

IN VITRO ANTIOXIDANT POTENTIAL OF THE METHANOLIC EXTRACT OF *Bacopa monnieri* L.

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Abstract

The crude methanolic extract of Bacopa monnieri was studied to evaluate its antioxidant activity by six in vitro methods. The extract was found to contain significant amount of phenol and flavonoid in Folin-Ciocalteu and total flavonoid content assay. Total antioxidant capacity of the extract was estimated to be 261.16 mg/g of ascorbic acid equivalent. Its IC₅₀ value in DPPH method was 456.07 µg/mL. The extract showed concomitant increase in reducing power with the increase of concentration of the extract. IC₅₀ value in NO scavenging activity of the extract was 21.29 µg/mL whereas ascorbic acid and quercetin showed the values of 5.47µg/mL 15.24µg/ml, respectively.

Key words: *Bacopa monnieri, Antioxidant, DPPH, Reducing power, Nitric oxide.*

***Bacopa monnieri* L. Metanollü Ekstresinin *In vitro* Antioksidan Potansiyeli**

Bacopa monnieri'nin ham metanollü ekstresinin antioksidan aktivitesi in vitro altı metotla değerlendirilmiştir. Ekstrenin Folin-Ciocalteu metodu ve total flavonoid içeriği tayinleri sonucunda önemli miktarda fenolik bileşen ve flavonoid taşıdığı belirlenmiştir. Ekstrenin total antioksidan kapasitesi 261.16 mg/g askorbik asite eşdeğer olarak bulunmuştur. DPPH metotunda IC₅₀ değeri 456.07 µg/mL'dir. Ekstre konsantrasyonunun artmasına bağlı olarak indirgeme kapasitesi artış göstermiştir. Ekstrenin NO temizleyici aktivitesi için IC₅₀ değeri 21.29 µg/mL olarak belirlenirken, askorbik asit ve kersetin için IC₅₀ değerleri sırasıyla 5.47µg/mL ve 15.24µg/mL olarak bulunmuştur.

Anahtar kelimeler: *Bacopa monnieri, Antioksidan, DPPH, İndirgeme kapasitesi, Nitrik oksit.*

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INTRODUCTION

Increased concentrations of free radicals in the body lead to various pathological conditions such as atherosclerosis, arthritis, Alzheimer's disease, cancers, etc. Detrimental effects resulted from the imbalance in the antioxidant-prooxidant ratio can be chiefly prevented by the intake of antioxidants (1). Antioxidants can be of both synthetic and natural origins. Natural antioxidants that are obtained from plants contain mainly phenolic compounds. Utilization of natural antioxidants from plants does not provoke adverse effects, while synthetic antioxidants are found to induce genotoxic effects (2).

Bacopa monnieri Linn. (*Scrophulariaceae*) is native in India, Bangladesh and Burma that commonly grows in marshy areas throughout India, Nepal, Sri Lanka, China, Taiwan, and Vietnam. The plant is also found in Florida and other southern states of the USA. *B. monnieri* has been used traditionally in the treatment of insanity, epilepsy and hysteria. The other reported activities include cardiac tonic, digestive, sedative, antiepileptic, vasoconstrictor and anti-inflammatory (3,4). The ethanol extract of *B. monnieri* was found to contain saponins, mainly bacoside A (5), that has been used for memory and intellectual improvement. Subsequent studies showed that bacoside A and B were responsible for the cognition facilitating effect (6). Other than facilitating learning and memory, these constituents in normal rats inhibited the amnesic effects of scopolamine, electroshock and immobilization stress (7). Heresaponin, herpestine brahmine and flavonoids were also found in *B. monnieri*. As both flavonoids and saponins possesses antioxidant property, in the present study we have made an effort to determine the total phenolic and flavonoid contents as well as to establish the antioxidant effect of *B. monnieri* by radical scavenging activity and ferric reducing power tests.

MATERIALS AND METHODS

Collection of plant material

B. monnieri was collected in January, 2009 from the botanical garden of Department of Pharmacy, Jahangirnagar University, Dhaka, Bangladesh. The plant was identified by Prof. Dr. A. B. M. Enayet Hossain of the Department of Botany, Jahangirnagar University, Dhaka, Bangladesh. A voucher herbarium specimen no. 32175 is maintained for future reference.

Extraction

The plant material was sun-dried first and then, dried in an oven at reduced temperature (< 70 °C) to make suitable for grinding. The powdered plant material was submerged in sufficient volume of methanol in an air-tight flat bottomed container for seven days, with occasional shaking and stirring. The organic phases were then filtered and dried on electrical water bath to give the crude methanol extract.

Chemicals & Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, quercetin and gallic acid were obtained from Sigma Chemical Co. (MO, USA). Folin-Ciocalteu's (FCR) and Griess reagents were purchased from Merck. All other chemicals and reagents were of analytical grade.

Determination of Total Phenol Content

Total phenol content of the extract was determined by Folin Ciocalteu's reagent (8). A diluted plant extract (0.5 mL of 1:10 g/mL diluted with distilled water) or gallic acid (standard) was mixed with Folin Ciocalteu's reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 mL, 1 M). The mixtures were allowed to stand for 15 minutes and the total phenol amount was determined by colorimeter at 765 nm. The standard curve was prepared

using 0, 50, 100, 150, 200, 250 mg/L solutions of gallic acid in methanol: water (50:50, v/v). Total phenol value is expressed in terms of gallic acid equivalent (mg/g of dry extract).

Determination of flavonoid content

Aluminum chloride colorimetric method was used for determination of total flavonoids (9). The plant extract (0.5 mL of 1:10 g/mL) in methanol was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. It remained at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 µg/mL in methanol.

Determination of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH described by Prieto *et al* (10). The antioxidant capacity is expressed as ascorbic acid equivalent (AAE). The plant extract (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm against methanol as blank. Total antioxidant capacity of the extract was measured from the regression equation prepared from the concentration versus optical density of ascorbic acid.

DPPH Scavenging Activity

DPPH scavenging activity of *B. monnieri* was measured by the method developed by Manzorro *et al* (11). Sample of the extract (0.2 mL) was diluted with methanol and 2 mL of DPPH solution (0.5 mM) was added. After 30 min, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated.

Total Reducing Power Determination

The reducing power of the extract was determined according to the method of Oyaizu (12). 10 mg of the extract in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 minutes. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Gallic acid, quercetin and ascorbic acid were used as the references. The analyses were performed in triplicate and the results were averaged. Increased absorbance of the reaction mixture indicated increasing reducing power.

NO Scavenging Activity

The scavenging effect was measured according to the method of Alisi *et al* (13). The extract solution (4 mL) at different concentrations was added in the test tubes to 1 mL of sodium nitroprusside solution (5 mM) and the tubes incubated at 29°C for 2 h. An aliquot (2 mL) of the incubation solution was removed and diluted with 1.2 mL of Griess reagent (1% Sulfanilamide in 5% H_3PO_4 and 0.1 % naphthylethylenediamine dihydrochloride). The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 550 nm and referred to the absorbance of standard solution of sodium nitrite salt treated in the same way with Griess reagent.

RESULTS AND DISCUSSION

Total phenolic content

The result was calculated from the regression equation of the calibration curve ($y=0.013x+0.127$, $r^2=0.988$). The content of phenolics in the extract of *B. monnieri* was 21.54 mg/g gallic acid equivalent (GAE). Earlier report of Ghosh *et al* (14) indicated that ethanolic extract of the aerial parts of *Bacopa monnieri* contained 47.7 mg/g pyrocatechol equivalent. Phenolic compounds in plants serve as one of the key roles as primary antioxidants or free radical scavengers. The antioxidant activity of the phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (15). It has been proposed that polyphenolic compounds provide antimutagenic and anticarcinogenic properties in humans, when ~1.0 g was daily consumed from a diet rich in vegetables and fruits (16). The content of total phenolics in the methanol plant extract was determined using the Folin-Ciocalteu reagent.

Total flavonoid content assay

Flavonoid content was calculated from the regression equation of the calibration curve ($y = 0.009x - 0.036$) and is expressed as quercetin equivalents (QE). Total flavonoid content was 24.36 mg/g quercetin equivalent in *B. monnieri*.

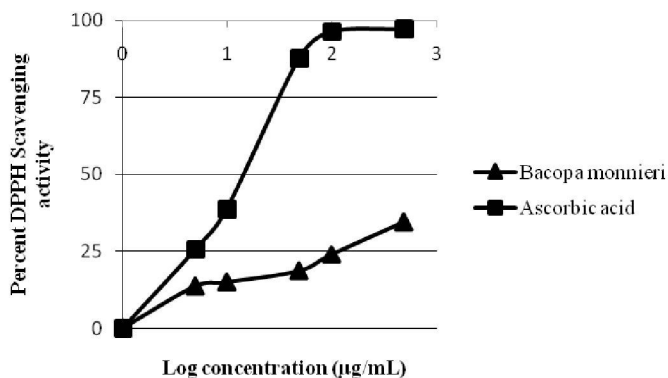
Total antioxidant assay

The total antioxidant capacity of the methanol extract of *B. monnieri* was determined from the calibration curve ($y=0.005x-0.028$, $r^2= 0.988$) established by ascorbic acid at 695 nm. Ascorbic acid equivalent of *B. monnieri* was 261.16 mg/g. Previous study gave similar result and explained there was no correlation between antioxidant activity and total phenol/flavonoid content (17). Significant antioxidant and protective activity of *B. monnieri* extract was also proved in diabetic rats by Kakkar *et al.* (18).

DPPH scavenging activity

DPPH acts as a stable free radical in methanol solution that easily accepts an electron or hydride radical and converted to a stable diamagnetic molecule. By reacting with suitable reducing agents DPPH radicals formed into the corresponding hydrazine. Depending on the number of electrons taken up, the solution therefore loses colour stoichiometrically. Figure 1 shows the amount of each extract needed for 50% inhibition. (IC_{50} value) IC_{50} value of *B. monnieri* was found to be 457.09 $\mu\text{g/mL}$ whereas ascorbic acid showed the value of 14.45 $\mu\text{g/mL}$. Earlier study conducted by Mahadik *et al.* showed the prominent DPPH scavenging capacity of solvent free lipid based extract, methanolic extract and Ayurvedic Ghrita of *B. monnieri* (19).

Figure 1. Comparative DPPH scavenging activity



Reducing power assessment

The antioxidant activity of the plant extract has been found to be associated with the advance of reducing power. The reducing power of the extract might be due to its hydrogen

donating ability (20). The reducing power of the extract and ascorbic acid, gallic acid and quercetin is shown in Table 1. The reducing power was found to be increased with the increase of the concentration of the extract, indicating the presence of some compounds in the extract that is both electron donors and could react with free radicals and to terminate free radical chain reactions.

Table 1. Comparative reducing power of the extract with references

Concentration. ($\mu\text{g/mL}$)	<i>Bacopa monnieri</i>	Ascorbic acid	Quercetin	Gallic acid
5	15.89 \pm 0.045	17.397	16.30	19.452
50	59.32 \pm 0.068	73.699	290.14	218.082
100	189.04 \pm 0.135	263.699	434.25	408.219
200	415.34 \pm 0.233	485.753	763.01	736.301

NO scavenging activity

Nitrosative stress results from the overproduction of reactive nitrogen species that may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralize and eliminate them (21). This may initiate nitrosylation reactions which can modify the protein structure and thus inhibit their normal function. Inactivation and nitration of human superoxide dismutase (SOD) by fluxes of nitric oxide radicals have been shown (22).

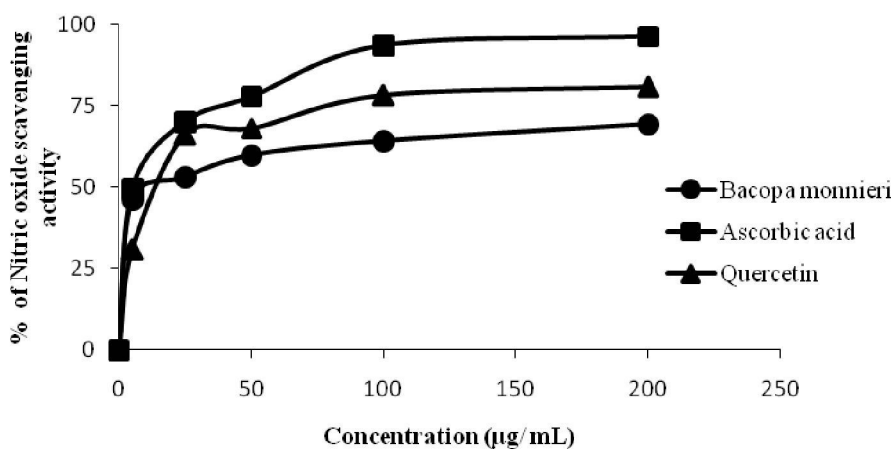


Figure 2. Comparative NO scavenging activity

It is also clear that excessive production of free radicals causes damage to biological material and is an essential event in the etiopathogenesis of various diseases (23). Incubation of solutions of sodium nitroprusside in PBS at 25 $^{\circ}$ C for 2 h resulted in linear time dependent nitrite production, which is reduced by the tested methanolic extract of the plant. NO scavenging capacity is determined by the decrease in the absorbance at 550 nm, induced by antioxidants. In order to evaluate the antioxidant potency through NO scavenging by the test samples, the change of optical density of NO was monitored. Figure 2 shows the comparative NO scavenging activity of the extract with standard antioxidants whereas IC₅₀ value of the methanolic extract of *B. monnieri* was found to be 7.29 $\mu\text{g/mL}$. The findings differ significantly from previous reports of Ghosh *et al*, 2007 where IC₅₀ value was 29.17 $\mu\text{g/mL}$ (14). During

peroxynitrite (ONOO⁻) scavenging activity of the methanolic extract of *B. monnieri* IC₅₀ value was estimated as 11.23 µg/mL by Haque *et al* (24).

CONCLUSION

From the above results, it can be concluded that the crude extract of *Bacopa monnieri* has potential antioxidant properties. The plant could be subjected for extensive chromatographic separation and purification processes to isolate bioactive lead compounds for the discovery of new therapeutic agents.

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