

## Neuroprotective Efficacy of A Combination of Fish Oil and Bacopa Monnieri Against 3-Nitropropionic Acid Induced Oxidative Stress in Rats

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### ABSTRACT

**AIM:** Despite the evidence on the beneficial effects of n-3 polyunsaturated fatty acids (n-3 PUFA) on neurodegenerative conditions, several concerns have been raised since PUFA may increase oxidative stress resulting in the formation of lipid peroxides. Hence addition of antioxidants/phytochemicals is recommended. In the present study, we have used fish oil (FO) in combination with *Bacopa monnieri* (BM) and examined the neuroprotective propensity of the combination (FO+BM) employing chronic 3-nitropropionic acid (NPA) model of neurotoxicity in rats.

**MATERIALS AND METHODS:** Growing male rats administered

NPA (10 mg/kg bw, 14d) were supplemented with FO (2 mL/kg bw), BM (100 mg/kg bw) or combination (FO+BM) for 14 days.

**RESULTS:** Higher degree of protection was discernible among FO+BM rats against NPA induced behavioral alterations. Further, NPA administration resulted in significantly elevated oxidative markers in brain regions (striatum- St, cerebellum- Cb) while robust protection was evident among rats supplemented with FO+BM. Depleted GSH levels in St were restored among all the supplemented groups. The combination also predominantly normalized the activity of antioxidant enzymes in brain regions. Further, significantly depleted dopamine levels in St were partially restored only among FO+BM rats. However, the combination did not offer protection against NPA induced mitochondrial dysfunction.

**CONCLUSION:** The present data clearly suggests that it is possible to enhance the degree of neuroprotection by a combination of FO with phytochemicals (such as BM) and thus provides an improved strategy for the treatment/ management of oxidative stress mediated neurodegenerative conditions.

**Key words:** Fish oil; *Bacopa monnieri*; 3-nitropropionic acid; Neurotoxicity; Oxidative stress

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### INTRODUCTION

Fish oil (FO) provides a rich source of n-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been recommended to be protective agents against neurological disorders. When consumed in the diet or via supplementation, these n-3 fatty acids are incorporated into the phospholipids that form cell membranes where they can influence the physicochemical and membrane-signaling properties of the cell<sup>[1]</sup>.

Most of the secondary mediators formed from EPA and DHA are anti-inflammatory and are shown to be neuroprotective<sup>[2]</sup>. Studies have shown that DHA and DHA derived metabolites promote cell survival through the induction of anti-apoptotic and neuroprotective gene expression<sup>[3]</sup>. Recent experimental evidence has also shown that n-3 fatty acids provide significant protection in animal models of Alzheimer's and Parkinson's disease<sup>[4,5]</sup>. Recent reports from our laboratory have shown the efficacy of FO supplements in modulating endogenous oxidative markers and activities of antioxidant enzymes in rat brain<sup>[6]</sup>.

*Bacopa monnieri* (BM), a traditional Ayurvedic medicinal plant is extensively used for centuries for treatment of epilepsy, insomnia, anxiety and also as a mild sedative and memory enhancer<sup>[7,8]</sup>. The major components of BM extract include two saponins, bacoside A and bacoside B<sup>[9]</sup>. The neuroprotective effects of BM extract have been assessed against various toxicants including glutamate, aluminium, beta amyloid, nitric oxide etc<sup>[7]</sup>. Alcoholic extract of BM has shown cognition facilitating effect and enhanced learning ability in rats<sup>[10]</sup>. BM extract significantly enhanced the GSH levels and activities of antioxidant enzymes in mice brain regions<sup>[11]</sup>.

3-Nitropropionic acid (NPA) is a well-known mitochondrial toxin that produces Huntington's disease (HD) like symptoms in both animals as well as humans. NPA irreversibly inhibits the mitochondrial enzyme succinate dehydrogenase (SDH) which further interferes with the electron transport cascade and oxidative phosphorylation that results cellular energy deficit and oxidative stress<sup>[12]</sup>. Among rats administered NPA, the neurons were shrunken and pyknotic, number of cells was decreased, and some cells displayed an irregular shape and a tangled appearance<sup>[13]</sup>. In rats, systemic administration of NPA results in striatal lesions, dystonia, and other abnormal motor behaviors secondary to a slowly progressive, excitotoxic striatal neuronal death<sup>[14]</sup>. Interestingly, recent findings from our laboratory employing *in vitro* and *in vivo* models have clearly shown that BM offered significant protection against NPA induced oxidative stress and cytotoxicity<sup>[15]</sup>.

The presence of unsaturated carbon chain in DHA/EPA makes them vulnerable to lipid peroxidation. Moreover, consumption of FO at higher doses for long duration increases the unsaturated fatty acids in membrane and renders them more susceptible to lipid peroxidation<sup>[16]</sup>. Hence massive doses of FO as a treatment for diseases may cause biological complications<sup>[17]</sup>. Recently co-supplementation with known antioxidant/s have been attempted to prevent the negative effects associated with FO<sup>[18]</sup>. However commercial antioxidants may promote DNA damage and may exert carcinogenic and cytotoxic effects<sup>[19]</sup>. Due to this, several plant based compounds have gained attention in recent years<sup>[20]</sup>.

Newer strategies such as incorporation of phytochemicals with FO or n-3 PUFA have yielded better protective effects in preventing diseases associated with oxidative stress<sup>[21]</sup>. In this regard, we have found enhanced neuroprotection among rats when FO was supplemented with Ferulic acid or quercetin against NPA induced neurotoxicity<sup>[22,23]</sup>. In continuation with this concept, in our laboratory we have been exploring the possibilities of enhancing the protective effects of FO with natural well established phytomedicines. Accordingly, in the present study, we have tested the hypothesis that a combination of moderate dose of FO (2 mL/kg bw for 14d) with a standard extract of BM is likely to provide better neuroprotection employing NPA model of neurotoxicity. The efficacy of the combination to protect against NPA induced behavioral alterations, oxidative markers and mitochondrial dysfunction was determined in brain regions.

## MATERIALS AND METHODS

### Chemicals

Thiobarbituric acid (TBA), 2',7'-dichlorofluorescein (DCF), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-nitropropionic acid (NPA) were procured from M/s Sigma Chemical Co. St. Louis, USA. All other chemicals used were of analytical grade.

### Animals and care

Growing male Wistar rats (4 wk-old) drawn from the stock colony of our animal house facility were housed in rectangular polypropylene cages kept on racks built of slotted angles, in a controlled atmosphere with a 12h light/dark cycle. They were acclimatized for 3 days prior to the start of the experiment. The animals were maintained on a commercial 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).

### Fish oil: composition and dosages

Cod liver oil from Seven seas (Seacod) was used for the experiment. Gas chromatography analysis showed the composition as 6.5% DHA and 7.5% EPA. FO at the dosage of 2 mL/kg bw/d, 14 d was employed for this study. Selection of this dosage was based on an earlier report<sup>[6]</sup>.

### BM: dosage selection

BM ethanolic extract was prepared as described previously<sup>[11]</sup>. The HPLC chromatogram of the extract has been reported earlier<sup>[11]</sup>. The selection of BM dosage was based on published literature and preliminary standardizations<sup>[24]</sup>. The alcoholic extract of BM is reported to contain various types of saponins which include Bacopa saponin A, B, C, D, pseudojujubogenin, bacosides (I, II, III, IV, and V), and other ingredients such as brahmine, herpestine, and monnierin<sup>[7]</sup>.

### Experimental design

For this study, selection of NPA dosage was based on literature and preliminary standardizations<sup>[25]</sup>. NPA was dissolved in physiological saline and intraperitoneally administered to rats for 14 days. FO, BM and FO+BM were supplemented by oral gavage. BM powder was suspended in distilled water. Separate FO and BM control groups were not included since their effects have been previously reported.

Growing rats were assigned to five groups of six animals each (n=6) and treated as follows: Group I: Vehicle control: Rats given physiological saline (i.p.) for 14d; Group II: NPA control: NPA (10 mg/kg bw/d) for 14d; Group III: NPA + FO: NPA administered rats + FO (oral, 2 mL/kg bw) for 14d; Group IV: BM+NPA: NPA administered rats + BM (oral, 100 mg/kg bw) for 14d; Group V: FO+BM+NPA: NPA administered rats given FO+BM supplements for 14d.

Behavioral assessments were carried out on day 7 and day 14 of the experiment. On day 15 all rats were autopsied, brain was excised and brain regions viz., cerebellum (Cb) and striatum (St) were dissected over ice. From each region, cytosol and mitochondrial fractions were prepared and subjected to quantification of various biochemical parameters.

**Behavioral assessment****Narrow beam test**

Narrow beam test was done as described with minor modifications<sup>[26]</sup>. Animals were trained to traverse a wooden beam (2.5 cm wide, 150 cm long), divided into three 50 cm segments (1, 2, 3) from a platform at one end to the animal's home cage at the other end. The beam was placed 60 cm above the floor horizontally. Each rat was tested 3 times; a score of 1 for a rat that traverse the beam without falling, a score of 2 if it fell off in the third segment, a score of 3 if it fell in second segment, 4 if it fell in first segment and 5 if the rat failed to even balance/sit down on the beam, was given. The average scores of three trials per rat were taken and mean value was calculated.

**Grip strength**

Grip strength was determined as described previously<sup>[27]</sup>. The experimental animals were suspended by its forelimbs on a wire stretched between 2 posts, 45 cm above a cushion support. The length of time for which the rat was able to hold the wire was recorded.

**Landing foot spread distance (LFSD)**

LFSD was measured among both control and treated rats as described previously<sup>[28]</sup>. In this behavior test, fourth digit of hind limbs of the rat was colored with ink and the animal was released from a horizontal position from a height of 30 cm, and the distance between the hind feet as the animal lands was recorded. This procedure was repeated three times for each rat and the average score was taken.

**Tail immersion (warm water) test- Hot hyperalgesia**

Thermal sensitivity was assessed for all the rats of various groups by tail immersion test. Tail of each rat was immersed in water maintained at a temperature of  $52 \pm 0.5^\circ\text{C}$ . The tail withdrawal latency (flicking response) or signs of struggle were observed and recorded. A cut-off time of 12 seconds was maintained in all the cases<sup>[29]</sup>.

**Tail immersion (cold water) test- Cold allodynia**

Tail of rat was immersed in cold water ( $10 \pm 0.5^\circ\text{C}$ ) until tail withdrawal (flicking response) or signs of struggle were observed. A cut-off time of 30 seconds was maintained in all cases<sup>[29]</sup>.

**Biochemical measurements****Preparation of cytosol and mitochondria**

Mitochondria were prepared by differential centrifugation as previously described with minor modifications<sup>[30]</sup>. Briefly a 10% homogenate of the brain regions was prepared in ice-cold Tris-Sucrose buffer (0.25 M, pH 7.4) using a glass-teflon grinder at  $4^\circ\text{C}$ . The homogenate was centrifuged at 1000 X g for 10 min at  $4^\circ\text{C}$  to obtain the nuclear pellet. The supernatant was further subjected to centrifugation at 10,000 X g for 20 min at  $4^\circ\text{C}$  to obtain the mitochondrial pellet and cytosol. The pellet was washed three times in Mannitol-Sucrose-HEPES buffer (pH 7.4) and resuspended in the same buffer.

**Markers of oxidative stress**

The extent of lipid peroxidation was measured using thiobarbituric acid (0.8% w/v) and quantified as malondialdehyde equivalents using 1,1,3,3-tetramethoxy propane as the standard<sup>[31]</sup>. For determination of reactive oxygen species, brain cytosol/mitochondrial protein (0.2 mg) suspended in Locke's solution was incubated with dihydrodichlorofluorescein diacetate (5 mM) for 30 min at room temperature. The fluorescence intensity measured at excitation (480

nm) and emission wavelengths (530 nm) were quantified from a dichlorofluorescein standard curve<sup>[15]</sup>.

**Protein oxidative damage: protein carbonyls**

Protein carbonyl content in the brain regions were determined as described earlier<sup>[32]</sup>. Brain cytosol (equivalent to 0.5 mg protein) was incubated with 2,4-dinitrophenyl hydrazine (DNPH) for 60 min at room temperature. Following precipitation by adding 20% trichloroacetic acid, the pellet was washed with acetone and dissolved in 1 mL of tris buffer containing sodium dodecyl sulphate (8% w/v, pH 7.4). The absorbance was measured at 360 nm and expressed as nmol carbonyls/ mg protein ( $\epsilon$ - 22000/ M/ cm).

**Determination of reduced glutathione (GSH)**

GSH levels were quantified by a fluorimetric method<sup>[33]</sup>. Brain cytosol (equivalent to 0.1 mg protein) was added to 1 mL formic acid (0.1 M) and centrifuged at 10,000 X g for 10 min. An aliquot of the supernatant was used for the assay. Concentration of GSH was calculated from the standard curve and the expressed as  $\mu\text{g}$  GSH/mg protein.

**Activities of antioxidant enzymes**

Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of quercetin auto-oxidation<sup>[34]</sup>. The activity of glutathione peroxidase (GPx) was determined using t-butyl hydroperoxide as the substrate and expressed as nmol of NADPH oxidized per min per mg protein ( $\epsilon$ -6.22/mM/cm)<sup>[35]</sup>. Glutathione reductase (GR) activity was measured as described previously<sup>[36]</sup>. Thioredoxin reductase (TRR) activity was measured as described previously<sup>[37]</sup>.

**Acetylcholinesterase (AChE) activity**

The activity of AChE was determined according to the method of Ellman *et al.* 1961<sup>[38]</sup>, by taking 1 mL reaction mixture containing phosphate buffer (0.1 M, pH 8.0), DTNB (10 mM), 50  $\mu\text{L}$  cytosol and acetylthiocholine iodide (150 mM). Change in absorbance was monitored at 412 nm for 3 min.

**Quantification of Dopamine (DA) levels by HPLC**

Dopamine (DA) levels in the cytosol of striatum were analyzed<sup>[39]</sup> using HPLC system (Model Shimadzu LC-10 Avp, UV visible detector (SPD-10 Avp), Pump (LC-10 ATvp), Shimadzu, Japan) linked to an injection valve with 20 $\mu\text{L}$  sample loop (Model 7125, Rheodyne, CA, USA). Sample was injected to a reversed-phase column (Make: 'Discovery' Supelco Sigma-Aldrich; C-18, 25 cm - 4.6 mm, 5  $\mu\text{m}$ ) accessorized with an ultraviolet detector (280 nm). Analyses were all performed at room temperature. The sample was eluted using the mobile phase 0.2% aqueous trifluoroacetic acid and methanol (70:30, v/v) at the flow rate of 1 mL/min. DA levels were calculated from an external standard curve.

**Succinate dehydrogenase and MTT reduction**

Succinate dehydrogenase activity (SDH) was determined as described earlier<sup>[40]</sup> with minor modifications. Briefly mitochondrial protein (0.05 mg) was incubated with 50 mM potassium phosphate (pH 7.4) containing sodium succinate (0.01 mol/L) and p-iodonitrotetrazolium violet (2.5  $\mu\text{g}/\text{mL}$ ) for 10 min. The reaction was stopped by addition of 10% TCA. The color obtained was extracted with ethyl acetate: ethanol: trichloroacetic acid (5:5:1, v:v:w) and measured at 490 nm. The activity was expressed as OD/mg protein. MTT reduction was determined as described previously<sup>[41]</sup>. Brain mitochondria were

incubated in a Mannitol-Sucrose-HEPES buffer (pH 7.4) containing sodium succinate (20 mM) and 15  $\mu$ L MTT (5 mg/mL) at 37°C for 60 min. The formazan crystals formed were dissolved in aqueous 10% SDS- 45% DMF buffer (v/v, pH 7.4). The absorbance of the clear solution was measured at 570 nm and expressed as OD/mg protein.

**Determination of protein**

Protein concentrations of the tissue homogenates and mitochondria were determined by the method of Lowry *et al.* 1951<sup>[42]</sup>, using bovine serum albumin as standard.

**Statistical analysis**

Results are represented as means  $\pm$  standard error (SE) for each experimental group. The data was analyzed by one-way ANOVA followed by a post hoc Tukey test to compare the control and treatment groups; p-values less than 0.05 were considered as statistically significant. All statistical analysis was performed using SPSS statistical software package version 17.0.

**RESULTS**

**Effect on narrow beam test and grip strength**

In the narrow beam test, the initial scores among various groups ranged between 1-1.2. However, measured on day 7 the scores were significantly increased among NPA administered rats and none of the supplements had any protective effect. The scores among NPA rats were further increased on day 14. While FO or BM supplements offered only marginal protection, FO+BM supplement significantly normalized the scores (Figure 1A).

Grip strength was also significantly reduced among NPA administered rats measured on day 7 and all the three supplements

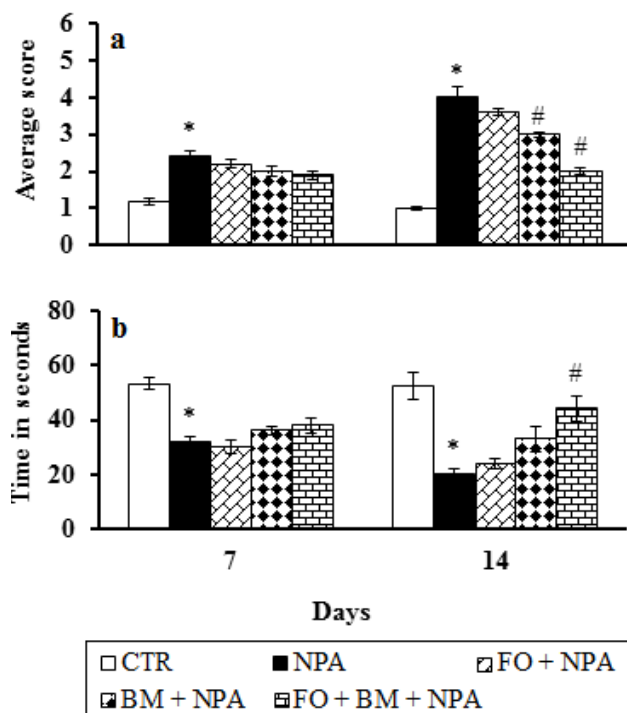


Figure 1 Modulatory effect of fish oil (FO), *Bacopa monnieri* (BM) or combination (FO+BM) supplements on narrow beam test (a) and grip strength (b) among rats administered with 3-nitropropionic acid (NPA). Data analyzed by one-way ANOVA followed by post hoc Tukey test. \* $p$ <0.05, Compared to Control group, # $p$ <0.05, Compared to NPA alone group.

had no effect. Grip strength was further reduced on day 14 and FO supplements had no effect, while BM supplements caused marginal improvement. However FO+BM supplement significantly improved the grip strength among NPA administered rats (Figure 1B).

**Effect on LFSD, heat hyperalgesia and cold allodynia**

LFSD values were marginally increased among NPA rats on day 7, while none of the supplements had any effect. Further, the LFSD values were significantly increased on day 14. FO or BM supplements offered marginal improvement while FO+BM supplement completely normalized the LFSD scores (Figure 2a).

In hot hyperalgesia test, NPA administered rats showed reduction (time dependent) in tail withdrawal latency period on both day 7 and day 14. Rats receiving FO, BM or FO+BM did not show any improvement (Figure 2b). In cold allodynia, NPA administered rats exhibited significant reduction in tail withdrawal latency period on day 7. FO, BM or FO+BM supplements had no effect on improving the withdrawal latency. However, the tail withdrawal latency was further reduced on day 14 among NPA administered rats. While FO supplements did not offer significant protection, BM supplements marginally improved the withdrawal latency period. However, FO+BM supplements significantly improved the tail withdrawal latency period (Figure 2c).

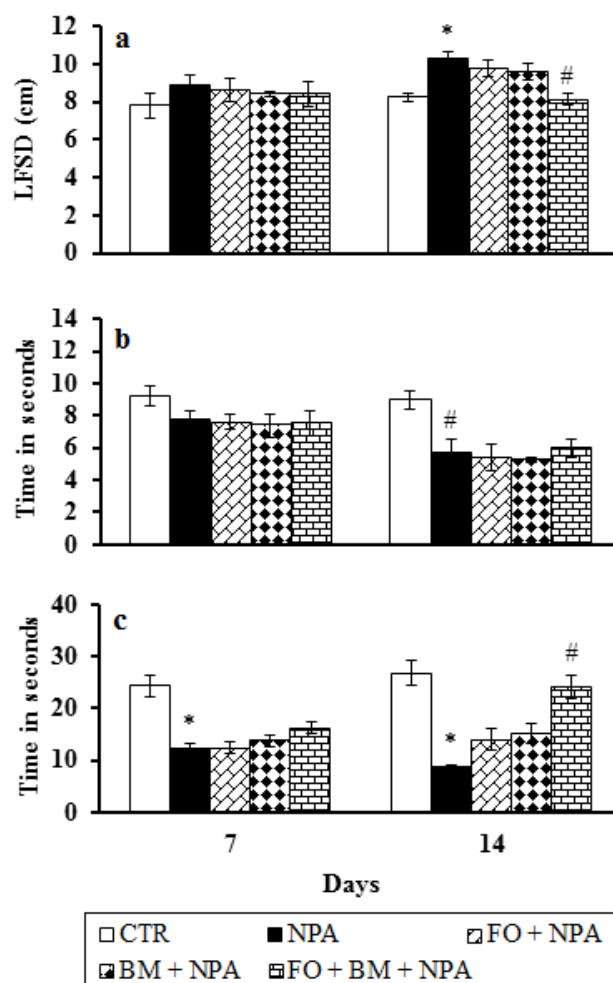


Figure 2 Modulatory effect of fish oil (FO), *Bacopa monnieri* (BM) or combination (FO+BM) supplements on landing foot spread distance (a), hot hyperalgesia (b) and cold allodynia (c) among rats administered with 3-nitropropionic acid (NPA). Data analyzed by one-way ANOVA followed by post hoc Tukey test. \* $p$ <0.05, Compared to Control group, # $p$ <0.05, Compared to NPA alone group.

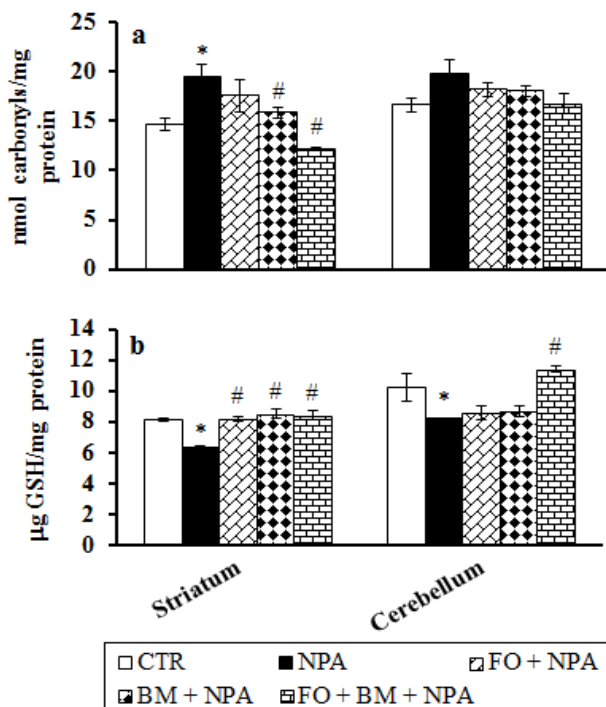
**Attenuation of oxidative markers in cytosol and mitochondria**

MDA levels were significantly elevated only in St among NPA administered rats. FO supplements did not offer protection, while BM partially normalized the MDA levels. However, the levels were completely normalized among FO+BM rats (Table 1). ROS levels were found to be elevated in both St and Cb among NPA administered rats. In St, ROS levels were marginally reduced among FO or BM supplemented rats. However, the levels were completely normalized among FO+BM rats. In Cb, FO or BM supplements did not offer protection while FO+BM supplements completely normalized the ROS levels (Table 1).

In mitochondrial fractions MDA levels were significantly elevated in both St and Cb among NPA rats. FO or BM supplements did not offer protection while FO+BM supplements significantly normalized the levels (Table 1). ROS levels were significantly enhanced only in Cb among NPA rats. FO supplements did not offer protection while BM supplements significantly lowered the ROS levels. However, the levels were significantly diminished below basal levels among FO+BM combination group (Table 1).

**Effect on protein oxidation and GSH levels**

NPA administration resulted in significantly elevated protein carbonyl levels only in St. FO supplements had no effect while BM supplements significantly normalized the levels. Interestingly, carbonyl levels were significantly diminished below basal levels among FO+BM supplemented rats (Figure 3a). Further, the GSH levels were significantly depleted both in St and in Cb among NPA administered rats. In St the levels were normalized among FO, BM or FO+BM supplemented rats, while in Cb restoration was evident only among FO+BM rats (Figure 3b).



**Figure 3** Effect of fish oil (FO), *Bacopa monnieri* (BM) or combination (FO+BM) supplements on protein carbonyl (a) and GSH levels (b) in cytosol of striatum and cerebellum among rats administered with 3-nitropropionic acid (NPA). Data analyzed by one-way ANOVA followed by post hoc Tukey test. \* $p < 0.05$ , Compared to Control group, # $p < 0.05$ , Compared to NPA alone group.

**Effect on antioxidant enzyme activities**

In general, NPA administration significantly reduced the activities of SOD and TRR in St, while GR and GPx was not affected. FO and BM supplements differentially affected the enzyme activity. The combination supplements restored the activity of SOD and TRR while the GPx and GR activity was significantly enhanced in St (Table 2). NPA administration did not have any significant effect on SOD activity in Cb. In Cb, NPA administration significantly reduced GR while the activity of GPx and TRR was not affected. However, the combination supplements enhanced the GPx and TRR activity and normalized GR activity (Table 2).

**Effect on AChE activity and dopamine levels**

In Cb, AChE activity was not affected among NPA administered rats. However, AChE activity was significantly elevated among NPA administered rats in St and the activity was completely normalized among FO, BM or FO+BM supplemented rats (Table 3).

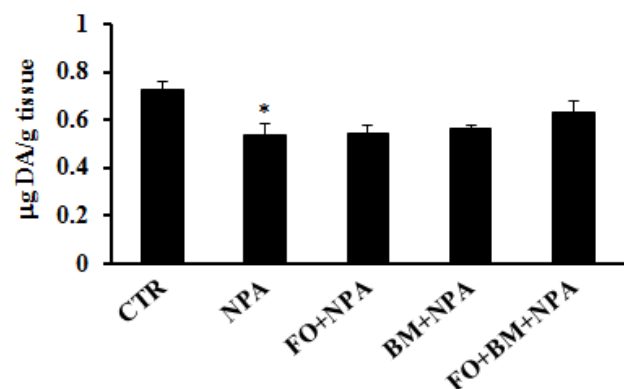
Dopamine levels were significantly depleted among NPA administered rats. The levels were marginally improved only among FO+BM rats (Figure 4).

**Effect on SDH activity and MTT reduction**

SDH activity and MTT reduction was significantly reduced among NPA administered rats in both St and Cb and none of the supplements offered protection (Table 4).

**DISCUSSION**

Though the neuroprotective effects of FO are well established, several experimental evidence suggests that higher doses of FO for longer duration induce lipid peroxidation<sup>[16]</sup>. However, the presence of antioxidants jointly with n-3 PUFA would prevent possible oxidative deterioration<sup>[43]</sup>. Hence, FO interventions coupled with natural antioxidants could enhance the potency of FO and provide dual benefits to the consumer<sup>[44,45]</sup>. In the present study, we selected *Bacopa monnieri* (BM), a traditional plant widely used in Ayurvedic system of medicine for its various neuropharmacological properties<sup>[46]</sup>. Clinical trials have shown that BM is beneficial especially in children and young adults owing to its antioxidant and neuroprotective properties<sup>[15]</sup>. Hence the present study examined the possibility of enhancing the neuroprotective efficacy of FO by enrichment with a standardized extract of BM in a shorter regimen of NPA model of neurotoxicity in rats.



**Figure 4** Dopamine levels in striatum among rats administered with 3-nitropropionic acid (NPA) and provided with oral supplements of fish oil (FO), *Bacopa monnieri* (BM) or combination (FO+BM). Data analyzed by one-way ANOVA followed by post hoc Tukey test. \* $p < 0.05$ , Compared to Control group, # $p < 0.05$ , Compared to NPA alone group.

In the present model, NPA administration significantly induced oxidative markers in both cytosolic and mitochondrial fractions of cerebellum and striatum. These results are in agreement with previous findings among NPA models of neurotoxicity<sup>[12,47,48]</sup>. Increased oxidative stress is one of the major deleterious events in both clinical and experimentally induced HD. Elevation of oxidative markers further leads to DNA damage, protein oxidation, lipid peroxidation and finally culminating into cell death and damage to brain<sup>[49]</sup>. Previously, we have shown the efficacy of FO supplements to protect against neurotoxicant induced oxidative stress and mitochondrial dysfunction in rat brain<sup>[6]</sup>. Interestingly, our earlier findings have shown that oral supplement of BM extract offered significant protection against NPA induced oxidative stress and mitochondrial dysfunction in mice brain<sup>[15]</sup>. The completely normalized levels of

oxidative markers among the combination (FO+BM) supplement can be attributed to the additive effect of both FO and bioactives present in BM extract.

In the present model, elevated protein carbonyl levels were observed in brain regions among NPA administered rats and the effects were found to be more pronounced in St. This correlates well with previous reports where elevated protein carbonyl levels were observed in brain regions among rodent models of neurotoxicity<sup>[50,51]</sup>. 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 lipid peroxidation or glycooxidation. Recently, the efficacy of FO supplements to reduce *in vivo* oxidative damage of proteins has been well demonstrated<sup>[52]</sup>. In the present study, we evidenced completely normalized protein carbonyl levels in St only among the FO+BM group, which further emphasizes their potential benefits against oxidative stress mediated neurotoxicity.

In the present study, GSH levels were significantly depleted among NPA administered rats in both striatum and cerebellum. Experimental evidence shows that oxidative stress is associated with neuronal loss and the most significant change in the antioxidant system is GSH depletion<sup>[24]</sup>. Recently, it was also shown that GSH depletion in a model of dopaminergic  $\alpha$ -synuclein overexpression leads to increased age-related loss in behavioral olfactory function as well as significant neurodegeneration of glomerular dopaminergic neurons<sup>[53]</sup>. GSH plays a critical role in protecting cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state in the central nervous system. Since depleted GSH levels are associated with the pathophysiology of neurodegenerative diseases, a number of researchers have attempted to find new potential approaches for maintaining or restoring the GSH levels<sup>[54]</sup>. In the present study, GSH

**Table 1** Ameliorative effect of fish oil (FO), *Bacopa monnieri* (BM) extract or combination (FO+BM) supplements on lipid peroxidation, ROS generation in cytosol and mitochondria of striatum and cerebellum among rats administered with 3-nitropropionic acid (NPA).

	CTR	NPA	FO+NPA	BM+NPA	FO+BM+NPA
<b>Striatum</b>					
<i>Cytosol</i>					
MDA <sup>1</sup>	5.1 ± 0.28	7.82 ± 0.46*	7.3 ± 0.52	6.7 ± 0.23	5.5 ± 0.46 <sup>#</sup>
ROS <sup>2</sup>	47.5 ± 0.3	75.0 ± 2.5*	63.0 ± 3.3	64.2 ± 1.6	45.1 ± 2.5 <sup>#</sup>
<i>Mitochondria</i>					
MDA <sup>1</sup>	1.6 ± 0.13	2.3 ± 0.16*	2.5 ± 0.16	2.2 ± 0.14	1.9 ± 0.11 <sup>#</sup>
ROS <sup>2</sup>	33.6 ± 4.2	37.2 ± 0.56	38.8 ± 5.3	38.7 ± 2.5	39.4 ± 0.6
<b>Cerebellum</b>					
<i>Cytosol</i>					
MDA <sup>1</sup>	4.0 ± 0.05	4.5 ± 0.28	4.3 ± 0.33	4.6 ± 0.35	4.4 ± 0.10
ROS <sup>2</sup>	153.3 ± 3.5	180.6 ± 8.0	200.9 ± 15.5	204.4 ± 7.4	165.9 ± 11.4
<i>Mitochondria</i>					
MDA <sup>1</sup>	1.7 ± 0.08	2.3 ± 0.17*	1.9 ± 0.16	1.9 ± 0.12	1.7 ± 0.16
ROS <sup>2</sup>	55.3 ± 3.5	74.1 ± 2.5*	67.5 ± 2.5	60.1 ± 0.5 <sup>#</sup>	49.0 ± 0.73 <sup>#</sup>

FO- 2 mL/kg bw/d, 14 d; BM- 100 mg/kg bw/d, 14 d; NPA- 10 mg/kg bw/d, 14 days. Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test. \*p < 0.05, Compared to control group, <sup>#</sup>p < 0.05, Compared to NPA alone group, <sup>1</sup>Malondialdehyde: nmol MDA/mg protein, <sup>2</sup>Reactive oxygen species:  $\mu$ molDCF/min/mg protein.

**Table 2** Effect of fish oil (FO), *Bacopa monnieri* (BM) extract and combination (FO+BM) supplements on the activities of antioxidant enzymes in cytosol of striatum and cerebellum among rats administered with 3-nitropropionic acid (NPA).

	CTR	NPA	FO+NPA	BM+NPA	FO+BM+NPA
<b>Striatum</b>					
SOD <sup>1</sup>	173.7 ± 9.2	149.3 ± 6.9*	136.6 ± 3.7	138.2 ± 1.4	181 ± 15.7 <sup>#</sup>
GR <sup>2</sup>	44.2 ± 0.4	49.2 ± 1.0	72.8 ± 10.0*, <sup>#</sup>	75.1 ± 1.2*, <sup>#</sup>	77.1 ± 5.1*, <sup>#</sup>
GPx <sup>3</sup>	28.8 ± 2.1	30.3 ± 1.5	32.4 ± 1.1	33.0 ± 1.8	42.7 ± 2.9*, <sup>#</sup>
TRR <sup>4</sup>	16.0 ± 1.7	12.1 ± 0.3*	16.5 ± 1.5 <sup>#</sup>	17.2 ± 1.2 <sup>#</sup>	21.5 ± 0.7 <sup>#</sup>
<b>Cerebellum</b>					
SOD <sup>1</sup>	236.1 ± 12.5	244.5 ± 11.6	244.2 ± 18.3	251.2 ± 9.2	240.1 ± 6.09
GR <sup>2</sup>	127.4 ± 4.9	90.2 ± 5.1*	119.1 ± 10.8 <sup>#</sup>	126.4 ± 2.1 <sup>#</sup>	147.6 ± 0.52 <sup>#</sup>
GPx <sup>3</sup>	39.2 ± 1.4	37.5 ± 3.5	39.9 ± 3.68	42.5 ± 2.5	49.7 ± 2.6*, <sup>#</sup>
TRR <sup>4</sup>	29.1 ± 1.1	29.2 ± 1.6	31.9 ± 1.9	31.3 ± 2.6	38.2 ± 2.01*, <sup>#</sup>

FO- 2 mL/kg bw/d, 14 d; BM- 100 mg/kg bw/d, 14 d; NPA- 10 mg/kg bw/d, 14 days. Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test. \*p < 0.05, Compared to control group, <sup>#</sup>p < 0.05, Compared to NPA alone group. <sup>1</sup>Superoxide dismutase: units/mg protein; <sup>2</sup>Glutathione reductase: nmol NADPH oxidized/min/mg protein; <sup>3</sup>Glutathione peroxidase: nmol NADPH oxidized/min/mg protein; <sup>4</sup>Thioredoxin reductase: nmol substrate/min/mg protein.

**Table 3** Activity levels of Acetylcholinesterase (AChE) enzyme in cytosol of striatum and cerebellum among rats administered with 3-nitropropionic acid (NPA) and provided with oral supplements of fish oil (FO), *Bacopa monnieri* (BM) extract or combination (FO+BM).

	Striatum	Cerebellum
CTR	576.1 ± 13.5	70.29 ± 0.63
NPA	810.0 ± 10.0*	67.71 ± 0.05
FO+NPA	656.2 ± 11.3 <sup>#</sup>	64.29 ± 2.65
BM+NPA	674.4 ± 42.2 <sup>#</sup>	66.86 ± 3.89
FO+BM+NPA	616.8 ± 1.36 <sup>#</sup>	68.86 ± 0.20

FO- 2 mL/kg bw/d, 14 d; BM- 100 mg/kg bw/d, 14 d; NPA- 10 mg/kg bw/d, 14 days. Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test. \*p < 0.05, Compared to control group, <sup>#</sup>p < 0.05, Compared to NPA alone group, AChE: nmol substrate hydrolyzed/min/mg protein.

**Table 4** Activity of succinate dehydrogenase enzyme and MTT reduction in striatum and cerebellum among rats administered with 3-nitropropionic acid (NPA) and provided with oral supplements of fish oil (FO), *Bacopa monnieri* (BM) extract or combination (FO+BM).

	CTR	NPA	FO+NPA	BM+NPA	FO+BM+NPA
<b>Striatum</b>					
SDH <sup>1</sup>	10.6 ± 1.9	3.23 ± 0.1*	3.67 ± 0.11	3.23 ± 0.08	4.12 ± 0.36
MTT <sup>2</sup>	14.2 ± 1.9	9.0 ± 0.3*	10.8 ± 0.7	8.13 ± 1.17	11.8 ± 0.17
<b>Cerebellum</b>					
SDH <sup>1</sup>	10.3 ± 1.18	4.80 ± 0.05*	5.05 ± 0.30	3.90 ± 0.38	6.50 ± 0.93
MTT <sup>2</sup>	14.5 ± 0.69	8.51 ± 1.22*	6.21 ± 0.46	8.28 ± 0.36	9.09 ± 0.69

FO- 2 mL/kg bw/d, 14 d; BM- 100 mg/kg bw/d, 14 d; NPA- 10 mg/kg bw/d, 14 days. Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test. \*p < 0.05, Compared to control group. <sup>1</sup>Succinate dehydrogenase- OD at 490 nm/mg protein; <sup>2</sup>MTT reduction-OD at 570 nm/mg protein.

levels were normalized to various levels among all the supplemented groups with the effect being more pronounced in the combination group. Hence, our approach presents an alternate way to enhance the GSH levels.

Further, administration of NPA caused significant alterations in the activity levels of antioxidant enzymes in brain regions clearly suggesting induction of oxidative stress *in vivo*<sup>[55]</sup>. NPA administration resulted in compromised activity levels of antioxidant enzymes such as SOD, GR and TRR in striatum. This data is consistent with our previous findings on the effect of NPA in a mice model of neurotoxicity<sup>[15]</sup>. Interestingly, activities of these enzymes were completely restored only among the combination group. Further, the activity levels of GPx and TRR were significantly enhanced among FO+BM rats in cerebellum under NPA exposure. Both of these selenium dependent enzymes are known to protect cells against oxidative injury<sup>[56]</sup>. These data allow us to speculate that the mechanism of increased neuroprotection offered by the combination supplement may be partly mediated through the normalization/enhancement of activities of antioxidant enzymes.

AChE, an acetylcholine-hydrolyzing enzyme, is implicated in cognitive functions and probably plays important roles in conditions such as neuropathy or neurodegenerative disorders<sup>[51]</sup>. Among NPA administered rats, AChE activity was significantly enhanced in striatum. This data corroborates well with the previous findings of enhanced AChE activity among neurotoxin administered rats<sup>[51]</sup>. Activation of AChE may be mediated by free radical production and consequent oxidative stress in brain. Evidence suggests that enhanced activation of AChE leads to faster acetylcholine degradation which impairs cholinergic neurotransmission and also affects other functions such as cell proliferation and promotes apoptosis<sup>[57]</sup>. It has been shown that acetylcholine has a potential neuroprotective role as a scavenger of superoxide anion and is able to reduce lipid peroxidation<sup>[58]</sup>. In the present study, complete restoration of AChE activity among FO+BM rats is suggestive of the increased efficacy of the combination to ameliorate cholinergic dysfunction.

In the present study, dopamine levels were significantly depleted in striatum among NPA administered rats, which is consistent with previous reports<sup>[27]</sup>. Dopaminergic neurons in the substantia nigra have been reported to be highly sensitive to NPA<sup>[59]</sup>. Studies on the transgenic models of HD clearly confirmed that decline in dopaminergic activity is accompanied by progression of the disease<sup>[60]</sup>. Oxidative stress plays a major role in NPA associated loss of dopaminergic neurons and depletion of dopamine levels<sup>[27]</sup>. In the present study, though oxidative markers were completely normalized with the supplements, the DA levels exhibited only marginal improvement among FO+BM rats. The underlying reason for this effect is not clear and further studies are required to delineate the mechanisms.

In the present model, chronic administration of NPA resulted in a significant decrease in SDH activity and MTT reduction. However the activity was not restored among any of the supplemented groups. Previously it was shown that FO supplements have the propensity to protect against toxin associated reduction in mitochondrial enzymes activities<sup>[6]</sup>. Further, BM supplements were found to offer protection against NPA induced mitochondrial dysfunction in mice model<sup>[15]</sup>. It is speculated that the failure to protect against mitochondrial dysfunction with FO+BM supplements may be attributed to the chronic NPA treatment paradigm.

Significant behavioral alterations were evident among NPA administered rats and these findings are in agreement with earlier reports<sup>[26]</sup>. The symptoms developed by chronic administration of

NPA are akin to juvenile onset and late hypokinetic stages of HD<sup>[60]</sup>. Furthermore, the behavioral defects are closely linked to the degree of neuronal dysfunction<sup>[61]</sup>. Balance and motor coordination skill was decreased in NPA administered rats as evident by the significantly elevated scores in narrow beam test. However the scores were reduced among FO, BM or FO+BM rats with the effect being more pronounced among the combination group. Similarly, the reduction in grip strength observed with NPA was significantly improved only among FO+BM rats suggesting the enhanced benefits of the combination.

In the present study, increased LFSD scores among NPA administered rats indicate the onset of peripheral neuropathy<sup>[28]</sup>. The effect of NPA administration on peripheral nervous system has been previously described<sup>[62]</sup>. The LFSD scores were significantly normalized among the combination group suggesting the protective effects on peripheral nervous system. Further, NPA administered rats exhibited significant reduction in tail withdrawal latency (hot hyperalgesia and cold allodynia) which indicates decrease in pain threshold<sup>[29]</sup>. The supplements did not offer protection in hot hyperalgesia while in cold allodynia FO+BM combination completely normalized the tail retention latency. The specific reasons responsible for these differential effects are not clear and merits further investigation to delineate the underlying mechanisms. However, the present data clearly suggests the beneficial effects of FO+BM combination supplements on peripheral nervous system.

In conclusion, these findings clearly support our working hypothesis that neuroprotective propensity of FO can be enhanced by enrichment with phytochemicals. The protective effects of the combination were discernible in terms of improvement in behavioral impairments and alleviation of oxidative stress. However, the specific mechanism/s by which the combination provides enhanced neuroprotection merits further investigation. Hence a combination of FO with extracts such as BM offers a useful experimental paradigm to achieve higher neuroprotective effect.

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