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Bacopa monnieri extracts prevent hydrogen peroxide-induced oxidative damage in a cellular model of neuroblastoma IMR32 cells

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[ABSTRACT] Neurodegenerative diseases are the consequences of imbalance between the production of oxidative stress and its nullification by cellular defense mechanisms. Hydrogen peroxide (H_2O_2), a precursor of deleterious reactive oxygen species, elicits oxidative stress, resulting in severe brain injuries. *Bacopa monnieri* is well known for its nerve relaxing and memory enhancing properties. The present study was designed to evaluate the protective effects of extracts from *Bacopa monnieri* against H_2O_2 induced oxidative stress using a cellular model, neuroblastoma IMR32 cell line. The protective potential of methanolic, ethanolic, and water extracts of *B. monnieri* (BM-MEx, BM-EEx, and BM-WEx) was evaluated using MTT assay. Although, all the *B. monnieri* extracts were found to protect cells against H_2O_2-mediated stress but BM-MEx showed significantly greater protection. UPLC analysis of BM-MEx revealed various polyphenols, including quercetin, catechin, umbelliferone, and caffeic acid predominance. Further, BM-MEx was found to possess considerable greater neuroprotective potential in comparison to the standard polyphenols such as quercetin, catechin, umbelliferone, and caffeic acid. The levels of antioxidant enzymes were significantly elevated after the pretreatment of BM-MEx and quercetin. The expression levels of oxidative stress markers, such as NF200, HSP70, and mortalin, were significantly alleviated after the pretreatment of BM-MEx as shown by immunofluorescence and RT-PCR. In conclusion, the present study demonstrated the protective effects of BM-MEx, suggesting that it could be a candidate for the development of neuropathological therapeutics.

[KEY WORDS] Antioxidant enzymes; *Bacopa monnieri*; H_2O_2; IMR32 neuroblastoma; NF200; HSP70; Mortalin

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Introduction

The ratio of oxidants and antioxidants determines the cellular redox status. Any imbalance between these two defines the oxidative state of the cell, leading to apoptosis or necrosis. The brain cells are highly susceptible to oxidative insults, mainly due to reactive oxygen species (ROS) [1]. Oxygen itself is a comparatively unreactive compound but can be metabolized *in vivo* to form highly reactive free radicals, which include superoxide anions, hydroxyl radicals, and many other reactive species [2]. These free radical species play an important role in the pathophysiology of many neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), ischemia, and aging [1-4]. Cellular defense mechanisms involving endogenous antioxidants and antioxidant enzymes such as superoxide dismutase, glutathione reductase, lipid peroxidase, catalase, glutathione peroxidase, and glutathione, help in detoxification [5-7]. The impairment of these defense mechanisms results in damage to vital biomolecules of the cell (lipid, DNA, and protein) and ultimately pushes the cell towards death [7-8].

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Today, the conventional medicines, including antibiotics, are ineffective against many serious diseases and therefore herbal renaissance is emerging all over the world. In Ayurvedic medicinal system, *Bacopa monnieri* Linn. (Scrophulariaceae), commonly known as "Brahmi", has been used as a well-known nerve relaxant and cognition-enhancer [9-12]. The bioactive components of Brahmi such as alkaloids, brahmine, herpestine, bacosides and bacosaponins are well known for their protective effects. The constituents responsible for Bacopa's cognitive effects are bacosides A and B [13-16]. Keeping in view of the general protective and cognitive effects of Bacopa in literature, the present study was designed to specifically investigate its possible neuroprotective and antioxidant potential in cellular models of neurodegenerative diseases, by inducing oxidative stress artificially by using hydrogen peroxide (H_2O_2). H_2O_2 per se is not a free radical but is very efficient in inducing oxidative stress by generating deleterious reactive oxygen species [7]. A number of polyphenolic compounds have been well characterized to attenuate the oxidative stress mediated damages at cellular level [17-22]. Quercetin, catechin, caffeic acid, and umbelliferone were used as the reference compounds in the current study.

IMR32 neuroblastoma cells used as cellular model system in the present study are transformed neural crest derived cells, capable of infinite proliferation *in vitro*. These cells show common neuronal cell properties like spontaneous or induced elaboration of neuritic processes, synthesis of neurotransmitter biosynthetic enzymes, expression of neurofilaments which make them suitable to be an *in vitro* model system for studying neurodegenerative diseases [23-26]. The markers used to study the effects of oxidative stress included NF200 (neurofilament), HSP70 (Heat Shock Protein), and mortalin/Grp75 (Glucose regulated protein). The expressions of these markers have been found to be upregulated under oxidative stress-induced neurotoxicity [27-32]. In addition, the levels of antioxidant enzymes, GSH content, and lipid peroxidation (antioxidant machinery) of the cells were also determined.

Materials and Methods

Chemicals and reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), quercetin, catechin, caffeic acid, umbelliferone, hydrogen peroxide, and primary antibodies used for immunofluorescence analysis; mouse monoclonal antibodies against NF200, and HSP70 (Clone BRM-22) were procured from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. Mouse monoclonal antibody against mortalin was procured from Abcam, NuLife Cons & Distr. Pvt. LTD., New Delhi, India. Anti-mouse Alexa Fluor 568 and 488 used as secondary antibodies were obtained from Invitrogen, Genex Life Sciences Pvt. Ltd., New Delhi, India. The PCR reagents including dNTP mix, random hexamer primer, 100bp ladder, reverse transcriptase, and Taq DNA polymerase were from Fermentas Life Sciences, Genex Life Sciences Pvt. Ltd., New Delhi, India. Primers for synthesis of cDNAs for NF200, HSP70, mortalin, and *β*-actin were from Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India. All other chemicals and reagents, including FC reagent, EDTA, and sodium hydroxide, and solvents were procured in their purest form available commercially from Qualigens (Trikamlal & Sons, Ahmedabad, India), Himedia laboratories (Mumbai, India), and Sisco Research Laboratories (Mumbai, India).

Preparation of plant extracts

The whole plant of *B. monnieri* was used in the present study. The plant was purchased from the local market and identified from the Head, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar. It was dried and powdered and then 10 g of plant powder was extracted with 100 mL each of methanol, ethanol and water at 30 ± 5 ºC using orbital shaker. The resultant extracts were named BM-MEx, BM-EEx and BM-WEx, respectively. The extracts were filtered using muslin cloth and centrifuged at 15 000 *g* for 10 min. The supernatant was air dried and then further diluted with growth medium to obtain final experimental concentrations ranging from 1.5−200 $\mu g \cdot mL^{-1}$.

Phytochemical screening

Three *B. monnieri* extracts used in the present study were tested for the presence of a range of bioactive constituents like flavonoids, saponins, tannins, amino acids, alkaloids, phytosterols, triterpenoids, and anthroquinones using standard protocols given by Harborne, 1998 [33].

Cell culture and administration of extracts

The neuroblastoma cell line IMR32 was procured from the National Center for Cell Sciences, Pune, India. The culture was maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (Life Technologies (India) Pvt. Ltd., Delhi, India) glutamine (20 $mmol \cdot L^{-1}$), penicillin (120 $\mu g \cdot mL^{-1}$), streptomycin (100 $U \cdot mL^{-1}$), and gentamycin (100 $\mu g \cdot mL^{-1}$) at 37 °C in humid atmosphere with 5% CO_2. To elucidate the protective role of extracts of *B. monnieri*, hydrogen peroxide was used as a stress generator. Three replicates per group were taken for each experiment. To calculate the 50% inhibitory concentration (IC_{50}) of H_2O_2, the cells were incubated with various concentrations of H_2O_2 (7.8 to 1 000 $\mu mol.L^{-1}$) in a serum-free medium at 50% confluency for 24 h. Similarly, to discover the maximum non-toxic concentration of *B. monnieri* extracts on the IMR32 cells, incubation at 50% confluency with different concentrations of extracts ranging from 1.5−200 $\mu g \cdot mL^{-1}$ for 24 h was done. To assess the neuroprotective effects, the cell culture was pretreated with non-toxic concentrations of BM-MEx, BM-EEx, and BM-WEx at 30% confluency stage for 24 h and then the cells were incubated with IC_{50}-level of H_2O_2 for additional 24 h. The negative control medium was deprived of H_2O_2 and extracts. To validate the neuroprotective efficacy of *B. monnieri* at molecular level, following techniques were used: enzyme assays, immunofluorescence and RT-PCR. Four different groups were included in subsequent experiments: BM-MEx-