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PEROXYNITRITE SCAVENGING AND TOXICITY POTENTIAL OF DIFFERENT FRACTIONS OF THE AERIAL PARTS OF *BACOPA MONNIERA* LINN.

M. B. Alam [1], M. S. Hossain [1], M. Asadujjaman [1], M. M. Islam [1], M. E. H. Mazumder [2] and M. E. Haque*[1]

Department of Pharmacy, Atish Dipankar University of Science & Technology[1], Dhaka, Bangladesh

Discipline of Biomedical Science, Faculty of Medicine, Cumberland Campus, University of Sydney[2], Australia

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Correspondence to Author:

Prof. (Dr.) Md. Ekramul Haque

Department of Pharmacy, Atish Dipankar University of Science & Technology, House # 83, Road # 4, Block # B, Banani, Dhaka, Bangladesh

ABSTRACT

Bacopa monniera is locally referred as Brahmi Shak, has been reputed to use in the Ayurvedic system of medicine for centuries. In our ongoing study to identify the scavenger of $ONOO^-$ from natural sources, the aerial parts of *Bacopa monniera* Linn. was evaluated to scavenge authentic peroxynitrites ($ONOO^-$) and to inhibit total reactive oxygen species (ROS) generation, in kidney homogenates using 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA). A methanol (MeOH) extract of the *B. monniera* showed strong $ONOO^-$ scavenging activity in the $ONOO^-$ system (IC_{50} = 11.23±2.14 µg/ml), and marginal activity in the total ROS systems (IC_{50} = 46.52±0.91µg/ml). So the MeOH extract was fractionated with several organic solvents, such as dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and n-butanol (n-BuOH). The EtOAc soluble fraction, showed strong activity in all the model systems tested. The Brine shrimp lethality bioassay method was used to determine the toxicity of the extracts where Vincristin sulphate was used as a positive control. The dichloromethane (CH_2Cl_2) fraction showed highest activity (LC_{50} = 19.02 ± 1.16 µg/ml) and other showed activity in the order of: CH_2Cl_2 fraction > MeOH extract > EtOAc fraction > n-BuOH fraction>H_2O layer.

INTRODUCTION: Besides the use of oxygen for combustion and respiration it has numerous benefit inside our body, such as metabolizing fats, proteins, and carbohydrates for energy; however, paradoxically reactive oxygen species (ROS) are formed as byproducts including superoxide anion radical ($\cdot O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), singlet oxygen ($^1O_2$) and free radicals of lipids such as alkoxyl radical (RO·) and peroxyl radical (ROO·) [1, 2]. In addition, Peroxynitrite ($ONOO^-$), the reactive nitrogen species (RNS), a product of the reaction of nitric oxide (NO) with superoxide anion (O^{-2}), is formed in tissues with perfusion injury and inflammation. $ONOO^-$ is a potent and versatile oxidant that can oxidize and nitrify DNA. These ROS and RNS may act as potent oxidizing and nitrating agents to damage several cellular components such as proteins, lipids and DNA. Also, these reactive species are likely to be involved in diseases such as Alzheimers disease and cancer [3], aging [4], arteriosclerosis, rheumatoid arthritis and allergies [5, 6, 7].

Undoubtedly, *in vivo* suppression of peroxynitrite is important for the human body to eliminate the peroxynitrite-induced toxicity. For several years, many researchers have investigated powerful and nontoxic peroxynitrite scavengers from natural sources, especially edible or medicinal plants because humans lack of a specific enzyme needed to decompose peroxynitrite[8]. Therefore, in addition to certain endogenous molecules, such as glutathione, ascorbate and albumin, the consumption of foods which are rich in peroxynitrite-scavengers appears to be a promising strategy to boost inherent protection against peroxynitrite damage in humans. *Bacopa monniera* Linn, (Family-*Scrophulariaceae*) is a small, creeping herb with numerous branches, small oblong leaves, and light purple flowers. In Bangladesh, India and the tropics it grows naturally in wet soil, shallow water and in marshy land. In Ayurveda, the plant has been used in the treatment of insanity, epilepsy and hysteria. It has been reported that, the plant have sedative, antiepileptic, vasoconstrictor and anti-inflammatory activities [9]. The plant is reported to contain tetracyclic triterpenoid saponins, bacosides A and B [10, 11] hersaponin, alkaloids viz, herpestine and brahmine and flavonoids [12].

Recently Tripathi [13] and Bhattacharya [14] have suggested an antioxidant property of *Bacopa monniera*. Based on these reports our studies have been designed to examine the natural scavenger of $ONOO^-$ as well as ROS of the methnol extract and its organic solvent soluble fractions, such as those from dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), n-butanol (n-BuOH), and the water (H_2O) layer, from the aerial parts of *Bacopa monniera,* were evaluated by the total ROS and ONOO- scavenging/inhibitory tests. In addition, the toxic potentiality of these fractions were also investigated.

MATERIALS AND METHODS:

Plant materials: The aerial parts of the plant of *Bacopa monniera* was collected from the botanical garden of Pharmacy department of Jahangirnagar University, Bangladesh during January 2009. The plant material was taxonomically identified by the National herbarium of Bangladesh. A voucher specimen no. 32175 is maintained in our laboratory for future reference.

Chemicals: The Trolox and DL-penicillamine (DL-2- amino- 3- mercapto- 3- methylbutanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The high quality DCFH-DA and DHR 123 (dihydrorhodamine 123), and $ONOO^-$ were purchased from Molecular Probes

(Eugene, Oregon, USA) and Cayman (Ann Arbor, MI, USA), respectively.

Preparation of plant extract: The plant material was shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The dried powder material (1 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40^0C to render the MeOH extract (260 g). This extract was suspended in H_2O and then successively partitioned with CH_2Cl_2, EtOAC, and n-BuOH to afford the CH_2Cl_2 (160 g), EtOAC (10 g), and n-BuOH (30 g) fractions and the H_2O residue (60 g).

Measurement of the inhibition of the total ROS generation: Rat kidney homogenates, prepared from the kidneys of freshly killed male Wistar rats, weighing 150-200 g, were mixed with or without a suspension of extracts, and then incubated with 12.5 µM DCFH-DA, at 37^0C for 30 min. 50 mM of pH 7.4 phosphate buffer was used. DCFH-DA is a stable compound, which easily diffuses into cells, and is hydrolyzed by intracellular esterase to yield a reduced non-fluorescent compound, DCFH, which is trapped within ceils. The ROS produced by cells oxidize the DCFH to the highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively[15]. IC_{50} was calculated from equation of line obtained by plotting a graph of concentration versus % inhibition.

Measurement of the $ONOO^-$ scavenging activity: The $ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123, by modifying the method of Kooy *et al*[16]. The DHR 123 (5 mM), in dimethylformamide, was purged with nitrogen, stored at -80^0C and used as a stock solution. This solution was then placed on ice, and kept from exposure to light, prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, at pH 7.4, and 100 mM diethylenetriaminepentaacetic acid (DTPA), each of which were prepared with high quality deionized water, and purged with nitrogen. The final concentration of the DHR 123 was 5 µM.

The background and final fluorescent intensities were measured 5 minutes after treatment, both with and without the addition of authentic $ONOO^-$. The DHR 123 was oxidized rapidly by authentic $ONOO^-$, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.), with excitation and emission wavelengths of 480 and 530 nm, respectively. The results were expressed as the mean ± standard error (n=3) of the final fluorescence intensity minus the background fluorescence. The effects were expressed as the percentage of inhibition of the DHR 123 oxidation. IC_{50} was calculated from the equation of line obtained by plotting a graph of concentration (µg/ml) versus % inhibition.

Brine Shrimp Lethality Bioassay: The toxic potentiality of the different fractions of the plant was evaluated using Brine Shrimp lethality bioassay method[17] where 6 graded doses (viz,. 5µg/ml, 10µg/ml, 20µg/ml, 50µg/ml, 100µg/ml, 200µg/ml) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment. DMSO was used as solvent and also

as a negative control. The median lethal concentration LC_{50} of the test sample after 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. Vincristine sulfate was used as a reference standard in this case.

Statistical analysis: All values were expressed as the mean ± standard error of three replicate experiments.

RESULTS AND DISCUSSION: It is well known that free radicals and ROS or RNS including H_2O_2, ·OH, NO· and $ONOO^-$ play a role in the etiology of a vast variety of human degenerative diseases [18, 19]. These reactive species are formed in the body as a consequence of aerobic metabolism, and damage all intracellular components, such as nucleic acids, proteins and lipids. ROS are also implicated in both aging and various degenerative disorders [20, 21].

Present study investigated the general antioxidant effects of the *Bacopa monniera* MeOH extract along with its solvent partitioned fractions such as CH_2Cl_2, EtOAc, n-BuOH, and H_2O soluble fractions for its potential to inhibit total ROS in kidney homogenates using DCFH-DA, and to scavenge the authentic peroxynitrites ($ONOO^-$). As summarized in **Table 1**, the total ROS system, the IC_{50} values of MeOH extract and its consequent CH_2Cl_2, EtOAc, n-BuOH, and H_2O fractions were 46.52±0.91, 98.45±1.33, 14.03±1.35, 35.29±0.39, 65.54±1.02 µg/mL, respectively. EtOAc fraction especially exhibiting more potent inhibitory activity than the positive control Trolox (IC_{50} 29.82±1.21 µg/mL). The MeOH extract, and all the fractions from the aerial parts of *B. monniera,* showed marked scavenging activity on the authentic $ONOO^-$ in the following order: EtOAc fraction (IC_{50} 1.03±0.06 µg/mL) > n-BuOH fraction (IC_{50} 3.29±0.26 µg/mL) > MeOH extract (IC_{50} 11.23±2.14 µg/mL) > H_2O layer (IC_{50} 25.54±1.21 µg/mL) > CH_2Cl_2 fraction (IC_{50} 55.52±1.01 µg/ mL). The $ONOO^-$ scavenging activity of the EtOAc fraction was equivalent with that of the positive control (penicillamine), having IC_{50} value of 1.17±0.06 µg/mL. These results suggest that there are likely to be many antioxidants in the EtOAc soluble fraction, so much attention should be given to the isolation of the natural $ONOO^-$ scavenger from this fraction.

TABLE 1: TOTAL TOTAL ROS IN KIDNEY HOMOGENATES USING DCFH-DA AND $ONOO^-$ SCAVENGING CAPACITY OF THE MEOH EXTRACT AND ITS SOLVENT SOLUBLE FRACTIONS OF THE AERIAL PARTS OF *BACOPA MONNIERA*

Sample	$ONOO^-$ [a] (IC_{50} µg/ml), Mean ± SE[c]	Total ROS[b] (IC_{50} µg/ml) Mean ± SE[c]
MeOH	11.23±2.14	46.52±0.91
CH_2Cl_2	55.52±1.01	98.45±1.33
EtOAc	1.03±0.06	14.03± 1.35
n-BuOH	3.29±0.26	35.29±0.39
H_2O	25.54±1.21	65.54±1.02
Trolox		29.82±1.21
L-penicillamine	1.17±0.06	

[a]ONOO is the inhibitory activity of authentic peroxynitrite (IC_{50} µg/ml), [b] Total ROS is the inhibitory activity of the total free radical generation in the kidney postmicrosomal fraction (IC_{50} µg/ml) [c]Values of $ONOO^-$ and total ROS were expressed as the mean±standard error of three experiments

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp, which could also provide an indication of possible toxicity of the test materials. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [17]. As summarized in **table 2**, the toxicity exhibited by the crude MeOH extract as well as the organic soluble fractions of the plant showed potent activity against with the positive control (vincristine sulphate). The toxicity of the MeOH extract and its fractions on the BSLA increased in the order of CH_2Cl_2> MeOH

> EtOAc > n-BuOH > H_2O and were 19.02 ± 1.16, 34.92 ± 2.56, 45.32 ± 2.13, 84.65 ± 3.03 and > 200 µg/mL in their LC_{50}, respectively. The variation in BSLA results (Table 2) may be due to the difference in the amount and kind of toxic substances (e.g. tannins, flavonoids, triterpenoids, or coumarins) present in the crude extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or µg/mL) to brine shrimp is indicative of the presence of potent toxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which crude plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

TABLE 2: LC_{50} DATA OF TEST SAMPLES OF *BACOPA MONNIERA* AND VINCRISTINE SULPHATE

Sample	LC_{50} (µg/ml) Mean ± SE[a]
MeOH	34.92 ± 2.56
CH_2Cl_2	19.02 ± 1.16
EtOAc	45.32 ± 2.13
n-BuOH	84.65 ± 3.03
H_2O	> 200
Vincristine sulphate	1.225 ± 0.11

[a]Values of toxicity (LC_{50}) were expressed as the mean±standard error of three experiments

In conclusion, the results of the present study, in agreement with other authors [13, 14] indicate that the MeOH extract and its various fractions extract exhibit interesting antioxidant properties, expressed by its capacity to scavenge $ONOO^-$ and to inhibit total ROS in kidney homogenates using DCFH-DA and also show potent toxicity. These results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity. Now our next aim is to explore the lead compound liable for aforementioned activity from this plant.

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