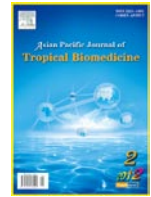




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### *In vitro* antioxidant activity of *Barleria noctiflora* L. f.

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#### ABSTRACT

**Objective:** To evaluate the antioxidant activity of defatted methanol extract of *Barleria noctiflora* (*B. noctiflora*) L.f. leaf and root using *in vitro* models. **Methods:** DPPH radical scavenging activity, ferrous reducing power, Fe<sup>2+</sup> chelating activity assay, nitric oxide radical scavenging activity, ABTS<sup>+</sup> radical cation decolourisation assay, superoxide anion and hydrogen peroxide radical scavenging activities were studied. **Results:** The extracts showed good antioxidant capacity in DPPH radical scavenging assay, when compared to other *in vitro* models and the IC<sub>50</sub> value were found to be 150 µg/mL in leaf extract and 140 µg/mL in root extract. The total phenolic content using Folin's-Ciocalteu reagent indicated that 1 mg of leaf and root extracts contain 368 µg and 481 µg with gallic acid equivalent and also the total flavonoid content found to be 240 µg and 410 µg respectively with quercetin equivalence. **Conclusion:** The results showed that the antioxidant potential of the extracts is high in root extract compared to the leaf extract. This is the first ever report of antioxidant studies in *B. noctiflora* L. f.

### 1. Introduction

Antioxidant play a major role in the living system and it prevent oxidative damage, when the oxidative damage occur in living system results in cancer, cardiovascular disease and diabetes<sup>[1]</sup>. The reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl, nitric oxide radical are various form of activated oxygen generated from biological reaction as oxidation product<sup>[2]</sup>. ROS are continuously produced during regular physiological process and it may cause cellular injuries, leading to the accumulation of lipid peroxides in biological membranes, damaging crucial biomolecules such as nucleic acids, lipids, proteins, polyunsaturated fatty acids and carbohydrates. The DNA damage can cause the mutation in living system. The oxidative stress leads to the pathogenesis of various lung disorders like asthma, chronic obstructive, pulmonary disorders, acute lung injury and lung cancer<sup>[3]</sup>. The ROS directly stimulate

histamine release from mast cells and mucus secretion from airway epithelial cells resulting in asthma. Most of the antioxidant present in a vascular plants such as Vitamin C and E, carotenoids, flavonoids and tannins<sup>[4]</sup>. Hence, research should focus on to improve the natural antioxidant from the natural resources.

Our aim in this study is to evaluate the antioxidant capacity of *Barleria noctiflora* (*B. noctiflora*) L.f, which belongs to the family Acanthaceae, which is being widely used as Folk medicine. It is widely distributed throughout tropical region of Africa, India, Sri Lanka and other parts of Asia<sup>[5]</sup>. *B. noctiflora* is a shrub and it grows up to 90 cm height. Many of the members of the Acanthaceae family are used as medication for asthma<sup>[6]</sup>.

The genus *Barleria* includes 28 taxa and 26 species. It has 3 unique characters calyx 4-partite with 2 large outer segments and 2 smaller inner ones, spheroidal, honey-combed pollen grains and the predominant with double cystoliths<sup>[7]</sup>. Most of the *Barleria* species are potent anti-inflammatory, analgesic, antileukemic, antitumor, anti hyperglycemic, anti-amoebic, virucidal and antibiotic<sup>[8–10]</sup>.

*B. prionitis* chemical constituents are barlerinoside, shanzhiside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetylshanzhiside methyl ester, barlerin, acetylbarlerin,

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7-methoxydiferroside and lupulinoside have possess enzyme inhibitory and free radicals scavenging activity and antifungal activity[11]. Furthermore, literature on *B. noctiflora* L. f. revealed that there is no study till now on the antioxidant and biological activities. Thus the present study was carried out to investigate the antioxidant activity of *B. noctiflora* L. f. leaf and root extracts using different *in vitro* models.

## 2. Material and methods

### 2.1. Collection of the plant samples

*B. noctiflora* L. f. was collected in and around Thanjavur District, Tamilnadu, India during winter season and the specimen was identified and authenticated by Dr. G. V. S. Murthy, Scientist 'F', Botanical Survey of India, Coimbatore-641 003, Tamilnadu, India. (Vide No. BSI/SRC/5/23/2010-11/Tech-1848 dated 8th February, 2011) and the voucher specimen was deposited at the same institute for future reference.

### 2.2. Chemicals

Petroleum ether, methanol, gallic acid, Folin's-Ciocalteu reagent, sodium carbonate, sodium dihydrogen phosphate dehydrate, disodium hydrogen phosphate dodeca hydrate, aluminium chloride, quercetin, L-ascorbic acid, 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, ferric chloride, ferrous chloride, ferrozine, EDTA, citric acid, sodium nitroprusside, sulfanilamide, o-phosphoric acid, naphthyl ethylene diamine dihydrochloride, 2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, phenazine methosulphate, nitro blue tetrazolium (NBT) and hydrogen peroxide were obtained from HiMedia laboratory and Qualigen, India. All the chemicals used in this study including the solvents were of analytical grade.

### 2.3. Extract preparation

The collected plant materials (leaf and root) were washed thoroughly in tap water, chopped, air dried for 2-3 weeks at 35-40 °C and pulverized in electric grinder. The 100 g dry powder obtained after defatted with petroleum ether and successively extracted with methanol (64-66 °C), finally 12.0 g and 8.0 g of extracts were obtained.

### 2.4. Phytochemical screening

Phytochemical screening was carried out to identify the secondary metabolites present in defatted methanolic extract of *B. noctiflora* L. f.[12-13].

### 2.5. Estimation of total phenolic content

The total phenolic content of the *B. noctiflora* L. f.

methanolic extracts was determined using Gallic acid equivalence (GAE)[14]. The dry extracts were diluted in methanol in the concentration of mg/ml and 50 µL of the samples was transferred to a 10 mL volumetric flask, to which 0.5 mL undiluted Folin's-Ciocalteu reagent was added. After one minute, 1.5 mL of 20% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added and the volume made up to 10 mL with distilled water. The reaction mixture incubated at 25 °C for one-hour and the absorbance was measured at 760 nm and compared with a pre-prepared gallic acid calibration curve. The blue colour formation is the end point of a reaction mixture.

### 2.6. Determination of total flavonoid content

The content of total flavonoid in methanolic extract of *B. noctiflora* L. f. leaves and root assessed followed by Marinova *et al*[15]. The 1 mL of extract (µg/mL) mixed with 0.5 mL of 2% AlCl<sub>3</sub> ethanol solution. After one-hour incubation at room temperature, the absorbance was measured at 510 nm. A yellow color indicated the presence of flavonoid. The total flavonoid content was calculated as quercetin equivalent (mg GE/g).

### 2.7. DPPH<sup>•</sup> radical scavenging activity

The ability of *B. noctiflora* L. f. extracts to scavenge DPPH radical was assessed using Mondal *et al*[16] method with modification. Briefly, aliquot of the extract 200-1 000 µg/mL was mixed with 3.0 mL DPPH (0.5 mmol/L in methanol), the resultant absorbance was recorded at 517 nm after 30 min. incubation at 37 °C. The percentage of scavenging activity was derived using the following formula, Percentage of inhibition (%) =  $(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$

Where  $A_{\text{control}}$  - absorbance of DPPH  
 $A_{\text{sample}}$  - absorbance reaction mixture (DPPH with Sample).

### 2.8. Ferrous reducing power

The reducing ability of *B. noctiflora* L. f. methanolic extracts was measured according to the method of Oyaizu[17]. Various concentrations (200 - 1 000 µg/mL) of the methanolic extracts (1.0 mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. with TCA (10%: 2.5 mL). Then mixture was centrifuged at 3 000 rpm for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (1%) and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. The reducing power of *B. noctiflora* L. f. methanolic extract was compared with that of standard antioxidant L-ascorbic acid (1 000 µg/mL).

### 2.9. Fe<sup>2+</sup> chelating activity assay

The chelating activity of *B. noctiflora* L. f. leaves and root extracts were evaluated by measuring the  $\text{Fe}^{2+}$  chelating activity according to the method of Dinis, *et al*[18]. Aliquot of 200–1 000  $\mu\text{g/mL}$  of extract, 1.6 mL of distilled water and 0.05 mL of  $\text{FeCl}_2$  (2 mM) were added and after 30 s, 0.1 mL ferrozine (5 mM) added. The reaction mixture was incubated for 10 min at 30 °C and the absorbance of the  $\text{Fe}^{2+}$  ferrozine complex was measured at 562 nm. A lower absorbance indicates a higher chelating power. The chelating activity of the extracts on  $\text{Fe}^{2+}$  was compared with that of EDTA (0.01 mM) and citric acid (0.025 M). The percentage of chelating activity calculated using the formula: % of chelating activity =  $(A_1 - A_2)/A_1 \times 100$

Where  $A_1$  – absorbance of the reaction mixture without extract and  $A_2$  – absorbance of the reaction mixture with extract.

### 2.10. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging was carried out as per the method of Sreejayan *et al*[19]. Nitric oxide radicals were generated from sodium nitroprusside solution. One mL of 10 mM sodium nitroprusside was mixed with 1 mL of methanolic extract of *B. noctiflora* L. f. aliquot 200–1 000  $\mu\text{g/mL}$  in phosphate buffer (0.2 M pH 7.4). The mixture was incubated at 25 °C for 150 min. After incubation the reaction mixture mixed with 1.0 mL of pre-prepared Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2% phosphoric acid). The absorbance was measured at 546 nm and percentage of inhibition was calculated using the same formula as above. The decreasing absorbance indicates a high nitric oxide scavenging activity.

### 2.11. ABTS.+ radical cation decolourisation assay

The *B. noctiflora* L. f. extracts were used evaluated their ABTS.+ radical capacity followed Re *et al*[20] method. The experiments were carried out using an improved ABTS decolourisation method.  $\text{ABTS}^+$  was generated by oxidation of  $\text{ABTS}^+$  with potassium persulfate. Three ml of generated  $\text{ABTS}^+$  solution were mixed with 30 mL of methanol extract solution in different concentration like 200 – 1 000  $\mu\text{g/mL}$ . The decreasing in absorption was measured during 6 min at 734 nm. The inhibition of the  $\text{ABTS}^+$  radical scavenging assay calculated using the above formula.

### 2.12. Superoxide anion scavenging activity

Superoxide anion scavenging activity was assessed according to the method of Ilhami *et al*[21]. The reaction mixture consisted of (200–1 000  $\mu\text{g/mL}$ ) dilution of *B. noctiflora* L. f. plant extract and L ascorbic acid was made upto 1 mL with respective solvent. The 1 mL of phenazine methosulphate (PMS) (60  $\mu\text{M}$ ), in phosphate buffer 0.1 M,

(pH 7.4) and 1 mL of nitro blue tetrazolium (NBT) (150  $\mu\text{M}$ ), in phosphate buffer 0.1 M, pH 7.4). The reaction mixture was incubated at 25 °C for 5 min. And the absorbance was measured at 560 nm. The abilities to scavenge the superoxide radical and the percentage inhibition were calculated using the above formula.

### 2.13. Hydrogen peroxide radical scavenging activity

The hydrogen peroxide radical scavenging activity was assessed by Ruch *et al*[22] method using methanolic extract of *B. noctiflora* L. f. leaves and root aliquots of 200– 1 000  $\mu\text{g/mL}$  were added to a 0.6 mL hydrogen peroxide (40 mM) with the already prepared phosphate buffer (pH 7.4). The reaction mixtures were incubated at room temperature for 10 mins. After incubation, the reaction mixture read at 230 nm against the blank solution with phosphate buffer (pH 7.4). The percentage of inhibition calculated based on the formula: % of inhibition =  $(A_1 - A_2)/A_1 \times 100$

Where  $A_1$  –absorbance of the  $\text{H}_2\text{O}_2$

$A_2$  –absorbance of the reaction mixture with extract

### 2.14. Statistical analysis

All experiments were repeated at least thrice. The results were expressed as mean  $\pm$  standard deviation.

## 3. Results

### 3.1. Phytochemical screening of *B. noctiflora* L.f.

The defatted methanolic extract of *B. noctiflora* L.f. leaf and root revealed the presence of alkaloids, terpenoids, flavonoids, steroids, cardioglycosides, tannins, aminoacids and proteins (Table 1).

**Table 1.**  
Phytochemical screening of the *B. noctiflora* L.f.

Extracts	Major secondary metabolites present								
	AL	SA	TER	FLA	S	CG	T	AA	P
Leaf	+	–	+	+	+	+	+	+	+
Root	+	–	+	+	+	–	+	+	+

Alkaloids – AL, Saponins – SA, Terpenoids – TER, Flavonoids – FLA, Steroids – S, Cardioglycosides – CG, Tannins – T, Aminoacids – AA and Protein – P.

### 3.2. Estimation of total phenolic content

The total phenolic content in *B. noctiflora* L. f. methanolic extract of leaf and root were obtained (Figure 1). The Gallic acid linear curve obtained using the  $Y=0.0030X + 0.0166$  ( $R^2 = 0.9963$ ). Using this gallic acid linear curve, *B. noctiflora* leaf and roots total phenolic content values were 368  $\mu\text{g/mL}$  and 481  $\mu\text{g/mL}$  respectively. The Total phenolic content was high in root extract compared to the leaf extract. It shows the *B. noctiflora* L. f. root posse's high antioxidant ability.

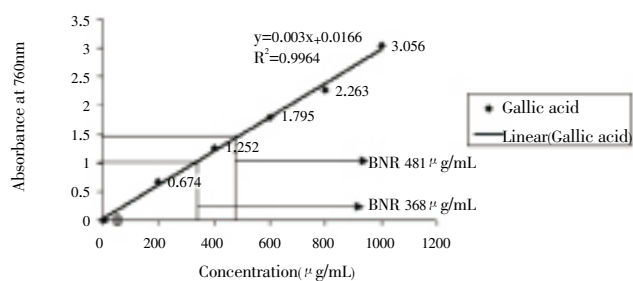


Figure 1. Total phenolic content.

### 3.3. Determination of total flavonoid content

The total flavonoid (mg/mL) content were obtained using the regression calibration curve  $Y=0.0006X-0.0129$ ,  $R^2=0.9955$  (Figure 2) with quercetin equivalent. The crude extract of *B. noctiflora* L. f. leaf and root contains  $240 \mu\text{g/mL}$  and  $410 \mu\text{g/mL}$  respectively. High amount of flavonoids were found in *B. noctiflora* L. f. root extract compared to the leaf extract.

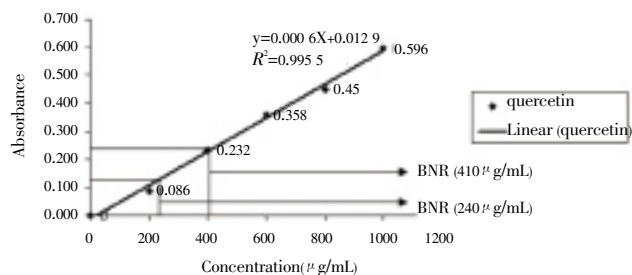


Figure 2. Total flavanoid content.

### 3.4. DPPH radical scavenging activity

The photometric evaluation of the antioxidant capacity of the methanolic extract of *B. noctiflora* L. f. leaf and root showed good antioxidant capacity (Figure 3). Significant decreases were observed in the DPPH radical activity due to the scavenging ability of the extracts.

The  $IC_{50}$  value of the methanolic extract of *B. noctiflora* L. f. leaf, root and standard antioxidant (ascorbic acid) such as  $150 \mu\text{g/mL}$ ,  $140 \mu\text{g/mL}$  and  $110 \mu\text{g/mL}$  respectively. A lower  $IC_{50}$  value indicates a higher free radical scavenging activity<sup>[30]</sup>. The ability of DPPH radical scavenging is higher in root extract compare to the leaf extract.

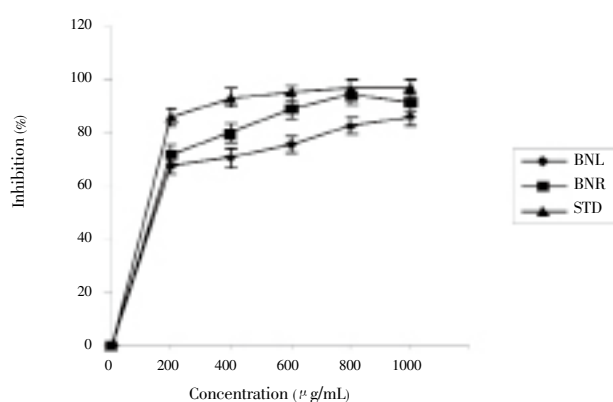


Figure 3. DPPH radical scavenging activity.

### 3.5. Ferrous reducing power

The reducing ability of *B. noctiflora* L. f. Leaf and Root extracts increased with increasing concentration of the sample. The higher absorbance value indicated that high antioxidant capacity of the extracts<sup>[31]</sup>. The result showed significant value in root extract compared to the leaf. The result showed the extract possess ferric ions ( $Fe^{3+}$ ) reducing ability (Figure 4).

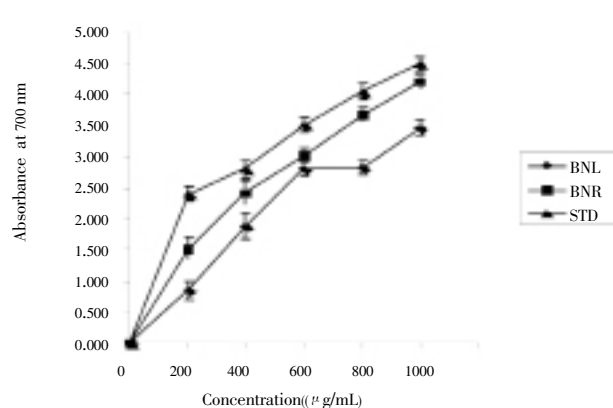


Figure 4. Ferrous reducing power.

### 3.6. $Fe^{2+}$ chelating activity assay

The  $Fe^{2+}$  chelating activity of *B. noctiflora* L. f. leaf and root shows potent chelating power and the  $IC_{50}$  value  $195 \mu\text{g/mL}$  in root,  $440 \mu\text{g/mL}$  in leaf and standard (ascorbic acid) found to be  $160 \mu\text{g/mL}$  (Figure 5). The iron generates free radicals through the fenton and Haber–Weiss reactions that prevent the oxidative damage.

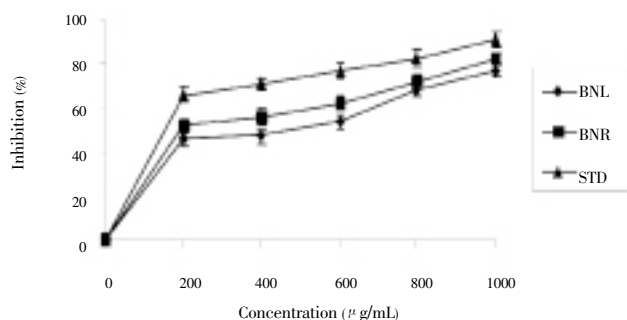


Figure 5. Fe<sup>2+</sup> chelating activity.

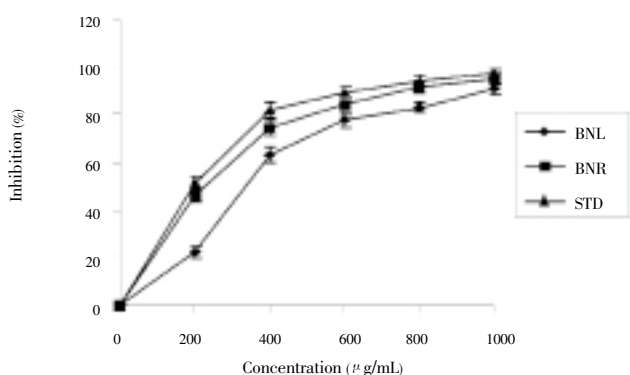


Figure 6. Nitric oxide radical scavenging activity.

### 3.7. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of *B. noctiflora* L. f. leaf and root extract performed by formation of nitric oxide using sodium nitroprusside. The sodium nitroprusside act as major source of nitric oxide radicals. The extracts scavenges the nitric oxide formed from the sodium nitroprusside by inhibiting the chromophore formation, hence absorbance decrease as the concentration increased extracts[35]. The IC<sub>50</sub> values were 220 µg/mL in root, 360 µg/mL in leaf (Figure 6).

### 3.8. ABTS.+ radical cation decolourisation assay

The ABTS<sup>•+</sup> radical scavenging ability found to be high in root compared to the leaf extract. The IC<sub>50</sub> value found 160, 150 and 140 µg/mL in leaf, root and L-ascorbic acid standard respectively (Figure 7).

### 3.9. Superoxide anion scavenging activity

The reduction of the yellow dye (NBT<sup>2+</sup>) to produce the blue formazan and it measured with spectrophotometrically at 560 nm. The *B. noctiflora* L. f. leaf and root extract have potent antioxidants capacity. The decrease of absorbance at 560nm indicates the high

antioxidant power and the IC<sub>50</sub> values of *B. noctiflora* L. f. leaf and root were 600 µg/mL and 430 µg/mL respectively (Figure 8).

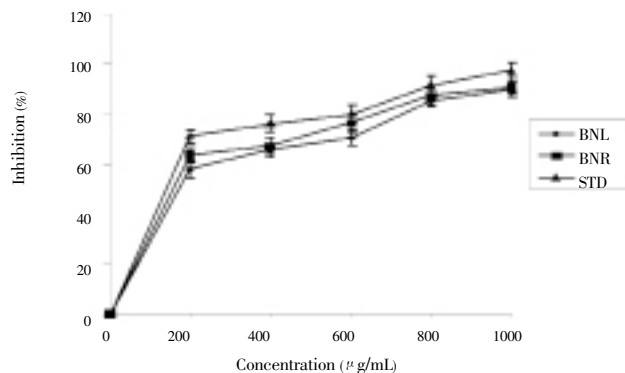


Figure 7. ABTS radical cation decolorization assay.

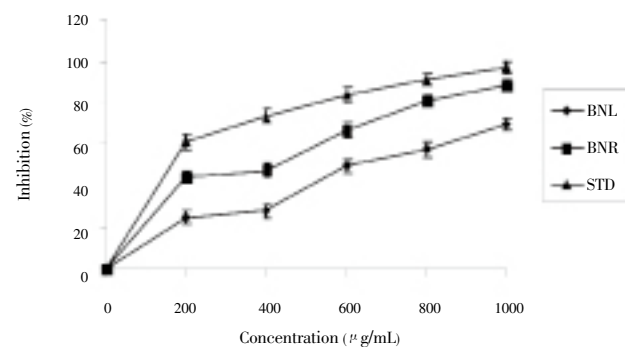


Figure 8. Superoxide radical scavenging activity.

### 3.10. Hydrogen peroxide radical scavenging activity

The scavenging ability of the *B. noctiflora* L. f leaf and root extracts are shown in Figure 9. The L-ascorbic acid was used as standard antioxidant and found the IC<sub>50</sub> value 400 µg/mL. The *B. noctiflora* L. f root posses scavenging activity in high compared to the leaf and IC<sub>50</sub> values were 600 µg/mL and 800 µg/mL respectively.

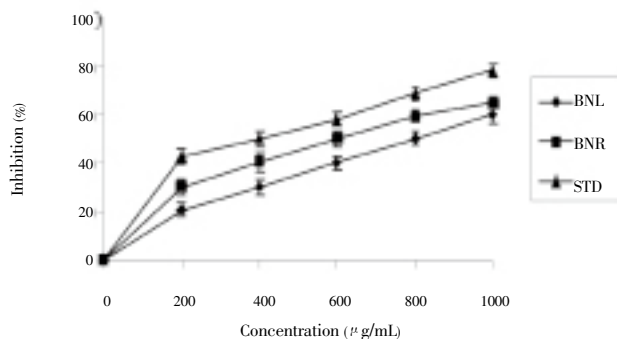


Figure 9. Hydrogen peroxide radical scavenging assay.



#### 4. Discussion

In most developing countries 80% of people utilize medicinal plants for the maintenance of good health as they have powerful natural antioxidants properties[23]. Nowadays most of the people include natural antioxidants as nutraceuticals or as food additives[24]. Many medicinal plants contain higher phenolic compounds such as flavonoids, monophenols and polyphenols[25]. The phenolic compounds was directly correlated with its antioxidant ability[26] and it is abundant in some fruits, vegetables and flowers[27]. Flavonoids are one of the most diverse and widespread groups of natural compounds[28]. The plant derived antioxidants especially polyphenols and flavonoids have been used to treat various disease such as cancer, diabetic, aging and prevention of cardiovascular disease.

The DPPH free radical scavenging activity of the methanolic extract of *B. noctiflora* L. f. leaf and root possess ability to scavenge DPPH free radicals as equal to the standard antioxidant L-ascorbic acid. It produced hydrazine by converting the unpaired electrons to paired electron due to the hydrogen donating ability of the extract[29]. The reduction ability was evaluated using the  $Fe^{3+}$ - $Fe^{2+}$  transformation using the extracts. The reduction capability of the extracts indicated them as potent antioxidant. The outcome of the reducing reaction is to terminate the radical chain reactions that may be very damaging to the tissues. The yellow color of the reaction mixture changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing ability of the extract can be measured by the direct reduction of  $Fe(CN)_6^{3-}$  to  $Fe(CN)_6^{4-}$ , the addition of free  $Fe^{3+}$  to the reduced product leads to the formation of the intense prussian blue complex,  $Fe_4[Fe(CN)_6]_3$ , which show strong absorbance at 700 nm[32]. It indicates the electron donating capacity of the extracts. The increasing absorbance of the reaction mixture indicates the reducing capacity due to the increasing blue colour complex formation.

Iron is an essential mineral for normal physiological activity of the human body, but excess can cause cellular damage and injury. The ferrous ions are the most effective pro-oxidants in food systems, the good chelating effect would be beneficial and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress-induced disease[33]. The chelating ability of the extracts evaluated using the metal chelation with ferrozine on ferrous ion by forming a stable iron chelation. The high chelating power reduces the free ferrous ion concentration thus decreasing the fenton reaction which is implicated in many disease[34].

The nitric oxides has important role in human body particularly in various types of inflammatory process[36], physiological process and it is also important as chemical mediator in endothelial cells, macrophages, neurons[37]. The excess concentration of Nitric oxide in human body may cause several diseases. The oxygen reacts with the excess nitric oxide to generate nitrate and peroxynitrite

anions, which act as free radicals[38]. DPPH radical scavenging activity involves H atom transfer and  $ABTS^{\bullet+}$  radical involve an electron transfer process[39].

The oxidative enzyme of a body produced superoxides from molecular oxygen through non enzymatic reaction. The superoxide generate more dangerous oxygen species in a human body such as singlet oxygen and hydroxyl radicals and these may cause the peroxidation of lipids[40].

Superoxide anions is a ancestor of active free radicals and may react with biological macromolecules leading to tissue damage[41]. Very slow reactive agents of the hydrogen peroxide some time may cause cell death due to the production of hydroxyl radical within the cells.

The presence of  $H_2O_2$  in the cell culture may lead to the oxidative DNA damage. Hence removing hydrogen peroxide is very essential for antioxidant defense in cells.

In conclusion, this is the first report on the antioxidant potentials of *B. noctiflora* L. f. leaf and root extracts. The defatted methanolic extract of *B. noctiflora* L.f. leaf and root revealed the presence of secondary metabolites such as alkaloids, terpenoids, flavonoids, steroids, cardioglycosides, tannins, aminoacids and proteins. *B. noctiflora* L. f. was also found to be an effective antioxidant in several in vitro assays including: DPPH $^{\bullet}$ , reducing power,  $Fe^{2+}$  chelating, ABTS radical scavenging, nitric oxide scavenging, superoxide scavenging and  $H_2O_2$  radical scavenging and posses good phenolic and flavonoid contents.

These results show the radical scavenging activity is high in root extract compared to the leaf extracts. The isolation of bioactive compounds from *B. noctiflora* L. f. will definitely serve as a good phytotherapeutic agent.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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