Evaluation of total phenolic content and free radical scavenging activity of Boerhavia erecta

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Abstract

Introduction: Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases such as cancer, atherosclerosis, and gastric ulcer. Antioxidants protect other molecules from oxidation when they are exposed to free radicals and reactive oxygen species, which have been implicated in the etiology of many diseases and in food deterioration and spoilage.

Aims: The present study was designed to evaluate the antioxidant effects of the ethanolic extract of Boerhavia erecta.

Methods: In vitro methods of assessment were used to determine the scavenging activity of the extract on 1,1-diphenyl-2-picryl hydrazyl, superoxide scavenging, reducing power, nitric oxide scavenging, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), hydroxyl radical, ferric reducing antioxidant power, and chelating ability of ethanolic extract of B. erecta.

Results: The antioxidant activity of B. erecta ethanolic extract is comparable to a certain extent with that of butylated hydroxytoluene, mannitol, EDTA, and ascorbic acid.

Conclusion: The in vitro antioxidant assays reveal potent antioxidant and free radical scavenging activity of the leaves of B. erecta.

Keywords: Antioxidant activity; Boerhavia erecta; 1,1-Diphenyl-2-picryl hydrazyl; Total phenol

1. Introduction

Free radicals are molecules containing one or more unpaired electrons in atomic or molecular orbitals. There is increasing evidence that abnormal production of free radicals leads to increased oxidative stress on cellular structures and causes changes in molecular pathways that underpins the pathogenesis of several important diseases, including cardiovascular diseases, neurological diseases, cancer, and in the process of physiological aging. Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress. Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer, and in the aging process. Inflammation is one of the manifestations of oxidative stress, and the pathways that generate the mediators of inflammation, such as adhesion molecules and interleukins, are all induced by oxidative stress. This concept is supported by increasing evidence that oxidative damage plays a role in the development of acute and chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease and thus a necessity arises to extract these antioxidants from the plant matrices. For several years, many researchers have been searching for powerful but nontoxic antioxidants from natural sources, especially edible or medicinal plants. The medicinal properties of plants have been investigated in the recent scientific world, because of their potent antioxidant activities, lack of side effects, and economic viability.

Boerhavia erecta is a weedy herb of the family Nyctaginaceae and is commonly available in almost all places. It
is used as a traditional medicinal plant in Africa. It has been found to possess diuretic action, anti-inflammatory, antifibrinolytic, anticonvulsant, and hepatoprotective activities. The aim of the present study was to evaluate the antioxidant activity, reducing power and free radical scavenging activity of the ethanolic extract of B. erecta.

2. Materials and methods

2.1. Sample collection and preparation

The leaves of B. erecta were collected from Kolli Hills in Namakkal District, Tamil Nadu, India. The plant was identified at the plant anatomy research center. A voucher specimen of the plant has been deposited (Accession No: PARC/2012/1388). The leaves were shade dried at room temperature and then ground to a fine powder in a mechanic grinder. The powdered material was then extracted using solvent ethanol in the ratio 1:10 using a Soxhlet apparatus. After extracting all coloring material, the filtrate was concentrated by evaporating in a water bath under normal pressure.

2.2. Chemicals

All chemicals used including the solvents were of analytical grade. Butylated hydroxytoluene, gallic acid, Folin–Ciocalteu reagent, and ascorbic acid were purchased from Merck (Bangalore, India). All other chemicals and reagents used were of the highest commercially available purity.

2.3. Estimation of total phenol and flavonoid content

Total phenolic content (TPC) was analyzed by the Folin–Ciocalteu colorimetric method using gallic acid as standard and expressed as mg/g gallic acid equivalent. Flavonoid content was analyzed using catechol as standard and this was expressed as mg/g catechol.

2.4. Determination of antioxidant capacity

2.4.1. 1,1 Diphenyl-2-picryl-hydrazyl radical quenching activity

Various concentrations of ethanolic extract of the sample (4.0 mL) were mixed with 1.0 mL of methanolic solution containing 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radicals, resulting in the final concentration of DPPH being 0.2mM. The mixture were shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as control. The percentage of DPPH decolorization of the sample was calculated according to the equation:

\[
\text{% decolorization} = \left[1 - \left(\frac{\text{ABS sample}}{\text{ABS control}}\right)\right] \times 100
\]

IC\textsubscript{50} value (mg extract/mL) was the inhibitory concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison.

2.4.2. Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) under aerobic condition. The 3 mL reaction mixture contained 50 μL of 1M NBT, 150 μL of 1M NADH with or without sample, and Tris buffer (0.02M, pH 8.0). The reaction was started by adding 15 μL of 1M PMS to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

2.4.3. Reducing power

The reaction mixture contained 2.5 mL of various concentrations of ethanolic extract of the sample, 2.5 mL of 1% potassium ferric cyanide and 2.5 mL of 0.2M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture was incubated at 50°C for 20 minutes, and was terminated by the addition of 2.5 mL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. A 2.5 mL sample of the supernatant upper layer was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride, and absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicated increased reducing power of the sample. Ascorbic acid was used for comparison.

2.4.4. Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured. Briefly, the reaction mixture (5.0 mL) containing sodium nitroprusside (5mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25 °C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30 minute intervals by mixing 1.0 mL of incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546 nm.

2.4.5. Ferric-reducing antioxidant power assay

A stock solution of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40mM HCL, 20mM FeCl\textsubscript{3}, 6H\textsubscript{2}O and 0.3M acetate buffer (pH 3.6) was prepared. The ferric-reducing antioxidant power (FRAP) reagent contained 2.5 mL TPTZ solution, 2.5 mL ferric chloride solution, and 25 mL acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 μL) was mixed with 90 μL water and 30 μL test ethanolic extract of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe\textsuperscript{3+}-TPTZ) complex was reduced to ferrous (Fe\textsuperscript{2+}) form. The absorption at 540 nm was recorded.
2.4.6. ABTS scavenging activity

Samples were diluted to produce 0.2–1.0 μg/mL. The reaction was initiated by the addition of 1.0 mL of diluted ABTS to 10 μL of different concentrations of ethanolic extract of the sample or 10 μL methanol as control. The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation $I = A_0 / A_1 \times 100$, where $A_0$ is the absorbance of control reaction and $A_1$ was the absorbance of test compound.

2.4.7. Hydroxy radical activity

The reaction mixture contained 1.0 mL of 1.5 mM FeSO₄, 0.7 mL of 6mM hydrogen peroxide, 0.3 mL of 20mM sodium salicylate, and varying concentrations of the extract. After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as: Scavenging activity = $[1 - (A_1 - A_2)/A_0] \times 100$, where $A_0$ was the absorbance of the control (without extract), $A_1$ was the absorbance of test compound, and $A_2$ was the absorbance without sodium salicylate.

2.4.8. Chelating activity

The reaction mixture contained 1.0 mL of various concentrations of the extract, 0.1 mL of 2mM FeCl₂, and 3.7 mL methanol. The reaction was initiated by the addition of 2.0 mL of 5mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability. The capacity to chelate the ferrous ion was calculated by % chelation = $[1 - (ABS_{sample}/ABS_{control})] \times 100$.

2.5. Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation of three parallel measurements. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan’s multiple range test using SPSS version 16.

3. Results

3.1. TPC and flavonoid content

The TPC of ethanolic extract of *B. erecta* measured by Folin–Ciocalteu reagent in terms of gallic acid equivalent is 18.4 ± 0.8 mg/mL. The flavonoid content of the plant sample calculated as catechol equivalent is 11.6 ± 0.6 mg/mL (Fig. 1). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The results strongly suggest that the phenolics are important components of this plant and some of the pharmacological effect could be attributed to the presence of this invaluable component.

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>B. erecta (%DPPH)</th>
<th>Ascorbic acid (%DPPH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>22.42 ± 0.44</td>
<td>32.23 ± 0.24</td>
</tr>
<tr>
<td>400</td>
<td>29.68 ± 0.38</td>
<td>38.16 ± 0.32</td>
</tr>
<tr>
<td>600</td>
<td>36.96 ± 0.47</td>
<td>48.32 ± 0.26</td>
</tr>
<tr>
<td>800</td>
<td>48.42 ± 0.27</td>
<td>58.16 ± 0.34</td>
</tr>
<tr>
<td>1000</td>
<td>59.88 ± 0.38</td>
<td>68.08 ± 0.46</td>
</tr>
</tbody>
</table>

DPPH radical scavenging activity of ethanolic extract of *B. erecta* and ascorbic acid values are expressed as mean ± standard deviation ($n = 3$).
Superoxide scavenging activity and reducing power by ethanolic extract of *Boerhavia erecta*.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Superoxide scavenging (%)</th>
<th>Reducing power (absorbance)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. erecta</em></td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>200</td>
<td>19.94 ± 0.14</td>
<td>22.98 ± 0.22</td>
</tr>
<tr>
<td>400</td>
<td>29.28 ± 0.19</td>
<td>34.01 ± 0.28</td>
</tr>
<tr>
<td>600</td>
<td>43.24 ± 0.38</td>
<td>45.98 ± 0.32</td>
</tr>
<tr>
<td>800</td>
<td>51.88 ± 0.34</td>
<td>55.63 ± 0.43</td>
</tr>
<tr>
<td>1000</td>
<td>59.16 ± 0.42</td>
<td>65.12 ± 0.48</td>
</tr>
</tbody>
</table>

Superoxide scavenging activity and reducing power by ethanolic extract of *B. erecta*, and ascorbic acid, values are expressed as mean ± standard deviation (n = 3).

The nitric oxide scavenging activity of the ethanolic extract of *B. erecta* was 23.82% at the minimum concentration of 200 µg/mL, whereas the maximum activity was 58.46% at 1000 µg/mL. Fig. 2 indicates the percentage inhibition was increased with increasing concentration of the extract. However, the activity of ascorbic acid was more pronounced than that of the extract of *B. erecta*.

3.2.4. Nitric oxide scavenging activity

The ability of the ethanolic extract of *B. erecta* to quench nitric oxide radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species. Fig. 5 shows that extract displayed potential inhibitory effect of nitric oxide scavenging activity. The *B. erecta* extract exhibited the maximum activity of 60.72% at 200 µg/mL and the maximum activity of 60.72% at 1000 µg/mL.

3.2.6. ABTS scavenging activity

The reduction capacity of ABTS radical was determined by the decrease in its absorbance at 734 nm, which is induced by antioxidants. The ABTS scavenging activity of *B. erecta* extract at the concentrations from 200 µg/mL to 1000 µg/mL were 23.28–58.44%. BHT at concentrations from 200 µg/mL to 1000 µg/mL were also found to produce a dose dependent inhibition of ABTS radicals (Fig. 4).

3.2.7. Hydroxyl radical scavenging

The extract at the concentrations from 200 µg/mL to 1000 µg/mL showed increased absorbance as the concentration increased. Table 2 shows the dose-response curves of the reducing power of the extract.

3.2.8. Chelating ability

The chelating ability of the ethanolic extract of *B. erecta* was evaluated for its chelating ability. The minimum chelating ability of the extract was 25.42% at 200 µg/mL, and the maximum was 68.78% at 1000 µg/mL. EDTA used as standard showed strong activity. Fig. 6 shows that the absorbance of Ferrozine complex was decreased dose dependently; that is, the activity increased as concentration increased.
3.3. Correlation between the TPC and total antioxidant activity

The correlation analysis revealed that a correlation exists between TPC and the antioxidant activity. The correlation coefficient ($R^2 = 0.9930$; Fig. 1) indicates a positive relationship between the total phenolics and the antioxidant activity. The strong correlation between the results of measuring antioxidant capacity and total phenolic content showed that phenol compounds largely contribute to the antioxidant activities of the plant.

4. Discussion

4.1. TPC and flavonoid content

Phenolic compounds from plants are known to be good natural antioxidants. However, the activity of synthetic antioxidants was often observed to be higher than that of natural antioxidants. Phenolic compounds at certain concentrations markedly slowed down the rate of conjugated diene formation. Interest in phenolics is increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for antioxidant effect in plants.

4.2. Antioxidant activity

4.2.1. DPPH free radical scavenging activity

DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or a plant extract. In the present study, ethanolic extract of B. erecta showed potential free radical scavenging activity. DPPH is a stable and nitrogen-centered violet colored free radical that upon reduction is converted to yellow by the electron- or hydrogen-donating ability of the antioxidant compound found in the extract. Thus an antioxidant candidate that proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective in vivo studies.

The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non radical form DPPH-H by the reaction. With this method it was possible to determine the antiradical power of an antioxidant by measuring of a decrease in the absorbance of DPPH at 517 nm. Ascorbic acid was used as standard.

4.2.2. Superoxide radical scavenging activity

The superoxide anion has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Superoxide anion plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA. Superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT$^{2-}$) to produce the blue formazan, which is measured spectrophotometrically at 560 nm, and antioxidants are able to inhibit the blue NBT formation. The superoxide scavenging activity of the ethanolic extract of B. erecta has the potential to scavenge superoxide anions. It was reported that the superoxide anion scavenging activity could be due to the action of a free hydroxyl group of phenolic compounds.

4.2.3. Reducing power

The reducing power of the extract was evaluated by the transformation of Fe$^{3+}$ to Fe$^{2+}$ through electron transfer ability, which serves as a significant indicator of its antioxidant activity. The reducing properties of the plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by donating a hydrogen atom by breaking the free radical chain. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The results showed the comparable reducing power towards ascorbic acid. The yellow color of the test solution changes to various shades of green.

Fig. 5. Hydroxyl radical scavenging activity of ethanolic extract of Boerhavia erecta and mannitol. Values are expressed as mean ± standard deviation ($n = 3$).

Fig. 6. Chelating ability of ethanolic extract of Boerhavia erecta and EDTA. Values are expressed as mean ± standard deviation ($n = 3$).
and blue depending upon the reducing power of the samples. The presence of antioxidant substances in the compound samples causes the reduction of the Fe^{3+}/ferri cyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl’s Prussian blue at 700 nm. The data obtained in the present study suggest that it is likely to contribute significantly towards the observed antioxidant effects.

4.2.4. Nitric oxide scavenging activity
Nitric oxide plays a vital role in various inflammatory processes. Higher levels of these radical are toxic to tissue and contribute to the vascular collapse, various carcinoma, and ulcerative colitis. The toxicity of nitric oxide increases when it reacts with superoxide radical forming highly reactive peroxy nitrate anion. B. erecta extract decreases the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro. This may be due to the antioxidant principle in B. erecta extract, which competes with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in vivo.

4.2.5. FRAP
The FRAP assay is based on the capability of antioxidant to reduce TPTZFe (III) complex to the TPTZFe (II) complex, forming a powerful blue Fe^{2+}eTPTZ complex with an absorption maximum at 593 nm. The antioxidant properties of the plant extract was evaluated through FRAP assays. It must also be noted that the antioxidant activities assessed are in direct relation with the polyphenolic content of the extract. Finally, a good correlation coefficient was found between the FRAP assay radical scavenging assay. The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power.

4.2.6. ABTS radical scavenging activity
Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm, which decreases with the scavenging of the proton radicals. The health promoting properties of B. erecta may be due to its antioxidant properties and also attributed to its multi therapeutic characteristics. Antioxidant properties are known to be concomitant with the development of reducing power. Reductones can react directly with peroxides and can also prevent peroxide formation by reacting with certain precursors. ABTS radical scavenging activity is relatively recent, and involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages, and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range has raised interest in the use of ABTS for the estimation of antioxidant activity. In the present study results, B. erecta showed a higher antioxidant capacity. The extract showed potent antioxidant activity in ABTS method, which is comparable to the standard used.

4.2.7. Hydroxyl radical scavenging activity
Hydrogen peroxide itself is not very reactive, but sometimes it is toxic to cells because it may give rise to hydroxyl radical. Therefore, removing hydrogen peroxide is very important for antioxidant defense in a cell system. Hydroxyl radical scavenging capacity of B. erecta extract is directly related to its antioxidant activity. This method involves in vitro generation of hydroxyl radicals using Fe^{3+}/ascorbate/EDTA/H_{2}O_{2} system using Fenton reaction. The oxygen-derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) cause the degradation of deoxyribose into malondialdehyde, which produces a pink chromogen with thiobarbituric acid. It is clear from the result that the extract have shown a concentration dependent radical scavenging activity.

4.2.8. Chelating ability
An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydrogen peroxide decomposition and Fenton-type reactions. Therefore, it was considered of importance to screen the iron (II) chelating ability to the extract. In the present study, there was overwhelming evidence that the ethanolic extract of B. erecta had high chelating activity in vitro. It has been reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion.

5. Conclusion
There has been an increased interest globally to identify antioxidant compounds from plant sources, which are pharmacologically potent and have low or no side effects for use in protective medicine and the food industry. Plants produce a large amount of antioxidants to prevent oxidative stress, they represent a potential source of new compounds with antioxidant activity. Nowadays, there is an increasing interest in detecting the natural antioxidants, which are safe and effective, in order to replace the commercial synthetic antioxidants. Therefore it is time for us, to explore and identify our traditional therapeutic knowledge and plant sources and interpret it according to the recent advancements to fight against oxidative stress, in order to give it a deserving place.

Conflicts of interest
We declare that we have no conflicts of interest.

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