

# Neuromodulatory Propensity of *Bacopa monnieri* Leaf Extract Against 3-Nitropropionic Acid-Induced Oxidative Stress: In Vitro and In Vivo Evidences

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**Abstract** We previously reported the propensity of *Bacopa monnieri* (BM) leaf powder to modulate endogenous levels of oxidative stress markers in the brain of prepubertal mice. In this study, we tested the hypothesis that pretreatment with an alcoholic extract of BM (BME) could provide neuroprotection against 3-nitropropionic acid (3-NPA)-induced oxidative stress under in vitro and in vivo conditions. In chemical systems, BME exhibited multiple free radical scavenging ability. Further, BME pretreatment completely abolished 3-NPA-induced oxidative stress response in brain (striatum, St) mitochondria in vitro. Likewise, pretreatment of dopaminergic (N27 cell lines) cells with BME not only abrogated the generation of reactive oxygen species (ROS) levels, but also offered marked protection against 3-NPA-mediated cytotoxicity. These findings were further validated employing a 3-NPA mice model in vivo. We determined the degree of oxidative stress induction, redox status, enzymic antioxidants, protein oxidation, and cholinergic function in various brain regions of male mice provided with BME for 10 days (prophylaxis) followed by 3-NPA challenge (75 mg/kg bw/day, i.p.). BME prophylaxis completely prevented 3-NPA-induced oxidative dysfunctions in St and other brain regions. 3-NPA-induced robust elevation of oxidative markers (malondialdehyde levels, ROS generation,

hydroperoxide levels and protein carbonyls) in cytosol of brain regions was predominantly abolished among mice given BME prophylaxis. Interestingly, BME prophylaxis also prevented the depletion of reduced glutathione, thiol levels, and perturbations in antioxidant enzymes caused by 3-NPA. Collectively these findings provide evidence on the significant prophylactic neuroprotective efficacy of BME in prepubertal mice brain. Based on these data, it is hypothesized that BME can serve as a useful adjuvant in protecting brain against oxidative-mediated neurodegenerative disorders involving oxidative stress conditions.

**Keywords** *Bacopa monnieri* · Oxidative stress · Brain regions · Prepubertal mice · 3-Nitropropionic acid

## Abbreviations

LPO	Lipid peroxidation
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
MDA	Malondialdehyde
DCF	2',7'-Dichloro-fluorescein
DCF-DA	2',7'-Dichloro-fluorescein diacetate
BM	<i>Bacopa monnieri</i>

## Introduction

*Bacopa monnieri* (BM, Brahmi, family—Scrophulariaceae) is well-known traditionally in Ayurvedic system of medicine for its various neuropharmacological properties (Chopra 1958). Used as a brain tonic, BM is known to enhance memory, cognitive functions, promote longevity, and provide relief to patients with anxiety or epileptic disorders (Gohil and Patel 2010). Major chemical components viz., steroidal saponins—bacoside A and B are

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speculated to be responsible for the memory-facilitating action of BM (Shinomol and Muralidhara 2011). In addition, the presence of other phytoactives such as dammarane-type triterpenoid saponins with jujubogenin and pseudo-jujubogenin as the aglycones, bacosides A1–A3 (Rastogi and Kulshreshtha 1999), bacopasaponins A–G (Mahato et al. 2000), and bacopasides I–V (Chakravarty et al. 2003) have been reported in alcoholic extract of BM.

The biological effects of BM have been reviewed periodically (Shinomol et al. 2011a; Gohil and Patel 2010; Russo and Borrelli 2005). Recently, BM has been reported to exert protective effects in an animal model of ischemia-induced brain injury (Saraf et al. 2011). Administration of bacosides attenuated experimentally induced anterograde amnesia, improved memory in mice (Kishore and Singh 2005) and enhanced spatial learning performance as well as memory retention in rats (Vollala et al. 2010). Earlier, alcoholic extract of BM was shown to facilitate cognition, inhibit amnesic effects of scopolamine, electroshock, and immobilization stress in rats (Singh and Dhawan 1997). Standardized BM extract (BME) was previously reported to normalize stress-mediated transient deregulation of plasma corticosterone and monoamine changes in brain (Rai et al. 2003). Further, BME was shown to inhibit multiple components of the  $\beta$ -amyloid-induced oxidative stress pathway that can contribute to Alzheimer's pathology and reduce  $\beta$ -amyloid levels in a transgenic mouse model (Dhanasekaran et al. 2007).

Various attributes such as metal ion chelation, upregulation of antioxidative enzymes, and scavenging of free radicals (Tripathi et al. 1996; Shinomol et al. 2011a) are believed to be responsible for the neuroprotective effects of BME. The antistress activity of BM in animal models is attributed to its potential to modulate the expression levels of Hsp70, cytochrome P450, superoxide dismutase (SOD), enhancement of kinase activity, neuronal synthesis, and restoration of synaptic activity (Chowdhuri et al. 2002; Kishore and Singh 2005). Despite the extensive usage of BM plant, its extracts and isolated bacosides, studies related to its prophylactic efficacy to render the brain tissue less vulnerable to neurotoxicant-induced oxidative dysfunctions are limited.

One of the long-term objectives of our laboratory has been to understand the potential of various natural compounds to modulate the endogenous antioxidant defenses *in vivo* and to exploit this ability to achieve neuroprotection. This approach would largely aid in identifying compounds which could be employed for targeting the pathways implicated in reactive oxygen species (ROS) production and oxidative stress (Dumont and Beal 2011). Earlier, we demonstrated the neuroprotective efficacy of BM against neurotoxicants *viz.*, rotenone and paraquat in *Drosophila* (Hosamani and Muralidhara 2009, 2010). Further, our recent findings in

prepubertal mice revealed the propensity of BM leaf powder to modulate the endogenous levels of oxidative markers in different brain regions (Shinomol and Muralidhara 2011). Hence, we hypothesized that BM prophylaxis is likely to render the brain less susceptible to oxidative stress-mediated neuronal dysfunctions. The hypothesis was tested in prepubertal mice administered with 3-NPA, a well-known fungal mitochondrial toxin which causes selective neuronal degeneration in the striatum (St). While the precise mechanism/s by which 3-NPA causes striatal degeneration is not known, various mechanisms including oxidative injury, glutamate receptor excitotoxicity, mitochondrial dysfunctions, and energy depletion have been proposed (Herrera-Mundo and Sitges 2010; Shinomol and Muralidhara 2008a, b; Beal and Ferrante 2004).

Accordingly, we examined the efficacy of BME to prevent 3-NPA-induced oxidative stress in cytosolic milieu in St and other brain regions. Initially, we established the antioxidant activity of BME in various chemical test systems as well as its potential to inhibit 3-NPA-induced oxidative stress in brain mitochondria *in vitro*. Further, the efficacy of BME to attenuate 3-NPA-induced oxidative stress response and cytotoxicity was evidenced in dopaminergic neuronal cells (N27cell line). To assess its *in vivo* neuroprotective efficacy, prepubertal mice were provided with BM prophylaxis (10 days) prior to the challenge with 3-NPA.

## Materials and Methods

### Chemicals

Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, 2',7'-dichloro-fluorescein (DCF), 2',7'-dichloro-fluorescein diacetate (DCF-DA), 3-NPA, and other fine chemicals were procured from M/s Sigma Chemical Co., St Louis, USA. All other chemicals used were of analytical grade.

### Animals and Care

Prepubertal male mice (CFT-Swiss, 4-week-old) drawn from the stock colony of the "institute animal house facility" were housed in polypropylene cages (three per cage) provided with dust free paddy husk as a bedding material. Mice were housed in a controlled atmosphere with a 12 h light/dark cycle. They were acclimatized for 1 week prior to the start of the experiment and were maintained on a powdered diet and tap water *ad libitum*. The experiments were conducted strictly in accordance with approved guidelines by the "Institute Animal Ethical Committee" regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA),

Ministry of Social justice and Empowerment, Government of India, India (Registration number; 49/1999/CPCSEA).

#### *Bacopa monnieri* Powder and Ethanolic Extract

*Bacopa monnieri* plant was collected during early summer from the state of Kerala, India and authenticated by Prof. C.M Joy, Department of Botany, Sacred Heart College Thevara, Mahatma Gandhi University, Kerala, India. In brief, fresh BM plant was shade dried, the leaves were separated from the stem and powdered (using a mill) without production of much heat and was sieved through a 400 micron sieve. The ethanolic extract was prepared according to the method of Jyoti and Sharma (2006) with minor modifications. The percent total yield of the ethanolic extract was 20%. The HPLC chromatogram of the extract has been previously reported (Shinomol and Muralidhara 2011).

#### Free Radical Scavenging Potency of BM Extract In Vitro

##### *Antioxidant Potential of BME: In Vitro Evidences*

**Chemical Systems:** The antioxidant potential of BME was assessed employing a battery of chemical systems following the methods described previously (Shinomol and Muralidhara 2008b). The assay systems used were DPPH radical scavenging assay (Cotelle et al. 1996); nitric oxide scavenging assay (Maccocci et al. 1994); hydroxyl radical scavenging assay (Chung et al. 1997); superoxide scavenging assay (Robak and Gryglewski 1998); deoxyribose oxidation assay (Halliwell et al. 1987); and iron chelation assay (Carter 1971).

##### *Inhibition of 3-NPA-Induced Oxidative Stress in Mitochondria of Brain Regions In Vitro*

Mitochondria isolated from different brain regions viz., cortex (Ct), cerebellum (Cb), hippocampus (Hc), and St were preincubated with BME (0.5 and 1  $\mu\text{g}/\text{ml}$ ) for 30 min. Further, untreated and BME pretreated mitochondria were exposed to 3-NPA (2 mM) for 1 h. The extent of oxidative damage was evaluated in terms of malondialdehyde (MDA) formation, ROS generation, and hydroperoxide levels.

##### *Experiments in N27 Cells*

Rat dopaminergic cell line 1RB<sub>3</sub>AN<sub>27</sub> (N27) cells used in this study was grown and maintained as described earlier (Jagatha et al. 2008). In brief, cells were grown in RPMI 1640 medium containing 10% fetal bovine serum,

penicillin (100 units/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Cells were subcultured once a week via trypsin treatment. Initially, confluent N27 cells (in 96-well plates) were exposed first to 3-NPA at various concentrations for 24 h to determine the LC<sub>50</sub> value. Further experiments were conducted by pretreating cells with BME at various concentrations for first 24 h followed by exposure to 3-NPA (LC<sub>50</sub>—4 mM) for an additional 24 h. At the end of treatment, the neuroprotective potency of BME against cell death was assessed by cell viability assay. Further the modulatory effect of BME (2, 4, and 6  $\mu\text{g}/\text{ml}$ ) was assessed employing a sublethal (3 mM) concentration of 3-NPA.

For cell survival studies, cells were seeded in 96-well plates at a density of 5,000 cells per well. Following treatment of untreated or BME pretreated cells with 3-NPA (3 mM), viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described earlier (Vali et al. 2007).

#### Prophylactic Efficacy of BME Against 3-NPA-Induced Oxidative Stress In Vivo

The criteria of dosage selection of 3-NPA was based on our previous studies (Shinomol and Muralidhara 2008b). The dosage of BME was selected based on a preliminary dose-determinative study. Prepubertal male mice were orally administered with BME (5 mg/kg bw) for a period of 10 days (prophylaxis group). Both untreated and mice given BME prophylaxis were administered 3-NPA (75 mg/kg bw, i.p.) on days 9 and 10 and killed 24 h after the last dose. Mice given physiological saline served as the untreated controls. Biochemical analysis was carried out in cytosolic preparations obtained from St and brain regions viz., Ct, Cb, and Hc.

##### *Preparation of Cytosolic and Mitochondrial Fractions*

**Cytosolic fractionation of N27 cells:** Cells were harvested by trypsin treatment and homogenates were prepared on ice using a glass homogenizer in ice-cold phosphate buffered saline (pH 7.4). Later the homogenates were sonicated for two cycles of 5 s each, centrifuged at 10,000 $\times g$  for 10 min and the supernatant was used for biochemical assays.

**Cytosolic fractionation of brain regions:** 10% homogenate of each brain region was prepared in ice-cold phosphate buffer (0.1 M; pH 7.4) using a glass–teflon grinder at 4°C, centrifuged at 1000 $\times g$  for 10 min at 4°C to obtain the nuclear pellet. Crude cytosol was prepared by centrifuging the post-nuclear supernatant at 10,000 $\times g$  for 20 min at 4°C. The pellet was discarded and the supernatant was used for assays.

Preparation of mitochondrial fractions: Mitochondria were prepared by differential centrifugation according to the method of Moreadith and Fiskum (1984) and Trounce et al. (1996) with minor modifications.

## Biochemical Analysis

### *Determination of Lipid Peroxidation*

Induction of oxidative damage was ascertained by measuring the extent of lipid peroxidation (LPO) in the cytosol of different brain regions (Ct, Cb, Hc, and St). LPO was quantified by measuring the formation of TBA reactive substances (TBARS) following the method described earlier (Ohkawa et al. 1979).

### *Measurement of ROS Generation*

ROS generation in brain regions or N27 cells was assayed using dihydro-dichlorofluorescein diacetate ( $H_2$  DCFH-DA), a non-polar compound that, after conversion to a polar derivative by intracellular esterases, can rapidly react with ROS to form the highly fluorescent compound dichlorofluorescein (Shinomol and Muralidhara 2007). ROS formation was calculated from a DCF-standard curve and the data was expressed as p mol DCF formed/min/mg protein.

### *Measurement of Hydroperoxide Levels*

The hydroperoxide levels were determined in cytosolic fractions following the method described previously (Wolff 1994) and results were expressed as  $\mu$ moles hydroperoxides (HP)/mg protein

### *Measurement of Protein Carbonyls*

Protein carbonyl content in cytosolic fractions was determined according to the method of Levine et al. (1990) and the results were expressed as nmoles carbonyls/mg protein using MEC  $22.0 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### *Determination of Reduced Glutathione*

Reduced glutathione (GSH) content was measured according to the fluorimetric method of Mokrasch and Teschke (1984). Concentration of GSH was calculated from a standard curve and the values were expressed as  $\mu$ g GSH/mg protein.

### *Quantification of Total Thiols and Non-Protein Thiols*

The levels of total thiols and non-protein thiols were determined according to the method of Ellman (1959). The

levels were calculated using MEC  $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$  and expressed as nmol oxidized DTNB formed/mg protein.

### *Determination of Ascorbic Acid*

Ascorbic acid (AA) content in different brain regions was analyzed by 2,4-dinitrophenyl-hydrazine method as described earlier (Omayya et al. 1979) and concentration was expressed as ng/mg tissue using AA standard.

### *Estimation of Iron Levels*

Iron content in the samples was determined by bathophenanthroline method (Peters et al. 1956) with minor modifications and expressed as OD/mg protein. This method is a sensitive colorimetric method for determining both the intrinsic and added iron. The method measures only the non-heme iron as compared to atomic absorption spectroscopy which determines both heme and non-heme iron.

### *Activities of Antioxidant Enzymes*

Each brain region was homogenized in phosphate buffer (50 mM, pH 7.4) and sonicated at  $4^\circ\text{C}$ . The activities of antioxidant enzymes viz., catalase, glutathione-S-transferase, glutathione peroxidase, and SOD were measured in cytosolic fractions with minor modifications (Aebi 1984; Flohe and Gunzler 1984; Guthenberg et al. 1985; McCord and Fridovich 1969).

### *Activities of Cholinergic Enzymes*

Activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were determined according to the method of Ellmann et al. (1961). The enzyme activities were expressed as nmoles of substrate hydrolyzed/min/mg protein.

### *Determination of Protein*

Protein content in mitochondria and cytosol were determined according to the method of Lowry et al. (1951), using bovine serum albumin as the standard.

### *Statistical Analysis*

Experimental data obtained were expressed as mean  $\pm$  standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) using SPSS software for windows (version 10.0.1., SPSS Inc, New York, 1999). The in vitro data were analyzed only by one-way ANOVA. Post hoc multiple comparisons were performed between groups using Duncan's Multiple Range Test (DMRT) in all the studies. A "*P*" value  $<0.05$  was taken as significant throughout the study.



## Results

### Free Radical Scavenging Potency of BME In Vitro

BME was able to reduce the stable free radical DPPH to the yellow colored, 1,1-diphenyl-2-picrylhydrazyl in a concentration-dependent manner ( $IC_{50}$ : 9.41  $\mu$ g/ml compared to BHT standard:  $IC_{50}$ : 4.16  $\mu$ g/ml). Likewise, BME was also found to scavenge nitric oxide radicals in a concentration-dependent manner ( $IC_{50}$ : 9.21  $\mu$ g/ml). Further, BME exerted a concentration-dependent superoxide radical ( $IC_{50}$ : 10  $\mu$ g/ml vs. standard caffeic acid:  $IC_{50}$ : 4.15  $\mu$ g/ml) and hydroxyl radical scavenging activity ( $IC_{50}$ : 436.77  $\mu$ g/ml compared to BHT standard:  $IC_{50}$ : 6.85  $\mu$ g/ml). In the deoxyribose oxidation assay, BME elicited a concentration-dependent inhibition of deoxyribose oxidation ( $IC_{50}$ : 217.34  $\mu$ g/ml). Interestingly, BME also showed concentration-dependent iron chelation effect ( $IC_{50}$ : 160  $\mu$ g/ml) in comparison to the chelating effect of the standard iron chelator, EDTA ( $IC_{50}$ : 50  $\mu$ g/ml).

### Inhibition of 3-NPA-Induced Oxidative Impairments in Brain Mitochondria In Vitro

BME per se caused significant diminution of basal MDA levels (39–49%) at both concentrations in striatum mitochondria, while 3-NPA (2 mM) markedly elevated the MDA levels (113%). BME pretreatment completely inhibited the 3-NPA-induced LPO. Likewise, 3-NPA exposure resulted in robust ROS generation (120%) and HP levels (126%) in striatal mitochondria which were also abolished by BME pretreatment (Fig. 1).

In mitochondria of other brain regions, BME alone significantly diminished the basal levels of MDA (38–67%), ROS (15–47%), and HP levels (10–37%). While, 3-NPA exposure markedly elevated the MDA levels, BME pretreatment completely inhibited the induction of LPO. Likewise, 3-NPA also caused marked enhancement in the levels of ROS and HP in mitochondria of other brain regions which were also abolished by BME pretreatment (Fig. 1).

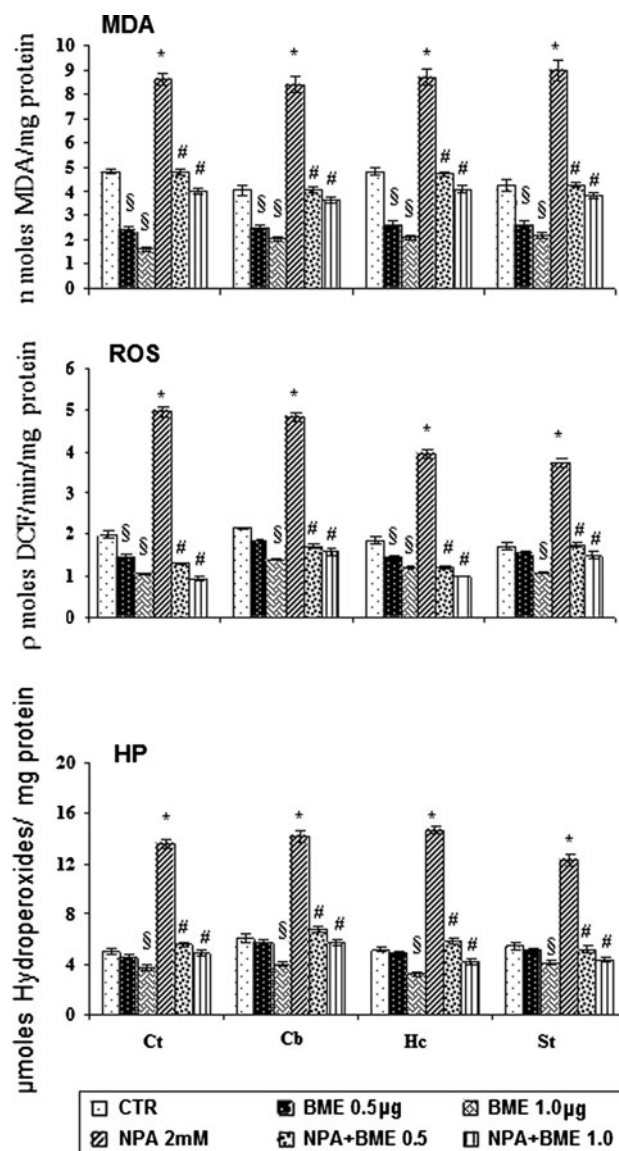
### Modulatory Effect of BME in N27 Cells

#### Toxicity Profile of 3-NPA in N27 Cells

From our preliminary study, we computed the 24 h LC<sub>50</sub> for 3-NPA in N27 cells as 4 mM.

#### Modulation of 3-NPA-mediated cytotoxicity

BME pretreatment conferred a concentration-dependent protective effect against 3-NPA (4 mM)-induced cell death

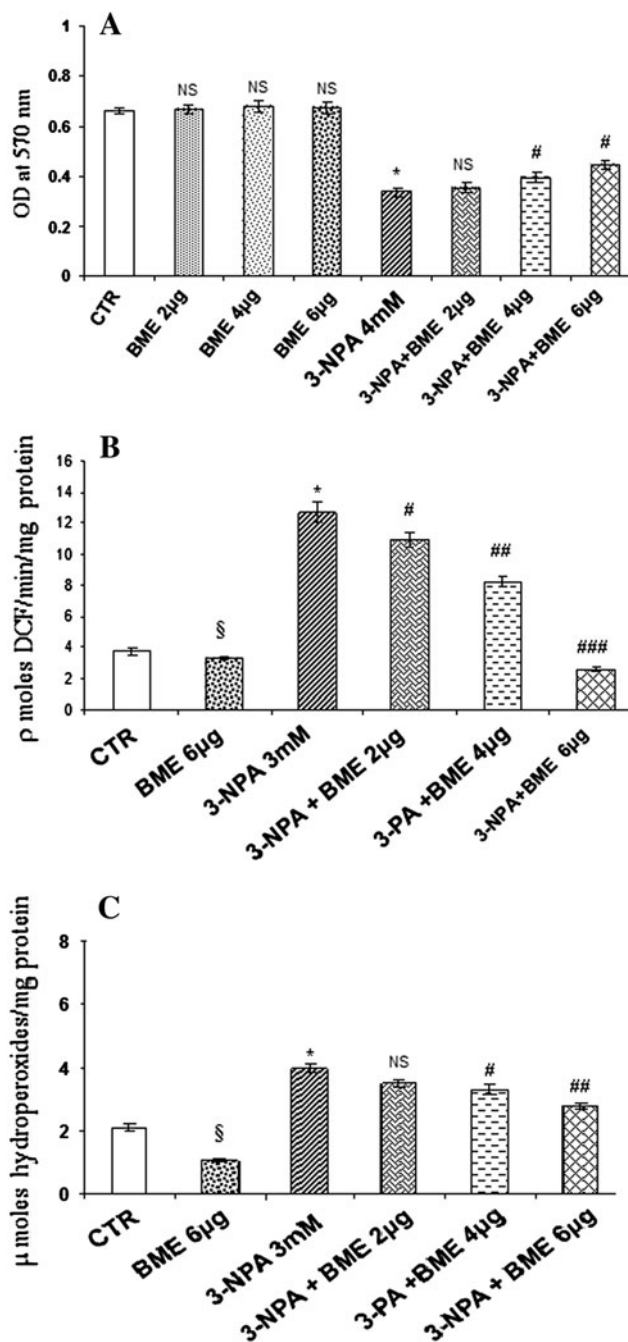


**Fig. 1** Attenuation of 3-NPA-induced oxidative stress by *Bacopa monnieri* ethanolic extract (BME) in mitochondria of brain regions of male mice in vitro. Data analyzed by one-way ANOVA (analysis were made between control vs. BME alone  $^{\$}P < 0.05$ ; control vs. 3-NPA  $^*P < 0.001$ ; 3-NPA vs. BME + 3-NPA  $^{\#}P < 0.005$ ). Ct Cortex, Cb cerebellum, Hc hippocampus, St striatum, MDA malondialdehyde, ROS reactive oxygen species, HP hydroperoxides, 3-NPA 3-nitropropionic acid (2 mM)

as evidenced in the MTT assay (Fig. 2a). While BME per se was devoid of cytotoxic effect even at the highest concentration (6  $\mu$ g), it offered significant concentration-dependent protection (10–35%) against 3-NPA-induced cell death.

#### Abrogation of 3-NPA-Induced ROS Generation and Hydroperoxides

Treatment of N27 cells with BME alone was associated with significantly (14–32%) lower levels of ROS. Further, 3-NPA



**Fig. 2** Neuroprotection against 3-nitropropionic acid (3-NPA)-induced cytotoxic response in N27 cells by pretreatment with *Bacopa monnieri* ethanolic extract (BME) as measured by MTT assay (a); ROS generation (b); and hydroperoxide levels (c). Values are mean  $\pm$  SD of six determinations each. Data analyzed by one-way ANOVA (analysis was made between control vs. BME alone § $P < 0.05$ ; control vs. 3-NPA \* $P < 0.001$ ; 3-NPA vs. BME + 3-NPA # $P < 0.05$ ; ## $P < 0.01$ ; ### $P < 0.001$ ). NS Not significant

treatment caused a dramatic (nearly 4-folds) elevation in the ROS generation (as reflected by increased fluorescence intensity) compared to untreated control cells. Interestingly, pretreatment of cells with BME extract offered varying

degree (20–100%) of protection against 3-NPA-induced ROS generation (Fig. 2b). Likewise, treatment of cells with BME alone resulted in a significant (10–40%) reduction in the basal levels of HP. While robust (nearly 3-folds) elevation was evident in the HP levels among cells exposed to 3-NPA, BME pretreatment caused significant diminution of 3-NPA-induced HP levels (Fig. 2c).

#### Prophylactic Efficacy Against 3-NPA-Induced Oxidative Dysfunctions In Vivo

##### Body Weight, Organ Weights, and Food Intake

3-NPA administration caused a marginal decrease (15%) in body weight and significant decrease in the weights of liver (21%), heart (20%), spleen (24%), and thymus (48%). 3-NPA caused only a marginal decrease in food intake (data not shown).

##### Attenuation of 3-NPA-Induced Oxidative Dysfunctions in Brain Regions

**Striatum:** BME treatment alone resulted in a significant decrease in the basal levels of oxidative markers in cytosol (MDA-23%; ROS-24%; HP-38%). Among mice administered 3-NPA, the cytosol of St exhibited robust elevation in MDA (70%), ROS (65%), and HP (57%) levels suggesting induction of oxidative stress in vivo. Interestingly, BME prophylaxis significantly diminished 3-NPA-mediated oxidative damage (Fig. 3).

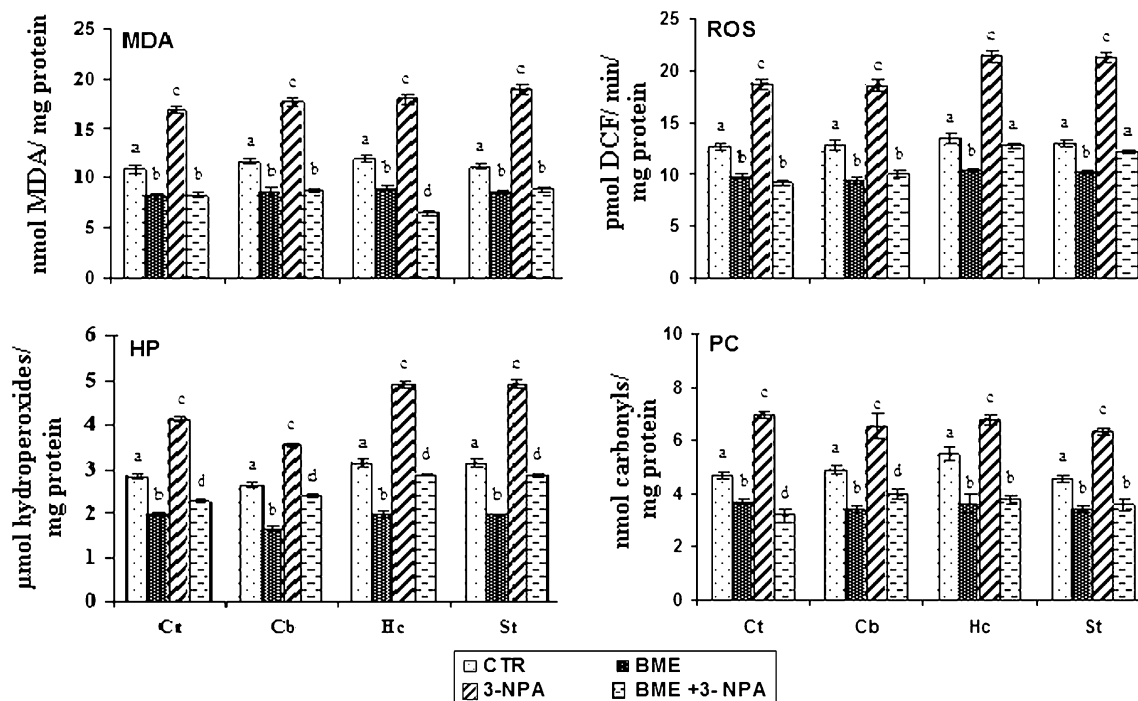
**Other Brain Regions:** BME treatment alone also caused diminution in the endogenous levels of oxidative markers in cytosol of other brain regions. 3-NPA administration caused a marked increase in the levels of MDA (56–71%), ROS (44–58%), and HP (34–45%) in all brain regions of untreated mice, which were significantly decreased among mice given BME prophylaxis (Fig. 3).

##### Protective Effect Against Protein Carbonyls Formation

BME treatment alone caused a significant (26%) decrease in protein carbonyls content in St and other brain regions (22–34%). On the other hand, 3-NPA administration significantly enhanced the protein carbonyls content in St (38%) and other brain regions (23–48%). However, brain regions of mice given BME prophylaxis were completely resistant to 3-NPA-induced protein oxidative damage (Fig. 3).

##### Effect of BME on Reduced Glutathione and Thiol Status

**Striatum:** BME treatment alone resulted in enhanced levels of GSH (25%), total thiols (21%), and non-protein thiols (20%). However, 3-NPA administration was associated



**Fig. 3** Modulatory effects of BM extract prophylaxis on 3-NPA-induced oxidative impairments in cytosol of brain regions of prepubertal male mice (*Ct* Cortex, *Cb* cerebellum, *Hc* hippocampus, *St* striatum). Values are mean  $\pm$  SD ( $n = 6$ ). Data analyzed by one-way ANOVA ( $P < 0.05$ ) followed by post hoc “Tukey” test. Means

followed by different letters differ significantly; BME-Bacopa monnieri ethanolic extract (5 mg/kg bw/day for 10 days); 3-NPA-3-nitropropionic acid (75 mg/kg bw/day for 2 days). MDA Malondialdehyde, ROS reactive oxygen species, HP hydroperoxide, PC protein carbonyls

significant depletion of GSH (23%), total thiols (21%), and non-protein thiols (19%) in striatum. However, BME prophylaxis prevented 3-NPA-mediated depletion of GSH and total thiols (Table 1).

**Other Brain Regions:** BME treatment alone also resulted in significant increase in GSH levels (19–30%), TSH (19–25%), and non-protein thiols (10–23%). On the other hand, 3-NPA administration resulted in varying levels of depletion in GSH, total thiols, and non-protein thiols (Table 1). A sustained increase (Ct-50%; Cb-33%; Hc-20%) was observed in GSSG levels on exposure to 3-NPA, where as marginal decrease (10–15%) was observed in BME prophylaxis group (data not shown). However, mice given BME prophylaxis were not susceptible to 3-NPA-induced depletion of antioxidant molecules, suggesting total protection (Table 1).

#### Effect on Antioxidant Enzymes

**Striatum:** BME treatment alone significantly enhanced the activities of antioxidant enzymes in St (CAT: 34%; GPx: 17%; SOD: 35%). However, 3-NPA administration resulted in significant decrease in catalase (37%), GPx (25%) and SOD (19%) activity levels, while increasing GST activity (15–27%). In contrast, mice provided with BME

prophylaxis exhibited normal levels of enzyme activities despite 3-NPA administration, indicating neuroprotective effect of BME (Table 2).

**Other Brain Regions:** In general, BME treatment alone enhanced the activities of antioxidant enzymes in other brain regions (Table 2). However, 3-NPA administration resulted in significant decrease in GPx (25%) and SOD (21–27%) activity levels, while increasing GST activity (14–27%). Further, varying degree of normalization in enzyme activities occurred among mice given BME prophylaxis (Table 2).

#### Status of Iron and Vitamin C Levels

BME treatment alone caused a general decrease in free iron levels in St (27%) and other brain regions (Ct-20%; Cb-14%; Hc-30%). In contrast, 3-NPA administration caused a significant increase in free iron levels in St (40%) and other brain regions (Ct-21%; Cb-47%; Hc-33%). Interestingly, BME prophylaxis caused in marked reduction in the free iron levels in St and other regions of mice administered 3-NPA (Table 3).

BME treatment alone did not have any effect on the vitamin C levels in any of the brain regions. However, 3-NPA administration caused a marginal decrease in the

**Table 1** Modulatory effect of *Bacopa monniera* extract (BME) prophylaxis on 3-NPA-induced perturbations in antioxidant molecules in cytosol of brain regions of prepubertal male mice

Group	Brain regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Reduced glutathione (GSH)*				
CTR	9.1 ± 0.13 <sup>a</sup>	9.9 ± 0.25 <sup>a</sup>	8.9 ± 0.14 <sup>a</sup>	9.2 ± 0.25 <sup>a</sup>
BME	11.9 ± 0.11 <sup>b</sup>	11.9 ± 0.13 <sup>b</sup>	10.6 ± 0.35 <sup>b</sup>	11.5 ± 0.13 <sup>b</sup>
3-NPA	7.9 ± 0.13 <sup>c</sup>	7.6 ± 0.51 <sup>c</sup>	7.7 ± 0.19 <sup>c</sup>	7.1 ± 0.51 <sup>c</sup>
BME + 3-NPA	10.9 ± 0.25 <sup>d</sup>	10.9 ± 0.22 <sup>d</sup>	10.8 ± 0.18 <sup>d</sup>	10.1 ± 0.22 <sup>a</sup>
Oxidized glutathione(GSSG)**				
CTR	0.32 ± 0.03 <sup>a</sup>	0.33 ± 0.05 <sup>a</sup>	0.31 ± 0.04 <sup>a</sup>	0.31 ± 0.04 <sup>a</sup>
BME	0.29 ± 0.01 <sup>b</sup>	0.26 ± 0.03 <sup>b</sup>	0.27 ± 0.05 <sup>b</sup>	0.28 ± 0.03 <sup>b</sup>
3-NPA	0.48 ± 0.03 <sup>c</sup>	0.44 ± 0.01 <sup>c</sup>	0.37 ± 0.03 <sup>c</sup>	0.45 ± 0.01 <sup>c</sup>
BME + 3-NPA	0.31 ± 0.05 <sup>a</sup>	0.29 ± 0.02 <sup>a</sup>	0.29 ± 0.04 <sup>a</sup>	0.30 ± 0.02 <sup>a</sup>
Total thiols (TSH) <sup>c</sup>				
CTR	8.7 ± 0.13 <sup>a</sup>	7.9 ± 0.25 <sup>a</sup>	8.5 ± 0.14 <sup>a</sup>	8.6 ± 0.13 <sup>a</sup>
BME	10.7 ± 0.15 <sup>b</sup>	9.4 ± 0.13 <sup>b</sup>	10.6 ± 0.35 <sup>b</sup>	10.4 ± 0.18 <sup>b</sup>
NPA	7.8 ± 0.16 <sup>c</sup>	6.8 ± 0.46 <sup>c</sup>	7.5 ± 0.17 <sup>c</sup>	6.8 ± 0.17 <sup>c</sup>
BME + 3-NPA	8.9 ± 0.15 <sup>a</sup>	8.6 ± 0.54 <sup>d</sup>	8.8 ± 0.19 <sup>a</sup>	8.8 ± 0.23 <sup>a</sup>
Non-protein thiols (npSH)***				
CTR	0.93 ± 0.05 <sup>a</sup>	0.99 ± 0.02 <sup>a</sup>	0.97 ± 0.03 <sup>a</sup>	0.97 ± 0.05 <sup>a</sup>
BME	1.01 ± 0.009 <sup>b</sup>	1.22 ± 0.06 <sup>b</sup>	1.14 ± 0.05 <sup>b</sup>	1.2 ± 0.06 <sup>b</sup>
3-NPA	0.67 ± 0.02 <sup>c</sup>	0.76 ± 0.04 <sup>c</sup>	0.68 ± 0.07 <sup>c</sup>	0.79 ± 0.04 <sup>c</sup>
BME + 3-NPA	0.91 ± 0.03 <sup>a</sup>	1.06 ± 0.05 <sup>a</sup>	0.98 ± 0.03 <sup>a</sup>	0.99 ± 0.04 <sup>a</sup>

Values are ± SD ( $n = 6$ )

Data analyzed by one-way ANOVA ( $P < 0.05$ ) followed by post hoc 'Tukey' test. Means followed by *different letters* differ significantly; BME: (5 mg/kg bw/day for 10 days)

3-NPA: 3-nitropropionic acid (75 mg/kg bw/day for 2 days)

\* μg GSH/mg protein; \*\* μg GSSG/mg protein; \*\*\* nmoles DTNB oxidized/mg protein

**Table 2** Activities of antioxidant enzymes in cytosol of brain regions of prepubertal mice given *Bacopa monniera* extract (BME) prophylaxis followed by 3-NPA administration

Group	Brain regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Catalase (CAT)*				
CTR	2.35 ± 0.06 <sup>a</sup>	2.02 ± 0.01 <sup>a</sup>	2.15 ± 0.05 <sup>a</sup>	2.05 ± 0.06 <sup>a</sup>
BME	2.95 ± 0.03 <sup>b</sup>	2.73 ± 0.03 <sup>b</sup>	2.42 ± 0.04 <sup>b</sup>	2.75 ± 0.03 <sup>b</sup>
3-NPA	1.70 ± 0.03 <sup>c</sup>	1.62 ± 0.02 <sup>c</sup>	1.79 ± 0.02 <sup>c</sup>	1.30 ± 0.03 <sup>c</sup>
BME + 3-NPA	2.19 ± 0.05 <sup>d</sup>	1.97 ± 0.03 <sup>a</sup>	2.44 ± 0.03 <sup>d</sup>	2.09 ± 0.05 <sup>a</sup>
Glutathione peroxidase (GPx)**				
CTR	19.72 ± 0.12 <sup>a</sup>	18.18 ± 0.34 <sup>a</sup>	18.89 ± 0.22 <sup>a</sup>	19.02 ± 0.10 <sup>a</sup>
BME	23.24 ± 0.13 <sup>b</sup>	23.56 ± 0.67 <sup>b</sup>	20.12 ± 0.31 <sup>b</sup>	22.24 ± 0.13 <sup>b</sup>
3-NPA	14.77 ± 0.34 <sup>c</sup>	13.45 ± 0.54 <sup>c</sup>	14.12 ± 0.11 <sup>c</sup>	25.77 ± 0.31 <sup>c</sup>
BME + 3-NPA	19.91 ± 0.78 <sup>a</sup>	18.56 ± 0.91 <sup>a</sup>	17.99 ± 0.78 <sup>a</sup>	19.21 ± 0.70 <sup>a</sup>
Glutathione S transferase (GST)***				
CTR	23.12 ± 1.56 <sup>a</sup>	25.21 ± 1.0 <sup>a</sup>	25.45 ± 1.11 <sup>a</sup>	23.12 ± 1.59 <sup>a</sup>
BME	25.12 ± 0.98 <sup>b</sup>	26.23 ± 0.83 <sup>b</sup>	26.67 ± 0.78 <sup>b</sup>	24.92 ± 0.58 <sup>b</sup>
3-NPA	29.32 ± 0.33 <sup>c</sup>	29.01 ± 0.45 <sup>c</sup>	29.11 ± 0.78 <sup>c</sup>	31.32 ± 0.35 <sup>c</sup>
BME + 3-NPA	24.12 ± 0.67 <sup>a</sup>	24.87 ± 0.54 <sup>a</sup>	24.15 ± 0.98 <sup>a</sup>	24.06 ± 0.60 <sup>ab</sup>
Superoxide dismutase (SOD) <sup>†</sup>				
CTR	40.44 ± 0.91 <sup>a</sup>	38.23 ± 0.67 <sup>a</sup>	35.99 ± 0.98 <sup>a</sup>	39.44 ± 0.91 <sup>a</sup>
BME	56.12 ± 0.89 <sup>b</sup>	50.45 ± 1.12 <sup>b</sup>	44.18 ± 0.99 <sup>b</sup>	53.12 ± 0.89 <sup>b</sup>
3-NPA	30.12 ± 1.56 <sup>c</sup>	28.21 ± 1.02 <sup>c</sup>	28.45 ± 1.11 <sup>c</sup>	52.12 ± 1.56 <sup>c</sup>
BME + 3-NPA	42.12 ± 0.98 <sup>a</sup>	40.23 ± 0.83 <sup>a</sup>	36.67 ± 0.78 <sup>a</sup>	40.12 ± 0.98 <sup>a</sup>

Values are mean ± SD ( $n = 6$ )

Data analyzed by one-way ANOVA ( $P < 0.05$ ) followed by post hoc 'Tukey' test. Means followed by *different letters* differ significantly

BME: 5 mg/kg bw/day for 10d; 3-NPA: 3-nitropropionic acid (75 mg/kg bw/day for 2 days)

\* μ moles H<sub>2</sub>O<sub>2</sub>/min/mg protein; \*\* nmoles NADPH/min/mg protein and \*\*\* n moles conjugate/min/mg protein; † Units/mg protein



**Table 3** Effect of *Bacopa monnieri* extract (BME) prophylaxis on 3-NPA-induced alterations in iron and vitamin C levels in cytosol of brain regions of prepubertal male mice

Group	Brain regions			
	Cortex	Cerebellum	Hippocampus	Striatum
<b>Iron*</b>				
CTR	2.31 ± 0.25 <sup>a</sup>	2.16 ± 0.35 <sup>a</sup>	1.80 ± 0.30 <sup>a</sup>	2.31 ± 0.29 <sup>a</sup>
BME	1.85 ± 0.20 <sup>b</sup>	1.86 ± 0.21 <sup>b</sup>	1.29 ± 0.21 <sup>b</sup>	1.67 ± 0.21 <sup>b</sup>
3-NPA	2.93 ± 0.18 <sup>c</sup>	3.18 ± 0.35 <sup>c</sup>	3.31 ± 0.23 <sup>c</sup>	3.23 ± 0.23 <sup>c</sup>
BME + 3-NPA	1.75 ± 0.21 <sup>d</sup>	1.84 ± 0.31 <sup>b</sup>	1.91 ± 0.25 <sup>d</sup>	2.03 ± 0.29 <sup>d</sup>
<b>Vitamin C**</b>				
CTR	0.210 ± 0.015 <sup>a</sup>	0.155 ± 0.005 <sup>a</sup>	0.171 ± 0.005 <sup>a</sup>	0.186 ± 0.006 <sup>a</sup>
BME	0.208 ± 0.010 <sup>a</sup>	0.149 ± 0.009 <sup>a</sup>	0.166 ± 0.008 <sup>a</sup>	0.193 ± 0.010 <sup>a</sup>
3-NPA	0.156 ± 0.011 <sup>b</sup>	0.211 ± 0.007 <sup>b</sup>	0.207 ± 0.009 <sup>b</sup>	0.167 ± 0.011 <sup>b</sup>
BME + 3-NPA	0.219 ± 0.012 <sup>a</sup>	0.235 ± 0.009	0.180 ± 0.004 <sup>a</sup>	0.187 ± 0.010 <sup>a</sup>

Values are mean ± SD ( $n = 6$ )

Data analyzed by one-way ANOVA ( $P < 0.05$ ) followed by post hoc ‘Tukey’ test. Means followed by *different letters* differ significantly

BME: 5 mg/kg bw/day for 10 days; 3-NPA: 3-nitropropionic acid (75 mg/kg bw/day for 2 days)

\* OD/mg protein; \*\* µg/mg tissue

vitamin C levels in St and Ct, while the levels were higher in Cb and Hc, and BME prophylaxis had no effect on 3-NPA-induced alterations (Table 3).

#### Activity of Cholinergic Enzymes

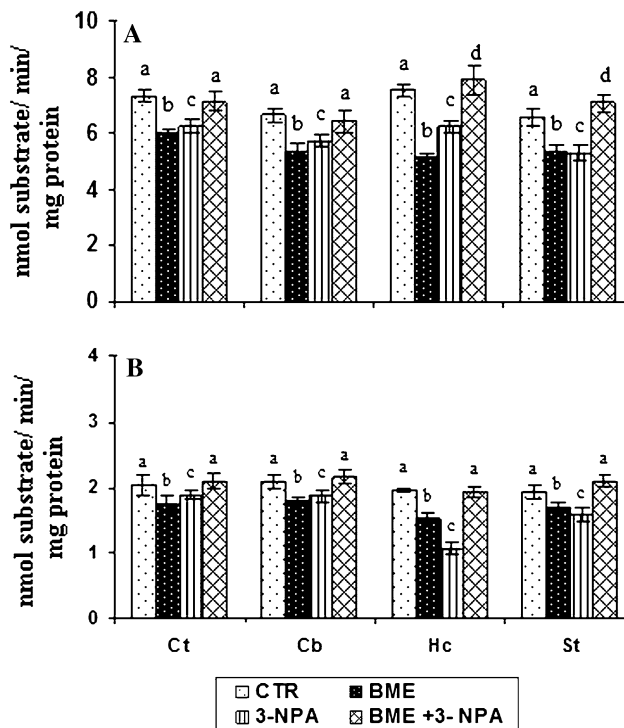
BME treatment alone caused significant decrease in the activities of AChE in St (18%) and other brain regions (18–32%). The activity of BChE was also decreased (13–23%) in all the brain regions. 3-NPA administration reduced the activities of AChE in St (20%) and other brain regions (14–32%). Likewise 3-NPA administration also significantly decreased the activities of BChE in all the brain regions (10–45%). However, among mice given BME prophylaxis and administered 3-NPA, the activity levels of AChE were higher (Fig. 4).

#### Discussion

Establishing new lines of evidence on the neuroprotective properties of BM assumes greater relevance since several over-the-counter formulations of BM are currently available resulting in increased human consumption. In general, BM is advocated for the treatment of anxiety, improvement of intellect/memory among children and adults as well as in the treatment of various neurological disorders (Singh and Dhawan 1997; Russo and Borrelli 2005; Shinomol et al., 2011a). Previously, we demonstrated the ability of BM-enriched diet to diminish endogenous levels of oxidative markers, enhance the redox status (reduced GSH and

total thiols) and increase in the activities of several antioxidant enzymes in brain regions of prepubertal mice (Shinomol and Muralidhara, 2011). In view of this, we tested the hypothesis that prophylaxis with BME could offset neurotoxicant-induced oxidative stress in prepubertal mice brain.

Alcoholic extract of BM has been tested by numerous researchers since it contains several types of saponins including Bacopa saponins (A, B, C, and D), pseudojujubenins, bacopaside I, II, III, IV, and V (Chakravarty et al. 2003; Deepak et al. 2005). Hence in this study, we employed an alcoholic extract of BM to obtain evidences in favor of our hypothesis. The HPLC chromatogram of our extract was highly identical with previously published data (Deepak et al. 2005; Shinomol and Muralidhara 2011). Our findings employing chemical systems with BME demonstrated the ability to scavenge various radicals, clearly suggesting its multiple mechanism of action in vitro. BME effectively scavenged DPPH, superoxide, and nitric oxide radicals and showed significant inhibition of deoxyribose oxidation. These data corroborate with earlier findings in which BME exhibited robust antioxidative activity against hydrogen peroxide-induced LPO and blocked the activity of lipoxygenase in vitro (Dhanasekaran et al. 2007). Further, in this study BME also exhibited significant metal chelation in vitro, indicating its potential to chelate divalent iron in vivo. The ability of BM to scavenge diverse free radicals as well as its chelating property indicates the presence of several neuroprotective antioxidant compounds in the extract which may be highly effective in regulating the dyshomeostasis of redox-active biometals including copper and iron which are



**Fig. 4** Effects of BM extract prophylaxis on 3-NPA-induced perturbations in the AChE and BuChE in brain regions of prepubertal male mice. Values are mean  $\pm$  SD ( $n = 6$ ). Data analyzed by one-way ANOVA ( $P < 0.05$ ) followed by post hoc “Tukey” test. Means followed by different letters differ significantly (Ct Cortex, Cb cerebellum, Hc hippocampus, St striatum); BME: *Bacopa monnieri* ethanolic extract (5 mg/kg bw/day for 10 days). 3-NPA: 3-nitropropionic acid (75 mg/kg bw/day for 2 days)

suggested to contribute to the neuropathology of Alzheimer’s disease (Mattson and Chan 2003; Dumont and Beal 2011).

Having established the potential of BME to scavenge free radicals in chemical systems, we further examined its efficacy to inhibit 3-NPA-induced oxidative stress in mitochondria isolated from different brain regions of mice in vitro. Striatal mitochondria were more sensitive to 3-NPA-induced oxidative damage. However, in the presence of BME, 3-NPA failed to enhance the levels of any oxidative markers suggesting total protection at the tested concentrations. This effect was consistent irrespective of the brain region indicating the ability of BME to protect different brain regions. Interestingly, these protective effects were highly reproducible in a dopaminergic cell model in which BME pretreatment effectively reduced 3-NPA-mediated cytotoxicity. In the cell model, BME pretreatment offered significant protection against 3-NPA-induced cell death and strongly inhibited oxidative stress induction response as evidenced by reduced formation of ROS and HP. Since 3-NPA toxicity is known to be mediated by oxidative damage, the protective effects of BME

observed could be a direct reflection of its antioxidative potency. Further mechanistic studies are in progress to identify specific mechanism/s by which BME offers neuroprotection in the cell model.

Based on these evidences, we examined the neuroprotective efficacy of BME employing a prophylactic approach in a prepubertal mice model. BM has been used in Ayurvedic medicine for several hundred years and the therapeutic doses are not associated with any known side-effects (Allan et al. 2007). In this study, BME was administered to prepubertal mice only for 10 days. As anticipated, administration of 3-NPA to prepubertal mice induced robust oxidative stress in both cytosolic and mitochondrial fractions of St and other brain regions (Shinomol and Muralidhara 2008b). Our salient findings viz., marked elevation in ROS and MDA levels, alterations in the activities of antioxidant enzymes, diminished GSH levels and enhanced protein carbonyl levels in St and other brain regions clearly suggested the existence of state of oxidative stress in vivo. These findings are in direct agreement with previous reports (Binienda et al. 1998; Shinomol and Muralidhara 2008a, b; Shinomol et al. 2009). Interestingly, BME prophylaxis completely attenuated 3-NPA-induced oxidative stress in cytosol in St as well as other brain regions suggesting its ability to render protection against neurotoxicant insult. These data corroborate our recent findings in which BM-enriched diet not only significantly diminished the basal levels of oxidative markers in *Drosophila melanogaster*, but also offered marked protection against rotenone as well as paraquat-induced oxidative stress, locomotor deficits, and mortality (Hosamani and Muralidhara 2009, 2010). Similarly, we have evidenced the prophylactic efficacy of BME against 3-NPA-induced oxidative stress and mitochondrial dysfunctions in St (Shinomol et al. 2011b).

GSH-associated metabolism is a major mechanism for cellular protection against toxicants which generate oxidative stress. It is becoming increasingly apparent that the glutathione tripeptide is central to a complex multifaceted detoxification system, where there is substantial interdependence between separate component members. GSH participates in detoxification at several levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, GSH provides the cell with multiple defenses not only against ROS, but also against their toxic products (Bharath and Andersen 2005; Aoyama et al. 2008). In this study, BME per se significantly enhanced the basal levels of GSH, total/non-protein thiols in cytosol of all brain regions in prepubertal mice. Interestingly, BME prophylaxis offered marked protection against 3-NPA-induced GSH depletion as evident by the restoration of GSH levels in St as well as other brain regions. Hence, we speculate that GSH-related mechanism/

s may play a significant role in the observed neuroprotective effect of BME against 3-NPA-induced oxidative stress. In addition, the observed enhanced levels of enzymatic antioxidant defenses in St and other brain regions could also have played a significant role in ameliorating the effects of 3-NPA. The ability of BME to uniformly upregulate the GSH levels and related defenses provides a highly useful paradigm to attenuate oxidative damage-mediated neurodegenerative disorders. Another important finding in the *in vivo* study was a general decrease in the free iron levels in cytoplasm of St and other brain regions of mice given BME prophylaxis. This suggests that the strong iron chelating ability of BME could complement its other antioxidant mechanisms in rendering brain less vulnerable to neurotoxic insults.

Elevated levels of protein carbonyls are considered as sensitive index of protein oxidation and have been implicated in pathophysiology of various neurodegenerative diseases (Butterfield et al. 2010). In the present model, we observed elevated levels of the protein carbonyls in cytosol of all brain regions among 3-NPA administered mice especially in the St, whereas BME prophylaxis completely prevented the protein oxidation. It is suggested that protein carbonyl formation might be due to ROS-mediated oxidation of amino acid side chains or by covalent binding to products of LPO or glycooxidation (Butterfield and Stadtman, 1997). The elevated levels probably reflect a very low rate of oxidized protein degradation and or low repair activity (Dalle-Donne et al. 2003). We speculate that the protective effect observed with BME prophylaxis is probably due to the high antioxidant capacity of the extract.

It is well known that inhibition of AChE is an important therapeutic target for the treatment of neurodegenerative diseases (Kaufer et al. 1999; Meshorer et al. 2002). Significant decrease in the activity of AChE indicates an increase in the acetylcholine levels in the synaptic cleft and is known to enable an improvement in cognitive functions such as learning and memory. In this study, brain regions of mice provided with BME prophylaxis exhibited consistent reduction in the activities of cholinergic enzymes viz., AChE and BChE clearly suggesting alterations in cholinergic functions *in vivo*. Previously, subchronic administration of bacosides-rich extract of BM was shown to reverse the experimentally induced cognitive deficits and reduction in acetylcholine concentration in frontal Ct/Hc (Bhattacharya et al. 1999). However, 3-NPA administration also caused a reduction in the activity levels of AChE among untreated mice, while the activity of levels were higher among mice given BME prophylaxis. Although speculative, the higher levels of AChE in the prophylaxis group are most likely to be result of the counter-regulatory mechanism to compensate the reduced levels. However, this needs to be experimentally verified.

In conclusion, our findings clearly demonstrate the prophylactic neuroprotective efficacy of BME as it markedly prevented 3-NPA-induced oxidative stress *in vivo*. Based on the data obtained in chemical systems and N27 cells, we hypothesize that the antioxidative property may be partly responsible for its neuroprotective action. Further, our findings in the mouse model give credence to the prophylactic neuroprotective efficacy of BME in offsetting the neurotoxicant-induced oxidative stress uniformly in all the brain regions. While the precise mechanism/s by which the bioactive components of BM protect brain against cytosolic oxidative stress and mitochondrial dysfunctions merit further study, we hypothesize that it may be predominantly related to its robust antioxidant potency, upregulation of enzymatic antioxidant defenses and enhanced GSH/thiol status. These findings besides illustrating the ability of BM to offset neurotoxicant-induced brain oxidative stress strongly emphasize the need to further identify the specific biochemical pathways responsible for the neuroprotective propensity of BM phytochemicals. This assumes importance as BM is widely consumed by general human population and is also being employed as a therapeutic agent to alleviate various neurodegenerative disorders.

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**Conflict of interest** None.

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