have the highest total phenolic and flavanoid contents with the values 73.2±5.01 expressed in mg gallic acid/gm dry extract and 56.51±4.11 expressed in mg rutin/gm dry extract respectively. Conclusion: The data obtained in the present study suggests that the *B.diffusa* extracts have potent antioxidant activity against free radicals, prevent damage to major biomolecules and afford significant protection against oxidative damage.

Keywords: *Boerhavia diffusa*, Scavenging activity, IC_{50} values, oxidative damage.

INTRODUCTION

Oxygen free radicals produced as a result of metabolic processes in our body, induce damage to biomembranes and genetic materials leading to many chronic degenerative diseases and aging. Oxidative stress, an unbalance between prooxidants and antioxidant mechanisms, directed the use of dietary or medicinal supplements particularly during disease attack. Scientific evidence suggested that antioxidants reduce the risk of chronic diseases including cancer and heart disease[8.] Recently, demand for naturally occurring antioxidants has increased considerably; replace synthetic antioxidants due to their adverse effects. Natural antioxidants act as effective free radical scavengers, by donating hydrogen to highly reactive radicals, inhibiting oxidation, and activating enzymes of the antioxidant defense systems[5]. Plant foods are known to protect against degenerative diseases and aging due to their antioxidant activity attributed to their high polyphenolic content. The use of medicinal plants with high antioxidant constituents has been proposed as an effective therapeutic approach for many degenerative diseases.

Boerhavia diffusa belongs to the family Nyctaginaceae commonly known as 'punarnava' and locally called 'thazuthama' which means rejuvenating the cells or renewing the body[4] by eliminating excess water from it make it highly advantageous for the kidney and the liver. The plant acts on all important organ systems and rejuvenate them, giving a new life. In India it has a long history of use by indigenous and tribal people, and in Ayurvedic or natural herbal medicine. Ayurveda classified this plant as 'rasayana' herb which is said to possess properties like antiaging, reestablishing youth, strengthening youth, strengthening life and brain power, and disease prevention like jaundice[25], all of which imply that they increase the resistance of the body against onslaught[14]. Pharmacological studies have demonstrated that *B. diffusa* known to possess diuretic[8], antifertility[2], antifibrinolytic[27], immunomodulatory[19], antidiabetic[22], antiviral[1], antistress[31], antimicrobial[12], anti-inflammatory, hepatoprotective[9], antioxidant[24], antiurethritis[23] and antimetastatic[18]. Phytochemical evaluation and GC MS analysis of the ethanolic extract of *B diffusa* carried out by the authors noticed the presence of dihydroxy anisole as the major compound with potent antioxidant activity besides high amount of phenolics and flavanoid[14]. This prompted us to study the antioxidant activity of the species, *Boerhavia diffusa* L.

MATERIALS AND METHODS

Preparation of plant extract

The fresh whole plants of *B. diffusa* collected from a wide population grown at the Botanical Garden, University of Kerala, during May 2014 were used as the experimental material. The voucher specimen (No: KUBH 5856) was deposited in the herbarium, Department of Botany, University of Kerala. The plant materials were washed thoroughly with distilled water and air dried in shade for two weeks at room temperature. Dried powdered sample (100gm) was extracted with ethanol, chloroform and petroleum ether (1000 ml) for 12 hrs and the extracts were denominated as BDE, BDC and BDP respectively. The filtered extracts were subjected to evaporation using a rotary evaporator, under reduced pressure at 50^0C.

Antioxidant activity of the plant extracts

Free radical scavenging activity on DPPH

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The 1, 1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assays are one of the most extensively used antioxidant assay for plant samples. The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The antioxidant activity was measured at 515nm after the thirty minutes incubation. In this method, a 0.1 nM solution of DPPH in methanol was prepared, and 4 ml of the solution was added to 1ml of the sample solution in methanol in varying concentrations. A large decrease in the absorbance of the reaction mixture indicates the significant free radical scavenging activity of the compound[6].

Hydroxyl radical scavenging assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction[11]. The reaction mixture contained 60µl of 1mM $FeCl_3$, 90µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2M phosphate buffer (pH 7.8), 150µl of 0.17 M H_2O_2, and 1.5mL of extract in various concentrations and it was incubated at room temperature for 5 min, absorbance was noted at 560nm. The % hydroxyl radical scavenging activity (HSRA) is calculated by the following formula:

% HSRA= $[(Abs_{control}-Abs_{sample})/Abs_{control}] \times 100$

Where $Abs_{control}$ is the absorbance of the control; Abs_{sample} is the absorbance of the extract/standard (ascorbic acid).

Nitric oxide scavenging assay

The assay is based on the principle that sodium nitropruside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions, which can be measured by a Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO[32]. The samples of different extracts were prepared in various concentrations in methanol and mixed with 3 ml of 10mM sodium nitroprusside. The same reaction mixture without the extract served as the control. The rection mixture was allowed to incubate at room temperature for 3hrs. Gallic acid was used as the standard for comparison. After incubation the samples were reacted with griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2% phosphoric acid), the absorbance of the chromophores formed were read at 546nm. The reactions were done in triplicate and % scavenging activity was caculated using the following formula:

% Scavenging= $[(Abs_{control}-Abs_{sample})/Abs_{control}] \times 100$

Where $Abs_{control}$ is the absorbance of the control; Abs_{sample} is the absorbance of the extract/standard (gallic acid).

Superoxide anion scavenging assay

The assay for superoxide anion radical scavenging activity was supported by riboflavin-light-NBT system. Briefly, 1ml of sample was taken at different concentration (25 to 200g/ml) and mixed with 0.5ml of phosphate buffer (50 mM, pH 7.6), 0.3 ml riboflavin (50mM), 0.25 ml PMS (20mM), 0.1 ml NBT (0.5 mM). After 5 min of incubation at 25^0C, the absorbance was measured at 560nm. Ascorbic acid was used as the standard[3].

Ferric reducing Antioxidant potential (FRAP)

This method is based on the reduction of colourless ferric complex (Fe^{3+} tripyridyltriazine) to blue coloured ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. FRAP values were obtained by comparing the absorbance change at 593 nm in test rection mixtures with those containing ferrous ion in known concentration. An aliquot(200µl) of the extract(with appropriate dilution) was added to 3ml of FRAP reagent (10 parts of 300mM sodium acetate buffer at pH 3.6, 1 part of 10mM TPTZ solution and 1 part of 20mM $FeCl_3.6H_2O$ solution) and the reaction mixture was incubated in a water bath at 37^0 C. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as µmol Trolox equivalent per gram of plant material on dry mass. All measurements were calculated from the value obtained from triplicate assays[4].

Phospho molybdenum assay (total antioxidant capacity)

The antioxidant activity of the samples was evaluated by the green phosphomolybdinum complex formation according to the method of Prieto *et al*[24]. An aliquot of 100µl of sample solution was combined with 1ml of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4 mM ammonium molybdate) and the mixture was incubated in a water bath at 95^0C for 90 min. After cooling the samples at room temperature, the absorbance of the mixture was measured at 695nm against a blank. Ascorbic acid (10mg/ml in DMSO) was used as the standard. The antioxidant capacity of the extract was evaluated as equivalents ascorbic acid (mg ascorbic acid/gm of dry extract).

Determination of reducing power

The reducing power was determined according to the method of Oyaizu[21]. Various concentrations of the extracts were mixed with 2.5ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5ml 0f 1% potassium ferricyanide. The mixture was incubated at 50^0C for 20 min. After 2.5 ml of 10% TCA (w/v) were added, the mixture was centrifuged and the upper layer was mixed with distilled water and ferric chloride and the absorbance was measured at 700nm. Higher absorbance indicates higher reducing power. The assays were carried out in triplicate and ascorbic acid was taken as the standard.

Non enzymatic antioxidants

Folin-Ciocalteau Total phenolic assay

Total phenolic constituents of plant extracts were estimated by Folin-Ciocalteau reagent[26]. The estimation was done spectrophotometrically at 760 nm and the results were expressed as gallic acid equivalents (GAE).

Estimation of Total flavanoid-Aluminium chloride Method

Aluminium chloride method was employed to quantify the total flavanoid content in the plant extracts. The results were expressed as rutin equivalents (QE)[7].

RESULTS AND DISCUSSION

A number of techniques have been done to evaluate the antioxidant activity of the plant *in vitro* in order to allow rapid screening of substances with low antioxidant activity *in vitro*, will probably show little activity *in vivo*. Free radicals generated during metabolic processes are known to play a definite role in a wide variety of pathological manifestations. Natural antioxidants either in the form of

Table 1: Quantitative phytochemical estimation of different extract of *B. diffusa*

Boerhavia diffusa extracts	Total Flavanoid	Total Phenol
Ethanol	73.2±5.01[a]	56.51±4.11[b]
Chloroform	56.3±4.05[a]	41.34±2.21[b]
Petroleum ether	24.1±1.12[a]	32.67±1.09[b]

[a] Flavanoid expressed in mg rutin/gm dry extract; [b] Phenolics expressed in mg gallic acid/gm dry extract;

Table 2: Determination of FRAP and total antioxidant activities of *B. diffusa* fractions.

FRAP assay	Total Antioxidant assay
19.48±1.02[c]	15.5±0.80[d]
14.67±0.89[c]	12±0.63[d]
11.67±0.51[c]	09±0.41[d]

[c] Ferric reducing activity expressed as µg trolox /100µm dry extract; [d] Total antioxidant capacity expressed in terms of µg Ascorbic acid/gm dry extract.

their chemical constituents or as raw extracts are very effective to prevent the harmful effects of free radicals.

Free radical scavenging activity on DPPH

DPPH (1, 1-diphenyl-2-picrylhydrazyl) analysis is one of the best known, accurate and frequently employed methods for evaluating antioxidant activity. It is a stable free radical because of its spare electron delocalization over the whole molecule. The donation of H^+ to the DPPH radicals made a corresponding change from violet colour to pale yellow in the solution. The degree of colour change is proportional to the potency and concentration of the antioxidants present in the extract[33]. Graph 1 showed the result of free radical (DPPH) scavenging activity of BDE, BDC and BDP, expressing the activity in percentage inhibition. The result revealed that the ethanol extract (BDE) exhibited the highest radical scavenging activity with an IC_{50} value of 82.12 µg/ml. Results of this assay suggest that the plant extract contain phytochemicals, capable of donating hydrogen to scavenge a free radical.

Hydroxyl radical Scavenging activity

The hydroxyl radicals are extremely reactive oxygen species that can react with every possible molecule in living organisms and can be quantified by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals generated by the Fenton reaction. They are capable of rapid initiation of the lipid peroxidation process by extracting hydrogen atoms from unsaturated fatty acids. The result showed (Graph 2) that different extracts of the plant have scavenging ability of OH^- free radicals in a dose dependent manner at the concentration 25-200 µg/ml. The IC_{50} values for BDE and ascorbic acid were 100.19µg/ml and 58.79µg/ml. Scavenging of hydroxyl radical is an important antioxidant activity because of its very high reactivity, which can cross the cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death. Thus, removing the hydroxyl radical is very important for the protection of living systems[31]. *Boerhavia diffusa* ethanolic extract showed strong hydroxyl radical scavenging ability.

Nitric oxide scavenging assay

Nitric oxide is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, antitumor activities etc. Nitric oxide or reactive nitrogen species, formed during their action are very reactive with oxygen or with superoxides. These compounds are responsible for altering the structural and functional behavior of many cellular components[28]. The nitric oxide scavenging activity of different extracts of the plant can be ranked as BDE > BDC >BDP indicating its strong nitric oxide scavenging capasity(Fig 3).

Superoxide radical scavenging activity

Superoxide generated as a result of numerous biological reactions, but they cannot directly initiate lipid peroxidation, they can only act as potential precursors of highly reactive species like dangerous hydroxyl radicals as well as singlet oxygen[30]. Results of superoxide radical scavenging activities of BDE, BDC and BDP were shown in the graph 4. The extracts demonstrated a dose response inhibition of the superoxide anion radicals. BDE exhibited good superoxide radical scavenging activity with an IC_{50} value of 99. 8µg/ml and was comparable with the standard ascorbic acid (109.09µg/ml). The results of our study revealed that BDE, BDC and BDP had effective capacity of scavenging for superoxide radical and correlated with total flavanoids content thus suggesting the antioxidant potential.

FRAP assay

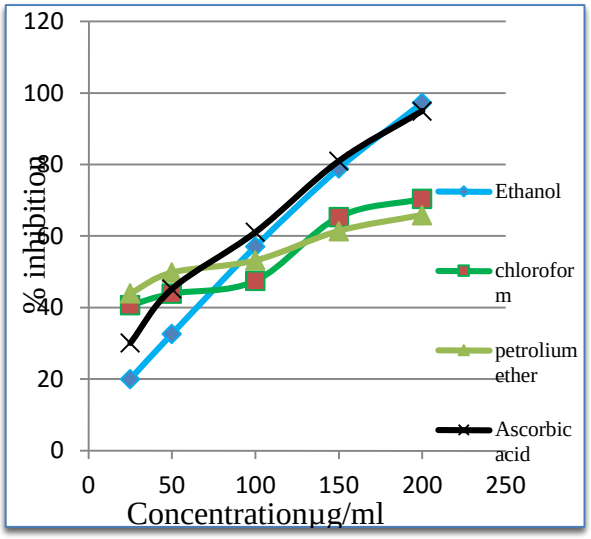
FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}- TPTZ)[13]. Ethanolic extract of *Boerhavia diffusa* showed greater FRAP value as 19.48±1.02 than chloroform (14.67±0.89) and petroleum ether (11.67±0.51) extracts Ferric reducing activity was expressed as µg trolox /100µm dry extract (Table 2). FRAP assay showed positive correlation between reducing power and phenolic content in *Boerhavia diffusa* extracts. The reducing properties associated with the presents of compounds exert their action by breaking the free radical chain through donating a hydrogen atom[9].

Phosphomolybdinum assay

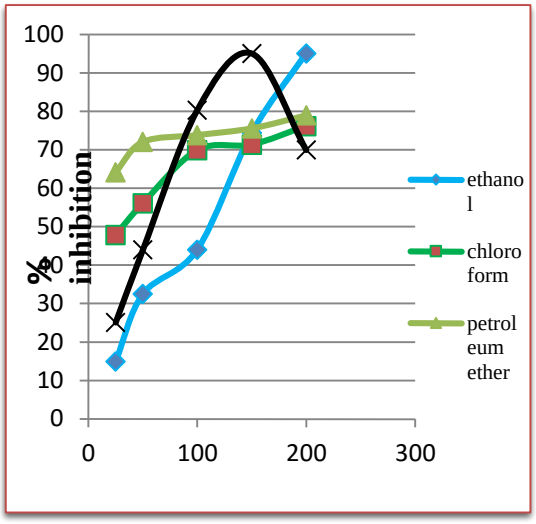
The antioxidant capacity of BDE, BDC and BDP were determined spectrophotometrically through phosphomolybdinum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and subsequent formation of green phosphate/Mo (V) complexes. The phosphomolybdinum reduction potential (PRP) of BDE was 15.5±0.80 and BDC was 12±0.63 and BDP was 09±0.41 (Table 2). The measure was expressed in terms of µg Ascorbic acid/gm dry extract. Phosphomolybdinum assay usually detects antioxidants such as ascorbic acid, some phenolics, a tocopherol and carotenoids[23]. However the result obtained by this assay confirms high reducing potency of BDE towards the transition metal ions.

Reducing power activity

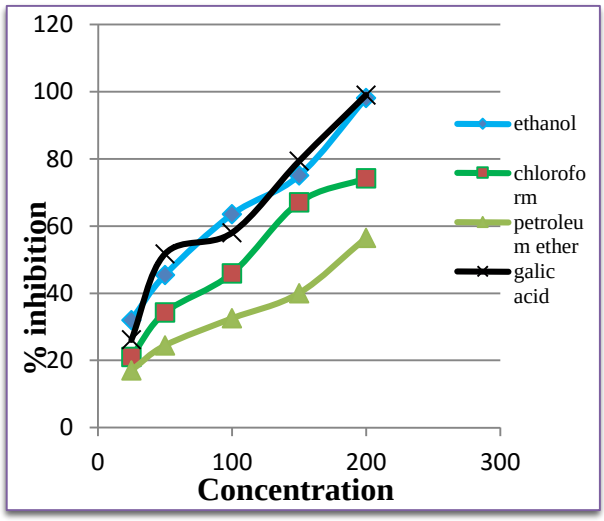
The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. In this assay, the ability of extracts to reduce fe^{3+} to fe^{2+} was determined.



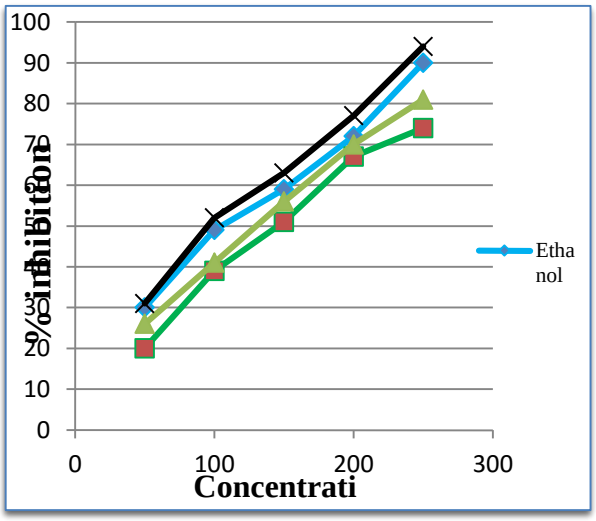
Graph 1: DPPH scavenging activity determination



Graph 2: Hydroxyl radical scavenging activity determination



Graph 3: Nitric oxide scavenging activity determination



Graph 4: Superoxide radical scavenging activity

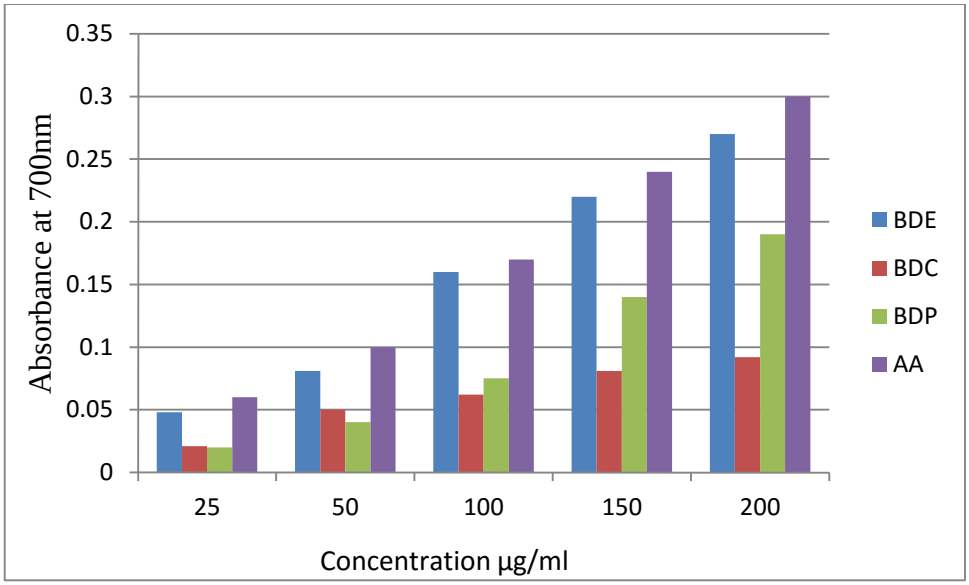
The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). Strong reducing agents, however, formed Perl's Prussian blue colour and absorbed at 700nm. Graph 5 showed the dose response curves for the reducing powers of all the extracts (25-200µg/ml) of *Boerhavia diffusa* in comparison with ascorbic acid as the standard. The ranking order for reducing power was BDE > BDP > BDC. Hence, ethanolic extract of the plant may act as electron donors and could react with free radicals to convert them into more stable products and then terminate the free radical chain reaction[29]. High reducing power observed in the extract (BDE) of *Boerhavia diffusa* suggests high antioxidant property as reported in sweet potato where a direct correlation was noticed between reducing power and antioxidant activity[17] (Graph 5).

Phytochemical estimation of extracts

Ethanol extract of *Boerhavia diffusa* registered for high levels of total free phenolics (73.2±0.01; phenolics expressed in mg gallic acid/gm dry extract), followed by total flavanoids (56.51±0.11; flavanoid expressed in mg rutin/gm dry extract). The order of total phenolics and flavanoid in different extracts was found to be in the order of BDE > BDC > BDP. Oki *et al*[20] observed that the radical scavenging activity increased with the increase of phenolic compound content. Result obtained in the present study revealed that the level of these phytochemicals in various extracts of *Boerhavia diffusa* were considerably higher in ethanol extract than that in other extracts, and this could be due to the different degree of polarity of solvents used for the extraction of phytochemicals. There is a highly positive correlation between polyphenols, flavanoids and antioxidant activities and it may be due to their redox properties, allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Furthermore, they scavenge free radicals and have a metal chelating potential.

CONCLUSION

The current study revealed that the whole plant extracts of *Boerhavia diffusa* had significant antioxidant and free radical scavenging activities. The species had the property to chelates iron and reducing power activity. The study



Graph 5: Reducing power activity determination

found out that ethanol extract of the plant was the strongest radical scavenger among the three screened. *In vitro* assay studies indicated that the extract of *Boerhavia diffusa* is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidant activity are currently unclear. Further investigations are needed to be carried out to isolate and identify the antioxidant compounds present in the plant extract. The *in vivo* antioxidant activity of this extract needs to be assessed prior to its introduction in both food and pharmaceutical industries.

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CONFLICT OF INTEREST
Declared none

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