BIOCHEMICAL INSIGHTS RELATED TO THE PROPENSITY OF PHYTOCHEMICALS IN FORESTALLING /REVERSING NEURONAL DYSFUNCTIONS

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by

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under the supervision of

Dr. Muralidhara, M.Sc. Ph.D.

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DECLARATION

I hereby declare that the thesis entitled " **BIOCHEMICAL INSIGHTS RELATED TO THE PROPENSITY OF PHYTOCHEMICALS IN FORESTALLING/REVERSING NEURONAL DYSFUNCTIONS**" submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy** in **Biochemistry** is the result of research work carried out by me under the guidance and supervision of Dr. Muralidhara, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period April 2003-April 2008.

I further declare that these results have not been submitted for any other degree or fellowship.

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Place: Mysore

Date: June 2008

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CERTIFICATE

I hereby certify that the thesis entitled "**BIOCHEMICAL INSIGHTS RELATED TO THE PROPENSITY OF PHYTOCHEMICALS IN FORESTALLING/REVERSING NEURONAL DYSFUNCTIONS"** submitted by **Ms Shinomol George. K** for the degree of **Doctor of philosophy** in **Biochemistry**, University of Mysore is the result of research work carried out by her at the Department of Biochemistry and Nutrition, CFTRI, Mysore under my Guidance and Supervision during the period of April 2003-April 2008.

Dr. MURALIDHARA

Place: Mysore

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Shinomol George K

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

OH	Hydroxyl radical
ºC	Degree celcius
AChE	Aetylcholinesterase
ATP	Adenosine tri phoshosphate
BChE	Butrylcholinesterase
BM	*Bacopa monnieri*
BSA	Bovine serum Albumin
CA	*Centella asiatica*
CAT	Catalase
CDNB	1-chloro-2-4-dinitrobenzene
CTR	control
Cb	Cerebellum
Ct	Cerebral cortex
Cyto	Cytosol
DCF	Dichlorofluoescein
DCF-DA	Dichlorofluorescein diacetate
DKD	Detoxified khesari dhal
EDTA	Ethylene diamene tetraacetic acid
GR	Glutathione reductase
GSH	Reduced Glutathione
GSH-Px	Glutathione peroxidase
GSSG	Glutathione oxidized
GST	Glutathione-S transferase
hrs	Hours
Hc	Hippocampus
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
i.p.	Intra peritoneal
KD	Khesari dhal
N	Normality
n	Number
mg	Milligram
Kg.	Kilogram
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
MDA	Malondialdehyde
MDH	Malate dehydrogenase
Mito	Mitochondria
Min	Minutes
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphare reduced
NDD	Neurodegenerative diseases
3-NPA	3-nitropropionic acid
nm	Nanometer
NPT	Non-protein thiols
O^{2-}	Superoxide radical
OS	Oxidative stress
PD	Parkinson's disease
ROS	Reactive oxygen species
Rot	Rotenone
RNS	Reactive nitrogen species
SD	Standard deviation
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SN	Substantia nigra
St	striatum
TBARS	Thiobarbituric acid reactive substances
tbHP	Tertiary butyl hydroperoxide
TRR	Thioredoxin reductase
TSH	Total thiols
v/v	Volume by volume
β	Beta
Wk	Week

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

ABSTRACT

The brain and nervous system are increasingly prone to oxidative stress since they are inadequately equipped with antioxidant defense systems to prevent ongoing oxidative damage, Despite the heterogeneity of neurodegenerative diseases (NDD), mitochondrial involvement is an important common theme in these diseases. Hence, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria, hold great promise. Animal models have made a substantial contribution towards the current understanding of the pathogenesis of various NDD which share many features with the mechanism/s underlying brain ageing including cognitive deficits. Based on the increasing recognition that adolescence period is a time of considerable neural structuring and sculpting of the brain, there has been a growing interest in understanding whether this developmental transition is a vulnerable period for neurotoxicity.

Numerous workers have investigated the putative positive benefits of antioxidant nutrients in altering, reversing or forestalling certain neuronal and behavioral dysfunctions in humans. Many studies have shown that nutritional antioxidants (especially vitamin E and polyphenols) can block neuronal death *in vitro* and likely to have therapeutic properties in animal models of NDD. In this context, we selected two medicinal plants *viz., Centella asiatica* and *Bacopa monnieri* and investigated their neuroprotective efficacy against neurotoxicant -induced early oxidative impairments and mitochondrial dysfunctions in the brain of prepubertal mice. The neurotoxicants selected were: 3-nitropropionic acid (3-NPA) and Rotenone. In addition, we examined whether consumption of Khesari dhal (*Lathyrus sativus* L) which contains β-N-oxalylamino-L-alanine (β-ODAP, a non-protein amino acid) causes early oxidative stress in growing male mice and if detoxification process would eliminate the induction of oxidative stress and associated oxidative implications in brain regions.

Centella asiatica (Family: Umbelliferae), a plant used in the ayurvedic system of medicine is reported to possess multiple therapeutic value and several neuropharmacological properties. Notable bioactive compounds of *C*. asiatica (CA) are the triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins *viz.,* madecassic and asiatic acid. CA contains contain numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempferol, catechin, rutin, and naringin, which are potent antioxidants. Our

understanding on the efficacy of CA to offset neurotoxicant induced oxidative stress and mitochondrial dysfunctions in prepubertal mice is poor. Results obtained in a dietary study clearly indicated the potential of CA to uniformly modulate the endogenous oxidative markers in cytosol/ mitochondria of brain regions of prepubertal male mice. Further, CA also showed significant neuroprotective ability and attenuated Khesari dhal induced oxidative stress and associated mitochondrial dysfunctions in brain regions of mice. Interestingly, mice given oral prophylaxis with an aqueous extract of CA afforded significant protection against 3-NPA induced early oxidative stress and mitochondrial impairments. Significant antioxidant activity demonstrable in chemical and biological systems *in vitro* suggested that the neuroprotective properties of CA may wholly or in part is related to its multiple free radical scavenging properties.

Bacopa monnieri, (family-Scrophulariaceae) also called Brahmi is extensively used in the ayurvedic system of medicine for treatment of various ailments like epilepsy, insomnia, anxiety and memory enhancer. Bacopa extract contains two prominent constituents namely, bacoside-A and bacoside-B and various other constituents. Comprehensive studies regarding the potential of Bacopa to modulate the levels of endogenous oxidative markers in prepubertal mice brain are lacking, though it has been extensively given to children. Further its neuroprotective efficacy against neurotoxin induced oxidative stress/ mitochondrial dysfunctions have not been investigated. Dietary studies with B.*monnieri* clearly indicated the potential to modulate the endogenous oxidative markers in mitochondria and cytosol of brain regions of mice. BM ethanolic extract showed high antioxidant activity in chemical and biological systems. Interestingly, prophylaxis with aqueous extract of BM conferred significant protection against 3-NPA induced early oxidative stress and mitochondrial dysfunctions. Further, BM extract treatment was able to afford marked protection against rotenone induced oxidative stress and mitochondrial dysfunctions. BM also provided significant protection against dopamine depletion and motor dysfunctions clearly demonstrating its neuroprotective properties.

In studies, utilizing N27 cells, BM extract exhibited marked neuroprotection against neurotoxicant induced cell death, membrane damage and oxidative stress. Further BM components could significantly modulate endogenous levels of oxidative markers and enhance glutathione and glutathione related enzyme activity. Additionally, it afforded varying degree of protection against mitochondrial dysfunctions (activity of ETC and TCA cycle enzymes), DNA damage and decreased protein expression.

PREFACE

In the first part of the thesis, an attempt has been made to review the relevant literature under **six** separate sections

In the **first section** of the general introduction, a brief account of current and general understanding on oxidative stress, general implications of excess free radicals, and an overview of the antioxidant defense mechanism/s (endogenous defense molecules and enzymes) has been presented.

The **second section** of the general introduction focuses on specific neurodegenerative diseases and the vital role of oxidative stress in the development of neurodegenerative diseases. A brief account of the four major neurodegenerative diseases- Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotropic lateral sclerosis has been presented. Further, aspects related to the motorneuron degenerative disease *viz.,* Neurolathyrism has also been covered briefly. In this section, current understanding on the role of mitochondria and biochemical targets of oxidative stress in these major and minor neurodegenerative diseases have also been dealt with.

In the **third section,** insights into the reasons underlying d the increased susceptibility of brain in general to oxidative stress have been provided. Further, this section discusses why the young brain is more susceptible to neurotoxicants and the importance of such studies.

The **fourth section** deals with the various animal models in neurodegenerative disease particularly related to basal ganglia disorders. This section provides a brief introduction to brain parts and their functions and path ways of neurodegeneration with respect to basal ganglia. Further, this section elaborates on rotenone and 3-nitropropionic acid, the two mitochondrial toxins used to mimic neurodegenerative diseases of basal ganglia.

In the **fifth section,** emphasis is placed on the importance of various natural dietary antioxidants in relation to the nervous system, brief description on major phytochemicals *viz.,* gingko biloba, red wine and green tea polyphenols. At the end, background literature on the two medicinal plants *viz., Bacopa monnieri* and *Centella asiatica* are presented briefly.

Oxidative stress, an overview

Humans need oxygen to survive, but hyperoxia produces toxicity, including neurotoxicity. In healthy aerobes there is a balance between the production of various reactive species and antioxidant defenses. In some species they are not immediately removed because they perform important biological roles like defense against infection and are also important co-ordinates of the inflammatory response. Free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. It is now evident that one of the central processes of neurodegeneration lies in the chronic damage induced by decades of free radical injury to neurons, their processes and to neuroglia. Oxidative stress, defined as the accumulation of damage caused by reactive oxygen species (ROS), is thought to contribute to cell death in many human diseases (Halliwell, 2006). Numerous studies have shown that free radical damage and /or accumulation is characteristic of Alzheimer's disease, Parkinson's disease, Amyotropic lateral sclerosis, Huntington's disease and many other neurodegenerative disorders (Esposito et al., 2002). Similarly, the effects of free radical induced damage is also evident in all these disorders as well, in the form of lipid peroxidation, DNA damage and protein oxidation.

The three tier antioxidant system in tissues

Under normal conditions, all cells produce free radicals as by products of cellular metabolism. Most of these radicals develop during the electron transport process, occurring within the mitochondria. It is known that, of all the oxygen transferred into the cell, 95% enters the mitochondria and of this amount, 2 to 5% is diverted into the generation of free radicals. Under normal conditions tissues are equipped with a three tier system of antioxidant defenses to protect the cells and tissues from such damage. The first tier includes the antioxidant vitamins (vitamins C E and D, and the carotenoids) and minerals (magnesium, zinc and manganese). Within this tier the antioxidants acts at different levels. Vitamins E and D functions within the lipid portions of the cell and vitamin c and minerals operate in the aqueous portions of the cells and tissues, primarily within cytosol .The carotenoids can operate within both lipid and aqueous systems.

The second antioxidant tier consists of the special antioxidant enzymes, such as the superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase. These enzymes act on different types of free radicals and free radical precursors, such as hydrogen

peroxide, in the case of catalase and glutathione peroxidase. The final but critically important tier is the thiol compounds, which include albumin, glutathione and alpha lipoic acid. The individual thiol compounds are found both intracellularly, extracellularly and dispersed throughout the tissue and operate within different compartments (Blaylock, 1998).

One of the critical reactions responsible for free radical accumulation and damage to tissues involves the conversion of superoxide radical to hydrogen peroxide by dismutase reaction. What makes this reaction important is that, hydrogen peroxide itself is not a free radical, but in presence of iron or copper gets converted to the dangerous and powerful hydroxyl radical. In fact iron and copper constitute two of the most powerful free radical generators known, especially in the presence of ascorbic acid, as ascorbic acid reduces ferrous iron to ferric form which catalyses the conversion of hydrogen peroxide to hydroxyl ion. Researchers have discovered an equally important free radical, which does not require iron or copper conversion, the peroxynitrite ion and is formed when nitric oxide is allowed to accumulate in the presence of superoxide ion. This is referred to as reactive nitrogen species (RNS) (Bolanos, 1998).

Iron and copper ions accelerate lipid peroxidation, by two mechanisms. First, they convert H_2O_2 to $OH^{\bullet}$ in the Fenton reaction by splitting the O–O bond. In an analogous reaction, they can split lipid Hydroperoxides giving alkoxyl ($LO^{\bullet}$) and more peroxyl ($LOO^{\bullet}$) radicals, both of which can abstract $H^{\bullet}$ to keep the chain reaction going. Metal-catalyzed decomposition of peroxides also generates a complex mixture of products, including hydrocarbon gases (e.g. ethane, pentane) and toxic products such as epoxides and aldehydes. An example is the unsaturated aldehyde 4-hydroxy-2-trans-nonenal (HNE), which binds avidly to membrane proteins, inactivating enzymes, ion channels and receptors.

Mitochondrial physiology and Role of mitochondria in free radical generation

In physiological conditions mitochondria generate cellular energy in the form of ATP by the process of oxidative phosphorylation. The electron transport chain, that is located within the mitochondrial inner membrane, contains several components such as NADH dehydrogenase (respiratory complex I) and succinate dehydrogenase (respiratory complex II) and is involved in the oxidative phosphorylation by oxidizing organic acids and fatty acids with atomic oxygen to generate water. Mitochondrial complexes I and II collect electrons from the catabolism of fats,

proteins, and carbohydrates and transfer them sequentially to coenzyme Q, complex III, and complex IV. Complexes I, III and IV use the energy in electron transfer to pump protons (H+) across the inner mitochondrial membrane, producing an electrochemical gradient (ΔΨ) that drives the condensation of ADP and inorganic phosphate (Pi) to generate ATP . Three of the main aspects of mitochondrial oxidative phosphorylation are implied in experimental neurodegeneration, (i) the production of cellular energy in the form of ATP, (ii) the reactive oxygen species production, and (iii) the regulation of apoptosis.

Many line of evidence suggest that mitochondria have a central role in ageing-related neurodegenerative diseases. Mitochondria are critical regulators of cell death, a key feature of neurodegeneration. Mitochondria are the seat of a number of important cellular functions, including essential pathways of intermediate metabolism, amino acid biosynthesis, fatty acid oxidation, steroid metabolism, and apoptosis. Oxidative damage mainly occurs via the mitochondrial electron transport chain. Mitochondria, which are cytoplasmic organelles, are responsible for the production of cellular ATP. Mitochondria are involved in three important functions such as (1) Producing ATP and regulating intracellular Ca^{2+} (2) Releasing proteins that activate the caspase family of proteases and (3) Altering the reduction-oxidation potential of cells.

Disruption of ETC has been recognized as an early characteristic of apoptotic cell death. ETC involves the reduction of hydrogen peroxide (H_2O_2) to H_2O and O_2 by catalase or glutathione peroxidase accepting electrons donated by NADH and $FaDH_2$ and then yielding energy for the generation of ATP from adenosine diphosphate and inorganic phosphate (Cadenas and Davies, 2000). The production of mitochondrial peroxide radicals ($O_2^{\cdot-}$) occurs primarily at discrete points in the ETC at complexes I and III and in components of tricarboxylic acid (TCA), including alpha-ketoglutarate dehydrogenase (Starkov et al., 2004). In addition mitochondrial $O_2^{\cdot-}$ are generated in the outer mitochondrial membrane, Monoamine oxidase (flavoportein), localized on the outer mitochondrial membrane, catalyzes the oxidative deamination of primary aromatic amines. This deamination is a quantitatively large source of H_2O_2 that contributes to an increase in the steady-state concentrations of ROS within both the mitochondrial matrix and the cytosol. These released H_2O_2 and O_2 are carried to the cytoplasm via voltage-dependent anion channels and, ultimately, lead to the oxidation of cytoplasmic proteins. The chronic exposure of ROS to cells can result in oxidative damage to mitochondrial and cellular proteins, lipids and nucleic acids and acute

exposure to ROS can activate the TCA cycle aconitase and the iron-sulfur centers of ETC at complexes 1, 2 and 3, resulting in a shutdown of mitochondrial energy production (Reddy and Beal, 2005; Reddy, 2006). The generation of free radicals can occur via several cellular insults, including ultraviolet irradiation, redox cycling quinines, the metabolism of xenobiotics, aging, environmental mitochondrial toxins and mutant toxic proteins.

Scavenging of Free Radicals: Role of GSH and related enzymes

The removal of free radicals is achieved through enzymatic and non-enzymatic reactions. NO is rapidly oxidized by oxyhemoglobin to form NO_3 (nitrate), the major end stable oxidation product of NO in the body. NO also reacts with glutathione (reduced; GSH) to form nitrosothiol or with heme to yield heme- NO. Physiologically, nitrosothiol can serve as a vehicle to transport NO in plasma, thereby increasing the biological half-life of physiologic concentrations of NO. In addition, tyrosine residues of proteins can be nitrosylated by NO or its derivative peroxynitrite. Moreover, GSH can scavenge $ONOO^-$ with the formation of oxidized glutathione (GS-SG), which is converted back to GSH by the NADPH-dependent glutathione reductase. The principal defense systems against oxygen free radicals are SOD, GSH, GSH peroxidases, glutathione reductase, catalase (a heme enzyme), and antioxidant nutrients. Vitamin E can transfer its phenolic hydrogen to a peroxyl free radical of a peroxidized PUFA, thereby breaking the radical chain reaction and preventing the peroxidation of PUFA in cellular and subcellular membrane phospholipids (Reviewed by Fang et al., 2002).

Glutathione, the most abundant thiol-containing substance of low molecular weight in cells, is synthesized from glutamate, cysteine, and glycine. *N*-acetylcysteine is a stable, effective precursor of cysteine for intracellular GSH synthesis. Interestingly, almost all dietary glutamate is catabolized by the small intestinal mucosa in the first pass. Thus, the hydrolysis of glutamine to glutamate by glutaminase and the production of glutamate from α ketoglutarate and branched-chain amino acids via transamination are two major sources of plasma and cellular glutamate for GSH synthesis. As a major component of the cellular antioxidant system, GSH has the following characteristics: 1) GSH in the diet can be partly absorbed from the small intestine and can be synthesized de novo, 2) although glutathione radical (GS•) formed from the oxidation of GSH is a pro-oxidant radical, GS• can react with another GS• to yield GS-SG, which is then reduced to

GSH by the NADPH-dependent glutathione reductase; 3) GSH can react with a variety of xenobiotic electrophilic compounds in the catalytic reaction of glutathione-S-transferase; 4) GSH effectively scavenges ROS (e.g., lipid peroxyl radical, peroxynitrite, and H_2O_2) directly and indirectly through enzymatic reactions; 5) GSH can conjugate with NO, resulting in the formation of a S-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO; and 6) GSH interacts with glutaredoxin and thioredoxin (thiol-proteins), which play important roles in the regulation of cellular redox homeostasis (Fang et al., 2002). Cytochrome C and SOD catalyze the formation of O_2 from $O^{2\cdot-}$. A coproduct of SOD is H_2O_2, which is converted to H_2O by catalase and the selenium-dependent GSH peroxidase. Lipid hydroperoxides are detoxified to alcohols by GSH peroxidase. Another type of GSH peroxidase (phospholipid peroxide GSH peroxidase) acts on phospholipid peroxides in membrane structures.

Neurodegenerative diseases, Oxidative stress and Role of mitochondria

Neurodegenerative disorders comprise a heterogeneous group of chronic, age-related conditions that are associated with a disease-specific topographic loss of neurons, astrogliosis and microgliosis. These conditions are inexorably progressive, of unknown etiology and unfortunately, without known cures. The various neurodegenerative diseases (NDD) have different symptoms, affect different parts of the brain, and have different causes. They have in common impaired mitochondrial function (Reddy and Beal, 2005; Zeevalk et al., 2005), increased oxidative damage (Halliwell, 2006), defects in the ubiquitin–proteasome system, the presence of abnormal, aggregated proteins (Bence et al., 2001), changes in iron metabolism, and some involvement of excitotoxicity and of inflammation. Oxidative damage is manifested as increases in lipid peroxidation end-products, DNA (and often RNA) base oxidation products and oxidative protein damage.

Damage to mitochondria generates more ROS from the electron transport chain and causes oxidative damage that modifies proteins and other biomolecules. Treatment of rats or monkeys with low-dose rotenone over long periods produces PD-like symptoms and neurodegeneration accompanied by oxidative damage, nitrotyrosine formation, and generation of protein aggregates containing α-synuclein. Unlike MPP^+, rotenone does not concentrate in dopaminergic neurons in this region, yet it can still induce fairly selective neurodegeneration in

the substantia nigra. It follows that the neurons here may be especially sensitive to inhibition of complex I. Another clue pointing to a key role for mitochondria is provided by the observation that early-onset PD can be caused by mutations in the nuclear gene encoding a mitochondrial protein, PINK1, a protein kinase that is somehow able to protect cells against apoptosis induced by proteasome inhibition (Moore et al., 2005).

Defects in mitochondria occur in the other common neurodegenerative disorders as well. Indeed, in AD the toxicity of amyloid peptides can involve direct mitochondrial damage (Yan and Stern, 2005). In addition, aggregating Aβs raise intracellular Ca^{2+}, increase NOX activity in astrocytes and directly produce ROS (as reviewed by Haliwell, 2006). If mitochondrial damage significantly depletes the ATP supply, this will interfere with removal of proteins by the ubiquitin-proteasome system (ATP dependent), and it may even cause cells to increase their rates of Ab production (Velliquette et al., 2005). Oxidized and nitrated proteins are usually removed by the proteasome; its inhibition allows abnormal proteins to accumulate and produces oxidative stress. Potential mechanisms include increased mitochondrial ROS production and increases in iNOS activity, producing more $NO^{\bullet}$ (Lee et al., 2001).

Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the leading cause of late-onset dementia in adults. About 10% of cases are familial with mutations in the amyloid precursor protein (APP), and presenilin-1 and -2, which may be part of the α-secretase complex. The pathological hallmarks of AD are extracellular amyloid ''neuritic'' plaques, which are composed of aggregates of β-amyloid (Aβ), a 39–42 amino acid peptide derived from the proteolytic breakdown of APP. Oxidative stress has been increasingly recognized as a factor in the pathogenesis of AD. There is abundant evidence that Aβ is toxic and plays a crucial role in AD pathogenesis, and that such toxicity is enhanced when the Aβ peptide becomes aggregated. A major aspect of Aβ toxicity is the promotion of oxidative stress. The Aβ peptide appears to be an important source of free radicals, which in turn, enhances Aβ aggregation , thereby making Aβ more toxic. Additional sources of free radicals in AD may come from the activation of NADPH oxidase, a source of superoxide anion and H_2O_2, possibly derived from glial activation, as well as from mitochondrial dysfunction (Norenberg, 2007). Aβ, including its aggregated form, is also

known to bring about a dysregulation of Ca^{2+} homeostasis (Sheehan et al., 1997). Such dysregulation may contribute to the mitochondrial impairment observed in AD (Lin and Beal, 2006) and after exposure of cells to Aβ. Recent studies have identified Aβ in mitochondria as well as presenilins and the α-secretase complex (Hansson et al., 2004). Several studies have shown that exposure of isolated mitochondria to neurotoxic Aβ peptides lead to matrix swelling and impaired respiration in the presence of Ca^{2+}. Additionally, use of PC12 cell lines expressing the presenilin-1 mutation (L286V) exhibited increased sensitivity to apoptosis when exposed to complex II inhibitors (Norenberg, 2007).

Parkinson's disease

Parkinson's disease (PD) is characterized by progressive rigidity, poverty of movement (bradykinesia), tremor, and postural instability. The condition is due to the loss of melanin containing dopaminergic neurons in the substantia nigra and at other sites. Other histopathological features include Lewy bodies, which are eosinophilic cytoplasmic inclusions composed largely of α-synuclein (Lang and Lozano, 1998). Environmental factors and genetic susceptibility, however, are strongly suspected to be involved, and oxidative stress and mitochondrial dysfunction have emerged as critical mediators of the neuronal damage in PD. There is also evidence for excitotoxicity in the mechanism of PD (Beal, 2003). Mitochondrial involvement in PD was initially proposed when it was shown that the parkinsonian toxin, 1-methyl-4- phenyl pyridinium ion (MPP^+) derived from 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibits complex I of the mitochondrial electron transport chain. Administration of MPTP to primates and rodents recapitulates many of the clinical, biochemical and pathological features of PD (As reviewed by Norenberg, 2007).

A reduction in complex I activity, impaired cellular energy metabolism and mitochondrial function, along with excessive production of ROS were identified in patients with PD. MPP^+ has been shown to cause mitochondrial depolarization (Cleren et al., 2005), and rotenone, another complex I inhibitor, has also been shown to cause similar clinical and pathological changes to that seen in humans with PD (Betarbet, 2002). A number of genes have been associated with PD including α-synuclein, parkin, UCH-L1, DJ-1 and others, and all appear to have important interactions with mitochondria (Reviewed by Lin and Beal, 2006). Both rotenone and MPP+ have

been shown to induce apoptosis in PC12 cells. A number of agents with mPT inhibitory effects have been shown to attenuate the toxic effects of parkinsonian toxins.

Huntington's disease

Huntington's disease (HD) is a hereditary autosomal dominant progressive neurodegenerative fatal disorder with an onset at 35–40 years and an average survival of 15–20 years after the onset. It is clinically characterized by progressive motor disorder (choreiform movements) and behavioral and cognitive impairments. The disease largely affects the striatum and to a lesser extent the cerebral cortex (Vonsattel and DiFiglia, 1998). The disease is due to a mutation in the huntingtin (htt) gene located on chromosome 4 resulting in expanded CAG repeats (coding for glutamine) (The Huntington's Disease Collaborative Research Group, 1993). Patients with HD have CAG repeats varying from 36 to 86 (average 46). The length of the polyglutamine extensions correlates with lower age of onset, severity of the disease and the higher density of ubiquitinpositive neuronal intranuclear inclusions. The prevailing hypothesis is that expanded glutamine repeats confers a toxic ''gain of function''. Functional disturbances in mitochondrial bioenergetics are a common feature of HD models and humans with PD. The complex II inhibitors 3-nitropropionic acid (3-NPA) and malonate cause striatal lesions and energy impairment similar to HD (Norenberg and Rao, 2007).

Interestingly, the severity of depolarization correlated with length of glutamine repeats and with the ability of mitochondria to depolarize at lower calcium concentration than control mitochondria (Panov et al., 2003). As in other neurodegenerative diseases, there is a substantial role for oxidative stress and excitotoxicity in HD (Whetsell and Shapira, 1993). Mutant Htt has been found to be associated with various cellular organelles, including mitochondria. Mutant Htt with expanded polyglutamine binds to p53 and upregulated levels of nuclear p53 in neuronal cultures. The p53 levels were also found to be increased in the brains of Htt transgenic mice and HD patients. Inhibiting p53 with pifithrin-alpha, RNA interference, or genetic deletion prevented the mitochondrial membrane depolarization and cytotoxicity in HD cells, as well as the decreased respiratory complex IV activity of transgenic mice. This important study provides a crucial link between nuclear and mitochondrial events in HD. Similarly, Choo et al., (2004) showed that the mutant Htt may directly interact with the outer mitochondrial membrane and affect its functions. They further demonstrated that mutant Htt induced the mPT in isolated mouse liver mitochondria

as well as promoted cytochrome c release. Mutant Htt also significantly decreased the Ca^{2+} threshold necessary to trigger mPT pore opening. Disturbances in calcium homeostasis have been identified in HD (Panov et al., 2003).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is an age-dependent progressive disorder resulting from degeneration of motor neurons in the ventral horns of the spinal cord, brainstem and motor cortex, along with associated degeneration of the corticospinal tract. This leads to progressive skeletal muscle atrophy, weakness, paralysis, and death frequently due to respiratory failure within 2–5 years of onset (Rowland and Shneider, 2001). About 10% of ALS cases are familial and approximately 20% of these have mutations in superoxide dismutase 1 (SOD1). Familial and sporadic forms have indistinguishable clinical and histopathological features. The cause of motor neuron death in ALS is unknown (Reviewed by Norenberg and Rao, 2007). The involvement of mitochondria in ALS was initially suggested by the widespread mitochondrial vacuolation identified in the early phase of motor neuron degeneration (Higgins et al., 2003). Similar structural mitochondrial abnormalities have been observed in humans with sporadic and familial ALS. Cells expressing the G93A SOD1 mutation show a significant loss of mitochondrial membrane potential, and an increase in cytosolic Ca^{2+} concentration. Mutant SOD1 (a cytosolic protein) has been shown to be imported into mitochondria, and it has been suggested that this localization may induce cell death found that the mitochondrial vacuolar patterns in transgenic mice expressing mutant SOD1G93A originate from the expansion of the mitochondrial intermembrane space and that these vacuoles were bounded by SOD1. As mutated SOD1 has a propensity to undergo aggregation, it is speculated that aggregation of mutant SOD1 may elicit mitochondrial degeneration (Norenberg and Rao, 2007).

Neurolathyrism

Grass pea (Lathyrus sativus L) is an annual crop cultivated in Eurasia, North America, temperate parts of South America and East Africa for animal and human consumption. Common name is grass pea or chickling pea or Khesari dhal. In humans, prolonged consumption of the seeds of *Lathyrus sativus* as a staple diet has been reported to result in a progressive neurodegenerative condition known as neurolathyrism (NL), a form of motor neuron disease clinically characterized by spastic paraparesis in the hind legs that mainly targets the Betz cells

and corticospinal tracts (Dwivedi, 1989; Haimanot and Kidane, 1990). Highly prevalent among young males after a continuous dietary intake (up to 2–4months), the neurotoxicity of L. sativus is believed to be due to the neuroexcitatory non-protein amino acid 3-N-oxalyl-L-2,3-diamin opropionic acid (β-ODAP) (Rao et al., 1964; Ross et al., 1985). The neurotoxic effects of β-ODAP have been demonstrated in several species of animals, such as mice, rats, guinea pigs and macaques (Rao et al., 1967; Rao, 1977; Spencer et al., 1986). Evidences gathered from both *in vitro* and *in vivo* studies suggest that the neurotoxin acts through an excitotoxic mechanism, potentiates glutamatergic neurotransmission and binds with AMPA receptors (Hugon et al., 1996; La Bella et al., 1996).

Previous studies showed that inhibition of mitochondrial complex I and consequent mitochondrial dysfunction may be a consequence of oxidation of protein thiol groups as a result of generation of reactive oxygen species (Ross et al., 1989). An interesting finding in mice *in vivo* was the selective loss of mitochondrial complex I in frontoparietal cortex and lumbosacral segment of spinal cord, following an acute dose of L-BOAA (Sriram et al., 1998). Further, recent evidence (Kenchappa et al., 2002) has convincingly demonstrated a critical role of thioltransferase in maintenance of mitochondrial function following subcutaneous administration of L-BOAA in mice. Furthermore, it is postulated that this natural toxin may act through multiple mechanisms and/ or sites of action in the CNS beyond those that are directly linked with its activity as a non-NMDA receptor agonist (Ravindranath, 2002).

Since the toxin responsible for the neurotoxicity is identified as b-ODAP, numerous attempts have been made to detoxify the L. sativus. Differences in mode of cooking can alter the toxicity of KD through isomerization of β-ODAP to the a-isomer which is considered non-toxic. In addition, other methods, such as overnight soaking, steeping and boiling have also been employed to detoxify the seeds (Lambein, 2000). At our institute, a process for detoxification of KD has been developed by which the neurotoxin can be removed by 85–90% (CFTRI Annual Report, 1994). Numerous attempts have been made to develop animal models for NL by administration (oral or i.p.) of larger doses of β-ODAP to mice, rats and monkeys (Spencer et al., 1986; Rao et al., 1967). While multiple mechanisms have been proposed, the occurrence of oxidative damage *in vivo* following dietary consumption of KD in mice (one of the most susceptible species to NL) has been less investigated. We hypothesize that dietary consumption

of KD (at doses equivalent to human consumption levels) may cause significant oxidative stress in brain regions of mice which is likely to be progressive and is likely to participate in the development of neuronal degeneration.

Why Brain is more susceptible to Oxidative stress?

All aerobic cells suffer oxidative damage, yet the mammalian brain is often said to be especially sensitive. One reason is its high O_2 consumption; in adult humans, the brain accounts for only a few percent of body weight, but about 20% of basal O2 consumption. Hence it processes a lot of O_2 per unit tissue mass. The discrepancy is even more striking in young children, who have much smaller bodies but not proportionately smaller brains. A major reason for the high O_2 uptake is the vast amounts of ATP needed to maintain neuronal intracellular ion homoeostasis in the face of all the openings and closings of ion channels associated with propagation of action potentials and neurosecretion. Thus interrupting mitochondrial function in neurons by toxins, or failing to supply O_2 or substrates for energy production, produces rapid damage. In particular, the high Ca^{2+} traffic across neuronal membranes means that interference with Ca^{2+} sequestration (e.g. by oxidative stress dependent damage to plasma membrane Ca^{2+} exporters, or to Ca^{2+} pumps in the endoplasmic reticulum) and/or disruption of the ATP supply produces especially rapid rises in intracellular free Ca^{2+} (Fonfria et al. 2005).

The major factors which render the brain more susceptible to oxidative stress are:

i) Presence of excitotoxic amino acids: Concentrations of glutamate in brain extracellular fluids are normally low (< 1 IM). The death of cells or collapse of normal ion gradients (e.g. owing to severe energy depletion) in neurons can cause massive glutamate release which can bind to receptors on adjacent neurons, leading to excessive and prolonged increases in intracellular free Ca^{2+} and Na^{+} within them. Neurons treated with excess glutamate or other excitotoxins swell rapidly and die, usually by necrosis. Oxidative stress can damage neurons and promote the release of excitatory amino acids, generating a 'vicious cycle' of events. Hydroxynonenal can readily damage glutamate transporters, slowing glutamate clearance (Mattson and Chan 2003).

(ii) Neuronal mitochondria generate O_{-2} (mostly from complex I). Levels of 8OHdG, mutations and deletions increase with age in brain mitochondrial DNA.

(iii) Several neurotransmitters (not glycine or glutamate) are autoxidizable. Dopamine, its precursor L-DOPA, serotonin and norepinephrine can react with O_2 to generate not only $O2^{\cdot -}$, but also quinones/semiquinones that can deplete GSH and bind to protein SH groups. Oxidation can be catalyzed by transition metal ions, as mentioned above, but if excess $O2^{\cdot -}$ is present it can react with norepinephrine, dopamine and serotonin to initiate their oxidation, which then continues with production of more ROS, quinones, etc. Dopamine–GSH conjugates are degraded by peptidase enzymes to produce dopamine-cysteine conjugates (e.g. 5S-cysteinyldopamine), which can be detected in several brain regions; levels are raised in Parkinson's disease (PD) (Spencer et al., 1998).

(iv) Iron is found throughout the brain (Zecca et al., 2004). Important iron-containing proteins include cytochromes, ferritin, aconitases, non-heme iron proteins in the mitochondrial electron transport chain, cytochromes P450, and tyrosine and tryptophan hydroxylases. There are about 60 mg of non-heme iron in the 'average' adult human brain. Several brain areas (e.g. substantia nigra, caudate nucleus, putamen, globus pallidus) have a high iron content, which can be detected by magnetic resonance imaging (Schenck and Zimmerman 2004). Transferrin delivers most of the required iron across the blood–brain barrier, utilizing receptors located on the brain microvasculature. However, there is a problem; damage to brain readily releases iron (and copper) ions in forms capable of catalyzing free radical reaction. 'Catalytic' iron released by brain damage can persist because CSF has little or no iron-binding capacity (Halliwell, 2006).

(v) Neuronal membrane lipids are rich in highly polyunsaturated fatty acid side-chains, especially DHA (C22: 6) residues. Homogenization of isolated brain tissue causes rapid lipid peroxidation, which can be largely inhibited by iron-chelating agents such as desferrioxamine. In addition, products of lipid peroxidation can injure the brain. 4-Hydroxynonenal is especially cytotoxic to neurons, increasing Ca^{2+} levels, inactivating glutamate transporters and damaging neurofilament proteins. It can also inactivate α-ketoglutarate dehydrogenase (αKGDH), a key enzyme of the tricarboxylic acid cycle (Halliwell, 2006).

(vi) Brain metabolism generates a lot of H_2O_2, not only via superoxide dismutases (SODs) (see below) but also by other enzymes. Especially important are monoamine oxidases A and B,

flavoprotein enzymes located in the outer mitochondrial membranes of neurons and glia. They catalyze the reaction

$$\underset{RCH_2NH_2}{\text{monoamine}} + O_2 + H_2O \rightarrow \underset{(RCHO)}{\text{aldehyde}} + H_2O_2 + NH_3$$

and generate substantial H_2O_2 in the brain (Gal et al. 2005). The ammonia is disposed of by several mechanisms, including its use by glutamine synthetase.

(vii) Antioxidant defenses are modest. In particular, catalase levels are low in most brain regions; levels are somewhat higher in hypothalamus and substantia nigra than in cortex or cerebellum. Brain catalase is located in microperoxisomes and its activity in rat or mouse brain is rapidly inhibited by aminotriazole .

(viii) Microglia, resident macrophage-type cells that arise from monocytes entering the brain during embryonic development. Normally, they help clear cellular debris (including apoptotic cells) and are alert for threats to neurons (Nimmerjahn et al., 2005). However, microglia can become activated to produce $O_2^{\cdot-}$, H_2O_2 and cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor α. In turn, such cytokines can cause microglia to generate more ROS upon activation and to produce inducible nitric oxide synthase (iNOS) and hence excess NO•. Cytokines can additionally be produced by activated astrocytes, which may again respond to them by iNOS induction. Thus microglia and astrocytes are major players in inflammatory processes in the brain (Duncan and Heales, 2005).

(ix) Cytochromes P450 (CYPs) are present in some brain regions (Miksys and Tyndale, 2004). For example, CYP46 metabolizes cholesterol, and CYP2D6 is present in several human brain regions (Miksys et al., 2002). Since CYP2E1 leaks electrons readily during its catalytic cycle, it produces more ROS than most other CYPs (Gonzalez, 2005). The magnitude of this may be small in normal brain because CYP2E1 levels are low. However, CYP2E1 metabolizes ethanol, acetone, halothane, related anesthetics and organic solvents such as CCl4 and CHCl3, and its levels may be increased in human brain by ethanol and smoking. (Howard et al., 2003).

(x) Reactive species, both directly (e.g. by decreasing the synthesis of proteins involved in tight junctions between cells) and/or by activation of matrix metalloproteinases, can contribute to

'opening up' the blood–brain barrier, allowing neurotoxins, endotoxin and inflammatory cells to enter the brain (Kim et al., 2003).

(xi) Like many other cells, neurons contain polyADPribose polymerase 1 (PARP-1), an enzyme that responds to DNA damage by cleaving NAD+ and attaching ADP-ribose residues to nuclear proteins to facilitate DNA repair. Over activation of PARP-1 can kill cells by depleting NAD+, preventing energy production and is involved in opening TRPM2 Ca2+) channels. Indeed, NAD+ is neuroprotective; NAD+ added to neurons can slow axonal degeneration, an effect that seems to require the sirtuin, silent information regulator-like protein 1 (SIRT1). Sirtuins are NAD+-dependent protein deacetylase enzymes, intimately involved in the regulation of gene expression and of lifespan reviewed by Halliwell, 2006).

(xii) Loss of trophic support can lead to oxidative stress and apoptosis in neurons, in part by activation of neuronal NADPH oxidase enzymes. NADPH oxidase (NOX) enzymes were first detected in phagocytes, but are now known to be widespread in animal tissues, seemingly producing O_2. - for defense and/or signaling purposes reviewed by Halliwell, (2006). Neuronal NOX enzymes may promote necessary apoptosis during development of the nervous system but, if trophic support is lost in the developed brain, they may be activated inappropriately, leading to neuronal death (Sanchez-Carbente et al., 2005).

(xiii) Hemoglobin is neurotoxic. This protein is normally safely transported in erythrocytes, which are rich in antioxidant defense enzymes. However, isolated hemoglobin is degraded on exposure to excess H_2O_2, with release of prooxidant iron ions from the heme ring (Halliwell, 2006). Heme can also be released and is a powerful promoter of lipid peroxidation by decomposing peroxides to peroxyl and alkoxyl radicals (Phumala et al., 2003). In addition, hemoglobin reacts with H_2O_2 and other peroxides to form oxidizing species (heme ferryl and various amino acid radicals) capable of stimulating lipid peroxidation. Hemoglobin also binds NO• avidly, producing vasoconstriction (Alayash, 2004). Both NO• binding and oxidative damage are important in the vasoconstriction that can sometimes follow bleeding in the brain (Reviewed by Halliwell, 2006).

Susceptibility of Young Brain to neurotoxins.

Most of what we know about neural and behavioral consequences of developmental exposure to drugs and other chemicals is based on exposures during the prenatal and early postnatal period, with little emphasis on exposure periods that subsume adolescence. With the increasing recognition that adolescence is a time of considerable neural restructuring and sculpting of the brain (Spear, 2000) there likewise has been a growing interest in assessing whether this developmental transition is a vulnerable period for neurotoxicity. Adolescence consists of a series of characteristic alterations that are seen during the transition from immaturity/dependence to maturity/independence. The brain of the adolescent also undergoes pronounced sculpting and modification. Adolescence is likewise characterized by expression of a number of typical behavioral features, such as increased novelty seeking and risk taking and a shift in social affiliation towards more peer-based social interactions (Spear, 2007).

The developmental stage of adolescence is not uniquely human. Developing organisms from other species likewise undergo adolescent-typical transitions that include pubertal changes and a growth-spurt, along with expression of certain adolescent-typical behavioral patterns (spear, 2000). Prominent among the neural alterations seen during adolescence is a substantial culling of synaptic connections, with close to 50% of the synaptic connections lost in some cortical regions. Synaptic pruning is more pronounced in prefrontal cortex (PFC) and other neocortical regions than subcortical areas (Rakic et al., 1994). Included among the synapses undergoing particularly notable pruning during adolescence are those providing excitatory input to the neocortex as well as synapses contributing to reverberating circuits within particular cortical regions. Adolescence is associated with a considerable decline in brain energy utilization in humans and other species, with the high rates of blood flow and the elevated rates of oxygen and glucose utilization seen during childhood gradually declining through adolescence to reach the lower rates of energy consumption characteristic of the adult brain. Progressive myelination of axons results in considerable developmental increases in cortical white matter through adolescence and into adulthood (Spear, 2007).

Although less prominent and consistent than developmental increases in white matter, ontogenetic declines in volume of gray matter (cellular regions) are seen in regions such as the

frontal cortex although some developmental increases in gray matter volume are reported in other areas (e.g., amygdala and hippocampus). Declines in relative volume of gray matter in particular brain regions may reflect both the culling of synapses as well as ontogenetic increases in white matter, with overall cerebral volume remaining about the same from 5 years of age onward in humans. Among the brain regions showing particularly marked ontogenetic alterations during adolescence is the PFC, with this region showing considerable synaptic culling and a prominent loss of excitatory input (Spear, 2007). Developmental studies using functional magnetic resonance imaging have revealed notable ontogenetic changes in PFC activation during performance of cognitive tasks thought to index various components of executive function (e.g., response inhibition, working memory, attention). Certain subcortical regions also undergo considerable remodeling during adolescence especially those regions that form part of an interconnecting network of circuitry with the PFC – e.g., the amygdala and extended amygdala, and other dopamine (DA) mesocorticolimbic terminal regions. For instance, projections from the amygdala to PFC continue to be elaborated through adolescence (Cunningham, 2002).

The amygdala of the adolescent also shows a different pattern of stress-induced activation than the adult with fMRI data as well often revealing ontogenetic differences in amygdalar activation to emotional stimuli (faces) between adolescents and adults. Among the numerous alterations seen in mesocorticolimbic brain regions during adolescence are regionally specific ontogenetic alterations in patterns of DA production and utilization, with estimates of DA synthesis and turnover in PFC being higher early in adolescence than later in adolescence and in adulthood, whereas DA synthesis and/or turnover estimates in nucleus accumbens and striatum conversely are lower earlier than late in adolescence. Stressors would likely exacerbate the shift in DA balance toward even greater mesocortical than mesolimbic/ striatal DA activity during early adolescence, given the greater sensitivity of the mesocortical DA projection system to activation by stressors. DA projections to mesolimbic brain regions and the PFC form part of the circuitry critical for modulating risk taking, novelty seeking, and social behaviors and for attaching motivational relevance to natural rewards (such as social stimuli, novelty, food) as well as alcohol and other drugs of abuse (Spear,2007).

The adolescent period in humans has been considered by a variety of developmental researchers to typically span the age range from 12 to 18, some emerging signs of adolescence

may begin as early as 8–10 (especially in females), with other characteristic features lasting until 25 years or later. The precise timing of adolescence defies absolute categorization in other species as well. In rats, the age range from 28 to 42 days postnatal has been conservatively classified as adolescence, based on timing of age-specific behavioral changes, neural changes in brain, puberty, and the growth spurt. (Spear, 2007). In mice, rapid brain growth occurs during the first 3–4 weeks of postnatal life, termed ‘‘brain growth spurt’’ (Davison et al., 1968). During this critical period of neuronal development, the essential processes of regional brain structure and function are established (Rosenszweig,et al., 1999). Although the division of pyramidal neurons in the hippocampus is completed well before birth, their structure is fairly simple at birth, and undergoes a process of postnatal maturation (Pokony et al.,1981). Structural maturation of the brain in mice, characterized by proximal dendrite formation, takes place within 3 weeks after birth, and elongation and bifurcation of apical dendrites extend to the 5th week after birth. During this period environmental conditions can exert profound effects on the process of neuron maturation (Bartesaghi et al., 2001). Treatment during this period with nootropic drugs can enhance the learning and memory performance (Tang, 2001)

Brain parts and their functions

The brain has many parts including the cerebral cortex, brain stem, and cerebellum. Cerebral cortex consists of 1) Frontal lobe: Most anterior, right under the forehead. Its main functions include how we know what we are doing within our environment (*Consciousness*), how we initiate activity in response to our environment and Judgments we make about what occurs in our daily activities. Controls our emotional response and expressive language. It assigns meaning to the words we choose, Involves word associations and Memory for habits and motor activities. 2) Parietal Lobe: is near the back and top of the head. Its functions include, Location for visual attention, Location for touch perception, goal directed voluntary movements and manipulation of objects and integration of different senses that allows for understanding a single concept. 3) Occipital Lobes: it is the most posterior, at the back of the head and its main function is vision. 4) Temporal Lobes are at the Side of head above ears. Functions include Hearing ability, Memory acquisition, some visual perceptions and catagorization of objects. 5) Brain stem, which is deep in Brain, leads to spinal cord. Functions are mainly Breathing, Heart Rate Swallowing Reflexes to seeing and hearing (Startle Response). Controls sweating, blood

pressure, digestion, temperature (Autonomic Nervous System). Affects level of alertness, ability to sleep and sense of balance 6) Cerebellum is located at the base of the skull. Functions for the Coordination of voluntary movement, balance and equilibrium and some memory for reflex motor acts.

Pathways of Neurodegeneration and experimental models of Basal ganglia disorders.

The basal ganglia consist of a set of neural structures buried deep inside the cortex. The main basal ganglia are the caudate nucleus, the putamen, and the globus pallidus. These ganglia are tightly interconnected and receive information from several different regions of the cerebral cortex. Once the basal ganglia have processed this information, they return it to the motor cortex via the thalamus. One of the likely functions of this loop, which operates in conjunction with another one involving the cerebellum, is to select and trigger well co-ordinated voluntary movements. The basal ganglia physiologically subserve motor and cognitive functions. In the basal ganglia circuit, cortical inputs reach separate subpopulations of striatal γ- aminobutyric acid (GABA)-containing medium sized spiny neurons. From the striatum, cortical information are transmitted to substantia nigra pars reticulata through parallel routes named “direct” and “indirect” pathways and to the others output structures of the basal ganglia (globus pallidus, subthalamic nucleus and thalamus) and finally link back to the frontal cortex (Di filippo et al, 2006). The selective neuronal loss in one of the structures within the circuit results in clinical syndromes characterized by motor and cognitive dysfunctions such as PD and HD. Parkinson's disease core pathological features are represented by the heterogenous loss of pigmented dopaminergic neurons in the substantia nigra and of their projecting fibers in the striatum , whereas in HD the neurodegenerative disorder primary involves the degeneration of striatal spiny neurons with sparing of striatal large cholinergic interneurons (Di filippo et al., 2006)

Role of mitochondria in Basal ganglia disorders

The pivotal role of mitochondria in controlling cell life and death has been in recent years . The disruption of electron transport leads to oxidative phosphorylation deficits and consequently to an impairment of ATP production and to a decline in energetic capacity (Green and Reed, 1998) triggering cellular necrosis. The mitochondria also provide a major switch for the initiation of apoptosis and their role as orchestrators of the apoptotic cellular death depends upon several

death pathways. Several death receptor-independent stimuli can trigger the translocation of pro-apoptotic molecules such as Bax to the mitochondria. Bax causes the opening of a non-specific mitochondrial inner membrane channel, the mitochondrial permeability transition pore (mtPTP) that leads to the mitochondrial outer membrane permeabilization. This dissipation of the mitochondrial innermembrane potential ($\Delta\Psi m$) causes the release of several molecules involved in caspase activation and in caspase-independent cell death. In addition, in mammals, many pro-apoptotic insults seem to impact directly upon the mitochondria, leading to the release of various pro-apoptotic polypeptides (Smac/Diablo , holocytochrome c , procaspase-3 and apoptosis-inducing factor (AIF) . One of these, cytosolic cyto c, forms an essential part of the “apoptosome” which is composed of cyto c, Apaf-1 (apoptosis protease-activating factor-1), and procaspase- 9 and that is responsible of caspase-9 activation. This last event leads to the cleavage and the activation of caspase-3 and finally to the biochemical execution of the cell (Di filippo et al., 2006)

Targeting mitochondrial complex I

Nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase (complex I) is the first enzyme of the mitochondrial respiratory chain. It catalyzes the transfer of two electrons from ADH to quinone, coupled to the translocation of about four protons across the membrane accounting for about 40% of the transmembrane proton gradient generated in NADH oxidation by the mitochondrial respiratory chain (Hinchliffe and Sazanov, 2005). Consistent findings suggest the role of mitochondrial complex I in sporadic Parkinson's disease and several toxins used to model human neurological disorders cause its selective inhibition.

6-hydroxydopamine (6-OHDA) : First agent used to model PD, 6-OHDA cannot cross the blood– brain barrier and is usually administered by local stereotaxic injection directly into the substantia nigra or in the striatum in order to destroy by a retrograde axonal transport the nigral neurons projecting to the injected area. After injection, the drug selectively accumulates in dopaminergic neurons and kills neurons owing to toxicity due to the inhibition of mitochondrial complex I and generation of free radicals (Di filippo et al., 2006). The 6-OHDA models can be either complete or partial. In the complete lesion model the nigral cell loss is severe and the model reproduces a late-stage parkinsonian syndrome. Conversely, partial lesions are believed to mimic intermediate stages of Parkinson's disease. None of the modes of 6-OHDA intoxication have led to the formation of Lewy body-like inclusions. The 6-OHDA model of PD has been

successfully used to study the electrophysiological consequences of the selective loss of the nigrostriatal pathway and to analyze the synaptic correlates of L-DOPA induced dyskinesia (Picconi et al., 2003)

MPTP: MPTP is shown to model Parkinson's disease both in mice and primates. MPTP is highly lipophilic and it readily crosses the blood–brain barrier. Once in the brain the pro-toxin MPTP is oxidized into its active metabolite, 1- methyl-4-phenylpiridinium (MPP+) by monoamine oxidase B (MAO-B), an enzyme physiologically involved in catecholamine degradation. MPP+ is taken up by the plasma-membrane dopamine transporter and it is concentrated in mitochondria where it inhibits complex I reducing ATP generation and causing increased free radical production (Di filippo et al., 2006). It has been demonstrated that continuous low-level exposure of mice to MPTP induces the development of a Parkinson-like syndrome (Fornai et al., 2005). MPTP toxicity in primates replicates all the clinical features of PD including tremor, rigidity, akinesia and postural instability and represents an excellent model to study the striatal circuitry and to monitor the response to L-DOPA. Administration of MPTP to mice leads to morphologically defined apoptosis of substantia nigra dopaminergic neurons and to the upregulation of Bax, a member of Bcl-2 family, implicated in the apoptotic death pathway. Bax upregulation coincides with its translocation to mitochondria, mitochondrial release of cytochrome c and activation of caspases-9 and -3. The MPTP-induced changes in Bcl-2 family members are probably secondary to the oxidative damage to DNA and the subsequent regulation of Bax expression by the tumor suppressor protein p53. Accordingly, p53 inhibition attenuates MPTP-induced Bax upregulation and the subsequent substantia nigra dopaminergic neuronal death (Di filippo et al., 2006).

Rotenone as a complex I inhibitor: Rotenone is a naturally occurring complex ketone, derived from the roots of Lonchocarpus species. It is a classical, high affinity inhibitor of complex I, which is typically used to define the specific activity of the complex, and interacts specifically with the ND1 and PSST subunits of complex I. Being extremely lipophilic, rotenone freely crosses cellular membranes independently of any transporters, easily crosses the blood–brain barrier, enters into the brain rapidly, and accumulates in subcellular organelles, such as mitochondria where it impairs oxidative phosphorylation by inhibiting complex I of the ETC (Uversky, 2004). It is well suited for the induction of the systemic inhibition of complex I in experimental animals (Betarbet et al., 2000). In rats, rotenone administration (i.p.) is capable of causing degeneration of

dopaminergic neurons and an induction of parkinsonian symptoms (Alam and Schmidt, 2002). Chronic exposure to low doses of rotenone was shown to result in the uniform inhibition of complex I throughout the rat brain and over a period of weeks, this chronic inhibition of complex I, uniformly distributed throughout the brain, caused selective degeneration of the dopaminergic neurons in the SN (Betarbet et al., 2000). The distribution of pathology precisely matched that seen in typical PD, and neurodegeneration began in the nerve terminals and progressed retrogradely to the cell bodies. Rotenone-treated rats developed all the pathological hallmarks of PD such as nigrostriatal dopaminergic neurodegeneration, formation of LB-like cytoplasmic inclusions, and oxidative damage. Rotenone-infused animals developed parkinsonian behavior, including bradykinesia and rigidity and severely affected rats also developed the flexed posture and motor freezing typical of advanced PD (Betarbet et al., 2000; Sherer et al., 2003c).

Molecular mechanisms of rotenone neurotoxicity

The mechanisms through which systemic dysfunction of complex I might produce neurotoxicity are as yet unknown. However, some conclusions have been drawn from the results of cellular model analysis and in vitro experiments. It has become obvious that the toxic effect of rotenone is multifactorial: the insecticide might express its toxicity via the inhibition of complex I, or the enhancement of activated microglia, or the increased production of ROS, or the increased oxidative damage of proteins, lipids and DNA, or the induction of apoptosis, or the direct interaction with α- synuclein, which accelerates fibrillation of this pre-synaptic protein, or a combination of all these factors.

Complex I inhibition, oxidative damage and rotenone toxicity:

Rotenone toxicity has been assumed to result from oxidative stress. Indeed, oxidative injury has been implicated in the pathogenesis of PD, and the brains of PD patients exhibit prominent evidence of oxidative stress, including decreased levels of reduced glutathione and oxidative modifications to DNA, lipids, and proteins. Although the precise source of this oxidative damage is unknown, several mechanisms could be involved. For example, ROS are known to be generated during dopamine metabolism and by mitochondrial respiration. Furthermore, upstream of the rotenone-binding site of complex I lies a site of electron leakage that produces ROS and impaired complex I activity might enhance ROS formation (Betarbet et al., 2000). Overall, there is

a belief that the primary mechanism by which chronic inhibition of complex I kills neurons is cumulative oxidative damage (Sherer et al., 2003b). In an in vitro culture system, chronic (4 weeks) exposure to 5 nM rotenone produces progressive depletion of glutathione, oxidative damage to proteins and DNA, release of cytochrome c from mitochondria to cytosol, activation of caspase 3, mitochondrial depolarization, and eventually apoptosis (as reviewed by Uversky, 2004). The hypothesis of the potential involvement of oxidative damage in rotenone neurotoxicity has been examined recently by using three model systems: SK-N-MC human neuroblastoma cells, a chronic midbrain slice culture model, and brains from rotenone-treated animals (Sherer et al., 2003b). Exposure of SK-N-MC human neuroblastoma cells to rotenone (in a range of 10–1,000 nM) causes dose dependent ATP depletion, oxidative damage, and cell death. Importantly, cell death is blocked in the presence of antioxidants.

The assumption that rotenone is able to inhibit complex I has been confirmed via the transfection of the cells with the rotenone-insensitive single-subunit NADH dehydrogenase of Saccharomyces cerevisiae (NDI1), which is known to be incorporated into the mammalian electron-transfer chain and to act as a "replacement" for endogenous complex I. These NDI1-transfected cells do not show any mitochondrial impairment, oxidative damage, or death in response to rotenone, directly demonstrating that all the mentioned outcomes of rotenone application are caused by the specific interaction of the insecticide with complex I (Sherer et al., 2003b). The chronic midbrain slice culture model, which has been used to determine the relevance of rotenone-induced oxidative damage to dopaminergic neuronal death, has revealed rotenone-induced oxidative damage and dopaminergic neuronal loss. Both of these effects are effectively blocked by an antioxidant, α-tocopherol. Finally, the brains from rotenone-treated rats demonstrate oxidative damage most notably in dopaminergic regions affected by PD. Based on the results from these three models of increasing complexity, oxidative damage has been concluded to be indeed involved in rotenone toxicity (Sherer et al., 2003b). In agreement with the above conclusions, the exposure of human cultured cells and HL-60 (promyeloma leukemia) and BJAB cells (B-cell lymphoma) to rotenone has been shown to be accompanied by internucleosomal DNA fragmentation and DNA-ladder formation, i.e., signs of oxidative damage (Tada-Oikawa et al., 2003).

Rotenone, microglia, and the production of reactive oxygen species

The widespread oxidative damage provoked by rotenone indicates that this reagent modulates ROS production. Indeed, flow cytometric experiments have revealed that rotenone is able to induce the generation of H2O2 in rotenone-treated HL-60 and NJAB cells. On the other hand, NAD(P)H oxidase inhibitors prevent the generation of H2O2, suggesting that rotenone induces superoxide (O2 •)-derived H2O2 generation through the inhibition of the NADH dehydrogenase complex and/or activation of NAD(P)H oxidase (Tada-Oikawa et al., 2003).

Recently, in primary mesencephalic cultures from NADPH oxidase-null (gp91phox−/−) or wildtype (gp91phox+/+) mice, rotenone and inflammogen lipopolysaccharide synergistically have been demonstrated to induce dopaminergic neurodegeneration (Gao et al., 2003b). The rotenone-induced degeneration of dopaminergic neurons may not be solely attributable to an impairment of neuronal mitochondrial complex I activity but may also involve the activation of microglia (Gao et al., 2002; Sherer et al., 2003a). Microglial cells, which are the resident macrophages in the brain, respond to many insults by rapid proliferation, hypertrophy, and expression of a number of cytokines. There is an increase in reactive microglia in the striatum and SN of patients with idiopathic PD. Activated microglia upregulate cell surface markers such as the macrophage antigen complex I and produce a variety of proinflammatory cytokines and results in the production of ROS (Uversky, 2004).

Rotenone and apoptosis

Rotenone is assumed to exert its cytotoxicity through the induction of apoptosis a form of programmed cell death that normally occurs during the development of the nervous system. There are two major pathways through which apoptosis is induced; one involves death receptors and is exemplified by Fas mediated caspase-8 activation, and the other is the stress-mediated or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change. Two cell models, human cultured cells HL-60 and BJAB, have shown that the exposure of cells to rotenone induces the generation of H_2O_2, which leads to significant changes in the mitochondrial membrane potential, and which is accompanied by the fragmentation of internucleosomal DNA and the formation of DNA-ladders. In the same study, the expression of antiapoptotic protein,

Bcl-2, in BJAB cells, was shown dramatically to inhibit rotenone-induced changes in the mitochondrial membrane potential and the formation of DNA ladders, confirming the involvement of mitochondrial dysfunction in apoptosis (Tada-Oikawa et al., 2003).

Rotenone and dopamine

Dopamine (3,4-dihydroxyphenethylamine,DA) has a variety of functions throughout the body. In addition to acting as a CNS neurotransmitter for neurons involved in regulating movement (nigrostriatal pathway) and motivated behavior (mesolimbic pathway), DA is a central component of neuroendocrine axes (hypothalamus) and serves as an intermediate in the synthesis of both norepinephrine and epinephrine in the peripheral and central nervous systems. A variety of *in vitro* and *in vivo* studies demonstrate that DA is a toxic molecule that may contribute to neurodegenerative disorders such as PD and ischemia induced striatal damage. The cytotoxic effect of DA has been assumed to originate either from the metabolism of dopamine via the production of different ROS (peroxide, superoxide, and hydroxyl radical) or as a direct effect of the neurotransmitter itself in the neurodegenerative process. Indeed, the oxidation of the DA molecule produces a reactive quinone moiety that is capable of covalently modifying and damaging cellular macromolecules. Macromolecular damage, combined with increased oxidant stress, may trigger cellular responses that eventually lead to cell death (reviewed by Stokes et al., 1999).

Rotenone causes the selective degeneration of dopaminergic neurons in animal and cell models. The involvement of O2 • and endogenous DA in rotenone-induced neurotoxicity has been analyzed in rat primary mesencephalic cultures (Sakka et al., 2003). Short-term exposure (24 h) to high concentrations of rotenone (100–300 nM) significantly reduces the viability of both dopaminergic and nondopaminergic neurons in a concentration-dependent manner and the toxicity is suppressed in both types of neurons by the addition of a membrane-permeable SOD mimetic, indicating the important role of superoxide production in rotenone toxicity. Interestingly, dopaminergic neurons are effectively protected from rotenone toxicity by α-methyl p- tyrosine, a compound that inhibits Tyrosine hydroxylase and leads to a reduction in the dopamine level .On the other hand, the addition of α- MT does not have any rescuing effects on non-dopaminergic neurons, suggesting that the dopamine neuronal loss induced by high concentrations of rotenone is partially mediated by endogenous dopamine. Chronic treatment of mesencephalic cultures with

low concentrations of rotenone (1–10 nM) causes the selective degeneration of dopaminergic neurons; this toxicity is blocked by the inhibition of dopamine synthesis *via* the addition of α-MT .On the other hand, chronic suppression of mitochondrial complex I by rotenone does not affect the viability of non-dopaminergic neurons (Uversky, 2004).

Rotenone and α-synuclein

α-Synuclein-positive inclusions have been described as a feature of the neurodegenerative process triggered by rotenone infusion in rats (Betarbet et al., 2000; Sherer et al., 2003c). Importantly, in vitro analysis has revealed that the fibrillation of α- synuclein is dramatically accelerated by several common pesticides, with rotenone being one of the most effective enhancers of fibril formation. Rotenone kills neurons by using a broad assassin arsenal that includes the inhibition of complex I, the activation of microglia, the enhancement of ROS production, oxidative damage, the induction of apoptosis, and the acceleration of α-synuclein aggregation and fibrillation (Uversky, 2004).

Targeting mitochondrial complex II

Succinate dehydrogenase (complex II) is a functional member of both the Krebs cycle and the aerobic respiratory chain. It couples the oxidation of succinate to fumarate in the mitochondrial matrix with the reduction of ubiquinone in the membrane (Yankovskaya et al., 2003). 3-nitropropionic acid (3-NP) is a suicide inhibitor of respiratory complex II was identified as a component of *Indigofera endecaphylla Jacq* and also found in fungal cultures of *Aspergillia* and *Penicillia*. The toxic effects of 3-NPA can be characterized as essentially due to neurological damage. Children in China who had consumed mildewed sugar cane containing 3-NPA developed an acute illness culminating in encephalopathy .Recently, a case of moldy sugarcane poisoning was reported, with dystonia and bilateral lenticular lucencies as the principal signs. Moreover, rats treated with 3-NPA developed damage in basal ganglia and the hippocampus, with cytological features reminiscent of excitotoxic lesions (Di Filippo et al., 2006). Chronic 3-NPA administration provides a useful experimental model of HD both in rodents and non-human primates which produces lesions confined to the striatum with loss of spiny projection neurons and relative sparing of NADPH-diaphorase inter-neurons and replicates most of the clinical hallmarks of HD including spontaneous choreiform and dystonic movements. 3- NPA produces impairment in energy metabolism, formation of reactive oxygen species and nitrogen species and

a decrease in intracellular ATP levels. The ATP depletion can lead to membrane depolarization and can produce NMDA toxicity via the relief of a voltage-dependent Mg^{2+} block (di Filippo et al., 2006). It has been demonstrated that 3-NPA induces a long-term potentiation (LTP) of the NMDA receptor-mediated corticostriatal transmission in spiny neurons mediated by the activation of mGluR1 (group I metabotropic glutamate receptors)/PKC (protein kinase C) pathway but not in cholinergic interneurons Gubellini et al., 2004), probably playing a role in the regional and cell-type-specific neuronal death observed in Huntington's disease. These two main mechanisms, oxidative stress and excitotoxicity, could provide a final common pathway to explain the selective cell vulnerability in the HD experimental model as well as in other NDD (di Filippo et al., 2006).

In striatal neurons exposed to 3-NPA the presence of nuclear DNA fragmentation and increased Bax/Bcl-2 ratio as apoptosis-related markers have been observed. The activation of the JNK pathway has also been demonstrated (Garcia et al., 2002). Interestingly, the administration of micromolar concentrations of 3-NPA altered NMDA-mediated synaptic transmission in spiny neurons while it did not modify the membrane potential. Conversely acute doses (mM conc.) caused an irreversible membrane depolarization/inward current in striatal spiny neurons and a hyperpolarization/ outward current in striatal cholinergic interneurons. The diverse membrane changes induced by succinate dehydrogenase inhibition may contribute to the cell-type-specific neuronal death in Huntington's disease (Saulle et al., 2004).

Final common pathways of neurodegeneration in Basal ganglia disorders

Oxidative phosphorylation is the major endogenous source of the reactive oxygen species (O2 −, H2O2, and OHU) which are toxic by-products of respiration. The production of superoxide radicals occurs primarily at two discrete points in the electron transport chain, precisely at the mitochondrial complex I (NADH dehydrogenase) and at complex III (ubiquinone–cytochrome c reductase) (Finkel and Holbrook, 2000). Most of the toxins used to model basal ganglia NDD uncouple the mitochondrial respiratory chain leading to an increase in intracellular reactive oxygen species concentration, perturbing the normal redox balance, and shifting the cell into a state of oxidative stress. Oxygen radicals can attack proteins, deoxynucleic acids and lipid membranes thereby disrupting cellular functions and integrity. Moreover, ROS production can disrupt glutamate transporters in the plasma membrane of both astrocites and neurons (Trotti et

al., 1998). The glutamate transporters are able to concentrate glutamate across the cell membrane, maintaining low resting levels of the transmitters extracellularly and thus protecting neurons against excitatory overstimulation. Excessive glutamate exposure is toxic to neurons, probably resulting in large part from glutamate-triggered Ca^{2+} entry .The Ca^{2+} –mediated effects of glutamate receptor activation may involve several pathways that cause oxidative stress thus promoting a vicious cycle. NMDA receptor-mediated stimulation of phospholipase A2 activates the arachidonic acid cascade system leading to the generation of oxygen radicals (Di Filippo et al., 2006) Moreover, elevated intraneuronal Ca2+ levels activate peptidases, such as calpain I, which can catalyze the enzymatic conversion of xanthine dehydrogenase to xanthine oxidase (Moldoveanu et al., 2002) leading to O2 −U production and induce the formation of nitric oxide from arginine by a Ca^{2+}-activated nitric oxide synthase. Increased intracellular Ca2+ can also activate the mitochondrial permeability transition pore leading to the alteration of the mitochondrial membrane potential ΔΨm and to a collapse of the protonmotive force Δp (Nicholls and Ward, 2000). The alteration of the mitochondrial membrane potential ΔΨm alters ATP synthesis, causing itself Ca^{2+} accumulation in the matrix and increasing reactive oxygen species production.

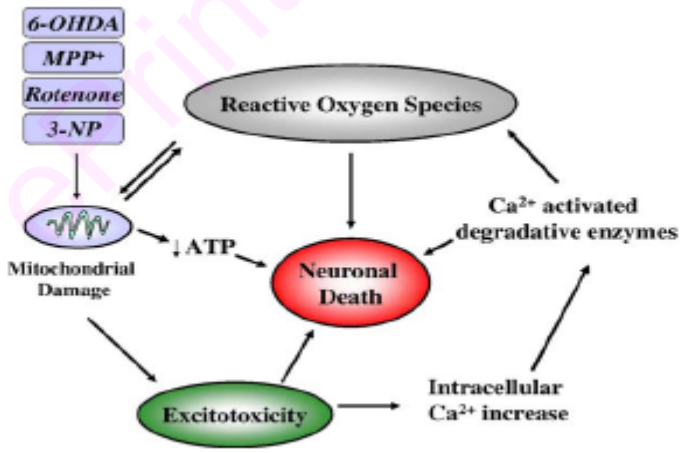

Fig. 1 The intracellular vicious cycle triggered by mitochondrial toxins.

Similarities between experimental neurodegeneration and disease pathogenesis

As described, the mitochondria are both the targets and important sources of reactive oxygen species and oxygen radicals generation is now recognised as playing a major role in both physiological aging and neurodegeneration. The nigrostriatal pathway seems to be particularly sensitive to free-radical attack and the role of mitochondria in Parkinson's disease pathogenesis is now generally accepted. Neuronal cell death in Parkinson's disease appears to occur either by apoptosis or by necrosis, although there is a debate over the extent to which this occurs (Tatton et al., 2003). The mitochondrial toxins used to model Parkinson's and Huntington's disease impair mitochondrial transport chain causing catastrophic effects on mitochondrial energetics and inducing neuronal death via necrosis and/or apoptosis . Oxidative damage has been implicated in the mechanisms of neuronal death and it contributes to the Parkinson's disease pathogenetic cascade showing several links with excitotoxicity (Jenner, 2003). The PD mitochondrial damage mimicked by the exogenous neurotoxins causes the impairment of several ATP-dependent cellular processes including the loading of dopamine into synaptic vesicles by the vesicular monoamine transporter. This results in increased cytoplasmic DA levels which might in turn result in oxidative stress through the generation of DA oxidation by-products (Andersen, 2004).

Several genetic forms of familial PD seem to show links with mitochondrial metabolism such as those due to PINK1 (Valente et al., 2004), DJ-1 (Canet-Avilés et al., 2004) and parkin (Moore et al., 2005) mutations. Parkin associates with outer mitochondrial membrane and protects against mitochondrial swelling and reactive oxygen species-induced release of cytochrome c (Darios et al., 2003). DJ-1 participates to the oxidative stress response and DJ-1 deficient mice show increased sensitivity to MPTP toxicity (Kim et al., 2005). Several evidence suggest the occurrence of mitochondrial dysfunctions In HD such as (i) studies showing an impairment in complex II–III activity and aconitase in the basal ganglia, (ii) an increased lactate production in cerebral cortex and basal ganglia and (iii) ultrastructural studies showing the presence of abnormal mitochondria in Huntington's disease patients (Beal, 2005). Mutant huntingtin, the protein responsible for Huntington's disease, seems to be associated with the outer mitochondrial membrane and to cause mitochondrial calcium abnormalities (Panov et al., 2002.

Natural dietary antioxidants

Flavonoids belong to a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, flowers, tea, and wine. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruits, and leaves. Based on their molecular structure, they are grouped as follows: (a) flavones; (b) flavanones; (c) catechins; (d) anthocyanins. The flavones are characterized by a planar structure because of a double bond in the central ring(eg., Quercetin). Quercetin is found in abundance in onions, apples, broccoli, and berries. The second group is the flavanones, which are mainly found in citrus fruit. Flavonoids belonging to the catechins are mainly found in green and black tea and in red wine, whereas anthocyanins are found in strawberries and other berries, grapes, wine, and tea. Another phenolic antioxidant is curcumin, derived from turmeric which is used as a food preservative and herbal medicine in India. Most flavonoids are glycosylated in their natural dietary forms with the exception of the catechins (Esposito et al., 2002).

Flavonoids can prevent injury caused by ROS in various ways like direct scavenging of free radicals. Flavonoids are oxidized by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize ROS by reacting with the compound of the radical. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical peroxynitrite. For example, flavanols are scavengers of superoxide anions, singlet oxygen and lipid peroxy radicals and they can sequester metal ions by chelation. Moreover, it has been demonstrated that (−)-epicatechin, (−)-epicatechin gallate and quercetin serve as powerful antioxidants against lipid peroxidation when phospholipid bilayers are exposed to ROS in vitro. There is also evidence that flavonoids can inhibit the activities of several enzymes, including lipoxygenase, cyclo-oxygenase, xanthine oxidase , phospholipase A2. These biological effects are believed to derive from the antioxidant properties of the related flavonoids (Esposito et al., 2002).

Several epidemiological evidences indicate the putative role of nutritional antioxidants in the prevention and attenuation of NDD and recently these are experimentally confirmed in a number of laboratory studies. Epidemiological studies have shown that moderate wine consumption can be protective against neurological disorders such as age-related macular

degeneration and AD. Moreover, *in vitro* and *in vivo* pre-clinical studies have shown the neuroprotective effect of lyophilized red wine , grape polyphenols , quercetin , *trans*-resveratrol , and (+)-catechin . Taken together, these findings raise the possibility that red wine constituents may be beneficial in the prevention of age-related neurodegenerative disorders. Esposito et al., 2002). There is also increasing interest for the role of tea (*Camellia sinensis*) in maintaining health and in treating disease. Although tea consists of several components, research has focused on polyphenols, especially those found in green tea. The green tea polyphenols include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin-3-gallate (EGCG). Of these, EGCG generally accounts for greater than 40% of the total (Hara, 1997). Green tea polyphenols are potent antioxidants. Many of the putative health benefits of tea are presumed to be caused by its antioxidant effects. Thus, the polyphenol epicatechin was shown to attenuate neurotoxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons (Schroeter et al., 2000). Recently, Ginkgo biloba extract, known to be enriched with flavonoids, has been shown to protect hippocampal neurons from nitric oxide or beta-amyloid derived peptide-induced neurotoxicity Esposito et al., 2002). In addition, the extract of Ginkgo biloba (Egb 761) is one of the most popular plant extracts used in Europe to alleviate symptoms associated with a range of cognitive disorders (Le Bars et al., 1997). The mechanism of action of Egb 761 in the CNS is only partially understood, the main effects seem to be related to its antioxidant properties, which require the synergistic action of the flavonoids, the terpenoids (ginkgolides, bilobalide), and the organic acids, principal constituents of Egb which act as scavengers of ROS (Esposito et al., 2002).

Bacopa monnieri

Bacopa monnieri (B. monnieri), also referred to as Bacopa monniera, Herpestis monniera, water hyssop, and ‘‘Brahmi,’’ has been in use since time immemorial as nerve tonic for improvement of memory. B. monnieri (BM) is a perennial creeping plant found throughout India in wet, damp and marshy areas. Traditionally, it was used as a brain tonic to enhance memory development, learning and concentration (Mukherjee and Dey, 1966) and to provide relief to patients with anxiety or epileptic disorders. The biological effects of B. monnieri are documented in both traditional and modern scientific literature. The plant, plant extracts and isolated bacosides have been extensively investigated for their memory and cognition enhancing effects.

Alcoholic extract of BM has shown cognition facilitating effect in normal rats and in different behavioural response studies and also inhibited the amnesic effects of scopolamine, electroshock and immobilization stress (Singh and Dhawan, 1997). The butanolic extract of BM has shown memory enhancing effect in rats by increasing recognition index in differential exploration of familiar and new objects test (Um et al., 2002). More recently, Kishore and Singh (2005) have reported that anterograde administration of alcoholic extract of BM in mice facilitated anterograde memory and attenuated anterograde experimental amnesia induced by scopolamine. Subchronic administration of standardized bacosides rich extract of BM reversed the cognitive deficits induced by colchicines/ibotenic acid and also reversed colchicine-induced reduction in frontal cortex and hippocampal acetylcholine concentration, choline acetyltransferase activity and muscarinic cholinergic receptors binding (Bhattacharya et al., 1999).

The standardized extract of BM exhibited cognitive enhancing activity by attenuating the dementia effect of scopolamine in passive avoidance test (Das et al., 2002). The major chemical entities shown to be responsible for the memory-facilitating action of BM are the steroidal saponins bacoside A and B (Singh et al., 1988). The other major chemical constituents isolated and characterized from the alcoholic extract are dammarane type triterpenoid saponins with jujubogenin and pseudojujubogenin as the aglycones, including bacosides A1–A3 (Rastogi and Kulshreshtha, 1999), bacopasaponins A–G (Garai et al., 1996; Mahato et al., 2000; Hou et al., 2002) and bacopasides I–V (Chakravarty et al., 2003). Sheikh et al., 2007 showed that the adaptogenic activity of BM might be due to the normalization of stress induced alteration in plasma corticosterone and levels of monoamines like NA, 5-HT and DA in cortex and hippocampus regions of the brain. Recent studies demonstrated that Bm extract reduced divalent metals, dose-dependently scavenged reactive oxygen species, decreased the formation of lipid peroxides and inhibited lipoxygenase activity (Dhanasekharan et al., 2007). These data combined with their earlier studies shows that BM extract treatment reduces beta-amyloid levels in the brain of an Alzheimer's disease doubly transgenic mouse model of rapid amyloid deposition (PSAPP mice) suggesting mechanisms of action relevant to the treatment of Alzheimer's disease. A summary of the studies done on *B.monnieri* is given in table below.

Table 1: Beneficial effects of Bacopa monnieri as reported in literature

OBSERVATIONS	REFERENCES
Reduces beta-amyloid deposits in the brain of an AD animal model	Dhanasekaran et al., 2007.
normalization of stress induced alteration in plasma corticosterone and levels of monoamines like NA, 5-HT and DA.	Sheikh et al., 2007.
BM ethanolic extract –antiinflammatory action in rodents	Channa et al., 2006.
BM methanolic extract protects against nitric oxide related toxicity in vitro	Russo et al., 2003.
BM ethanolic extract has free radical scavenging and protective against DNA damage in vitro	Russo et al., 2003.
BM extract have anti stress effects	Chowdhuri et al., 2002.
Antidepressant activity in adult rats	Sairam et al., 2002.
Mast cell stabilizing activity	Samuilla et al., 2001
BM extract improves cognitive function in healthy humans	Nathan et al., 2001. . Stough et al., 2001. .
BM ethanolic extract increases Activity of antioxidant enzymes in brain of adult rats	Bhattacharya et al.,2000.
Protection from phenytoin-induced cognitive deficits	Vohora et al., 2000.
Anxiolytic activity in rodent models	Bhattacharya and Ghosal. 1998.
BM extract has in vitro anticancer activities	Elangovan et al., 1995.
BM Revitalising intellectual functions in childrens	Sharma et al., 1987.

Centella asiatica

Centella asiatica (Umbelliferae) is an ancient Ayurvedic remedy, which is used as a revitalizing herb that strengthens nervous function and memory. It is reported to restore youth, memory and longevity (Kapoor, 1990). A formulation composed of four herbs, including *C. asiatica*, is used to retard age and prevent dementia, and the herb combined with milk is given to improve memory in ayurvedic medicine (Manyam, 1999). The herb is also taken as a tonic for poor digestion and rheumatism. *C. asiatica* is used in TCM for combating physical and mental exhaustion (Brinkhaus et al., 2000). C.asiatica is a psychoactive medicinal plant that has been used for centuries in Ayurvedic medicine to alleviate symptoms of anxiety and to promote a deep state of relaxation and mental calmness during meditation practices. Recent investigations using human and animal models of anxiety have confirmed that Gotukola does indeed possess anxiolytic activity. The crude extract of C. asiatica and the products derived from glycoside were used as oral antifertility agents.The extract of C. asiatica extract possesses antioxidant (Gupta

and Flora, 2006), anti-inflammatory, immunomodulating , antitumor, antiproliferative, radioprotective and antigenotoxic properties as reviewed by Howes et al., 2003).

It is reported that a single 12 g dose of Gotukola administered orally was more effective than placebo in decreasing acoustic startle response in healthy humans. This effect was most pronounced 60 min after treatment. In animals, Gotukola increases pentobarbitone-induced sleeping time and decreases immobility in the forced swim test (Sakina and Dandiya, 1990). Gotukola also elicits anti-anxiety effects in the elevated plus maze (Lucia et al., 1997) and an aqueous extract of Gotukola was reported to have cognitive-enhancing as well as antioxidant effects in rats . The extract of C. asiatica L. has certain bioactive terpene acids such as asiatic acid, madecassic acid and their respective glycoside, asiaticoside and madecassoside .The most prominent group of biologically active compounds isolated from Gotukola is the terpenes . Asiaticoside is the most abundant triterpene glycoside, which is effective in wound healing and apparently acts by enhancing the induction of antioxidant levels at an early stage of wound healing . Asiaticoside is transformed into its aglycone asiatic acid in vivo by hydrolysis. Several derivatives of asiaticoside and asiatic acid were found to show protective effect against beta amyloid-induced neurotoxicity massociated with the dementia of Alzheimer's disease (as reviewed by Howes et al., 2003)

Asiatic acid decreased the viability and induced apoptosis in human melanoma cells (Park et al., 2005). Asiaticoside and madecassoside have anti-psoriatic properties (Sampson et al., 2001). Asiaticoside, has wound healing activity and promotes fibroblast proliferation (Lu et al., 2004). The crude extract of C. asiatica was shown to be non-toxic in normal human lymphocytes (Babu et al., 1995) and reduced the genotoxic effects of methyl methanesulphonate and cyclophosphamide in cultured human lymphocytes (Siddique et al., 2007). C. asiatica has been reported to contain numerous caffeic acid derivatives and flavonols and in particular, quercetin and kaempferol catechin, rutin and naringin (Zainol et al., 2003), some of which have been shown to be potent antioxidants. Recent studies (Hussin et al., 2007) have revealed that CA extract and powder may ameliorate H_2O_2-induced oxidative stress in blood by decreasing lipid peroxidation via alteration of the antioxidant defense system of the rats.

An alcohol extract of the leaves is reported to be tranquillizing in rats, an activity that was attributed to a triterpene, brahmoside (Kapoor, 1990; Sakina and Dandiya, 1990). In mice, an extract of *C. asiatica* leaf was sedative, antidepressant and showed cholinomimetic activity, which was blocked by atropine (Sakina and Dandiya, 1990). These results indicate that *CA* may be appropriate to treat symptoms of depression and anxiety in AD, and may also enhance cholinergic activity and thus, cognitive function. An aqueous extract of *CA* leaf improved learning and memory processes in rats, and modulated dopamine, 5-hydroxytryptamine (5-HT) and noradrenaline systems in rat brain *in vivo* (Nalini et al., 1992) indicating that more polar compounds (perhaps triterpene saponins) present in CA may enhance cognitive function by influencing neurotransmitter systems in the CNS. The triterpene asiatic acid and its derivatives have been shown to protect cortical neurons from glutamate-induced excitotoxicity *in vitro*.

Table 2: Beneficial effects of Centella asiatica as described in literature

OBSERVATIONS	REFERENCES
Positive modulation of cognition and mood in healthy elderly volunteers following administration of CA	Wattanathorn et al., 2008.
Antigenotoxic role of CA extract against cyproterone acetate induced genotoxic damage in cultured human lymphocytes	Siddique et al., 2007
Protective effect of CA extract and powder on oxidative stress in rats	Hussin et al., 2007.
CA extract has Anxiolytic property in rat behavioral study models	Wijeweera et al., 2006.
CA aqueous extract treatment during post natal period enhances learning and memory in mice	Rao et al., 2005.
Improving age-related neurological antioxidant status in rats	Subathra et al., 2005.
CA extract and asiaticoside on acetic acid induced gastric ulcers in rats	Cheng et al., 2004.
Inhibitory effects of CA on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats	Bunpo et al., 2004.
Protective against pentylenetetrazole-induced kindling, cognition and oxidative stress in rats	Gupta et al., 2003.
Improves cognition and protects against oxidative stress in healthy rats	Veerendra kumar and Gupta J. 2002.(Dhanasekharan et al., 2007)

SCOPE OF THE PRESENT INVESTIGATION

Neurodegenerative disorders (NDD) comprise a heterogeneous group of chronic, age-related conditions that are associated with a disease-specific topographic loss of neurons, astrogliosis and microgliosis. The various NDD have different symptoms, affect different parts of the brain, and have different causes and have in common impaired mitochondrial function increased oxidative damage defects in the ubiquitin–proteasome system, the presence of abnormal aggregated proteins, impaired iron metabolism and some involvement of excitotoxicity and of inflammation. Oxidative damage is manifested as enhanced lipid peroxidation end-products, DNA (and often RNA) base oxidation products and oxidative protein damage.

The brain and nervous system are prone to oxidative stress and are inadequately equipped with antioxidant defense systems to prevent the ongoing oxidative damage, let alone the extra oxidative damage imposed by NDD. In all major examples of NDD, there is a strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis. Hence, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria, hold great promise.

Animal models have made a substantial contribution towards the current understanding of the pathogenesis of various NDD which shares many features with the mechanism underlying brain ageing. Further, large amount of evidence implicates oxidative stress as being intimately involved in the various cognitive impairments associated with NDD. Understanding the implications of such cognitive deficits in the young assumes paramount importance since young and the aged are known to be relatively more susceptible to specific neurodegenerative conditions. Currently the need and relevance of the assessment of adolescent neurotoxicity is receiving wide attention. Experimental studies have focused on neural and behavioral consequences of neurotoxicant exposure during the prenatal and early postnatal periods with little emphasis on exposure periods that subsume adolescence. Based on the increasing recognition that adolescence period is a time of considerable neural structuring and sculpting of the brain, there has been a growing interest in understanding whether this developmental transition is a vulnerable period for neurotoxicity.

Current epidemiological evidences indicate that diets rich in fruits and vegetables substantially reduce the incidence of certain types of cancer and cardiovascular diseases. Hence, numerous workers have investigated the putative positive benefits of antioxidant nutrients in altering, reversing or forestalling certain neuronal and behavioral dysfunctions in humans. Experimental evidences suggest that dietary supplements (vitamins A, C, E) may be beneficial in altering the neuronal/behavioral deficits in aging and experimentally –induced neurotoxicity. Many studies have shown that nutritional antioxidants (especially vitamin E and polyphenols) can block neuronal death in vitro and likely to have therapeutic properties in animal models of neurodegenerative diseases. Interestingly, bioflavonoids have been demonstrated to be more effective than vitamin E in antagonizing the effects of aging or oxidative challenges.

Several natural products, often part of human diet, are sources of antioxidants and are of potential interest in prevention, and treatment of neuropathologies related to oxidative stress. Among several natural products considered to be of potential therapeutic utility, those containing compounds belonging to the flavanoid and to the related polyphenolic class (whose daily human intake is substantial) have recently gained importance. Further, antioxidants which have a mitochondria targeted action are gaining substantial significance as the role of mitochondria in NDD is brought to day light. It is in this context, that we chose two medicinal plants *viz., Centella asiatica* and *Bacopa monnieri* and investigated their neuroprotective efficacy against neurotoxicant -induced oxidative impairments and mitochondrial dysfunctions in the brain regions of prepubertal mice.

Centella asiatica (Family: Umbelliferae), a plant used in the ayurvedic system of medicine is reported to possess multiple therapeutic value and several neuropharmacological properties. Notable bioactive compounds of *C*. asiatica (CA) are the triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins *viz.,* madecassic and asiatic acid. CA contains contain numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempferol, catechin, rutin, and naringin, which are potent antioxidants. To the best of our knowledge, data on the efficacy of CA to offset neurotoxicant induced oxidative stress and mitochondrial dysfunctions in prepubertal mice are lacking.

Bacopa monnieri, (family-Scrophulariaceae) also called Brahmi is extensively used in the ayurvedic system of medicine for treatment of various ailments like epilepsy, insomnia, anxiety and memory enhancer. It is known to have antioxidant properties and is reported to improve the performance of rats in various learning situations depicting its cognitive enhancement properties. Bacopa extract contains two prominent constituents namely, bacoside-A and bacoside-B and various other constituents. To the best of our knowledge there have been no studies regarding the potential of Bacopa to attenuate endogenous oxidative markers in prepubertal mice brain, though it has been extensively given to children. Further the efficacy of BM to protect against neurotoxin induced oxidative stress and mitochondrial dysfunctions have not been comprehensively investigated .

The neurotoxicants selected for our studies were 3-nitropropionic acid (3-NPA) and Rotenone. 3-NPA is a major mitochondrial toxin that has been found to effectively induce specific behavioral changes and selective striatal lesions in rats and non-human primates and brain lesions produced by a systemic administration of 3-NPA are more or less specific of the striatum, although hippocampus, thalamus and brain cortex are also affected . For this reason, 3-NPA has been widely used as a suitable model for disruption of energy metabolism, and more specifically as a model of HD. The primary mechanism of action of this toxin involves the inhibition of complex II (succinate dehydrogenase (SDH) at the mitochondrial electron transport chain and irreversible blocking decreases the levels of ATP leading to neuronal cell death. The impaired energy metabolism can produce oxidative stress as well as formation of reactive oxygen and nitrogen species which are suspected to be critically involved in neuronal death. However studies describing the susceptibility of prepubertal brain to 3-NPA induced early oxidative impairments and associated mitochondrial dysfunctions are scarce.

Rotenone is a lipophylic compound and is a specific inhibitor of mitochondrial complex I. Inhibitors of complex I increase ROS formation and this may affect the vital mitochondrial parameters like ATP production. In all the models used to reproduce Parkinson's disease symptoms including rotenone, oxidative stress was of the major factors that contributed to dopaminergic neuronal lesions. The rotenone model suggests that dopaminergic neurons may be more vulnerable than other cell populations to the effects of complex I inhibition, and several

observations are consistent with a high sensitivity of dopaminergic neurons to oxidative stress The mechanisms of selective damage may be inter-related, because inhibition of mitochondrial complex I is likely to result in augmented production of ROS. The feature that distinguishes dopaminergic cells from other neuronal cell types *i.e* their dopamine content—might contribute to their susceptibility to oxidative injury owing to the participation of dopamine in harmful oxidative reactions. Though extensively studied, the effects of rotenone on the induction of early oxidative damage in prepubertal mice brain regions are not well known.

Neurolathyrism is a neurodegenerative disease caused by the chronic consumption of Khesari dhal (Lathyrus sativus L). It is generally accepted that β-N-oxalylamino-L-alanine (b-ODAP), a non-protein amino acid present in the seeds is the primary causative agent. Based on in vitro studies with b-ODAP, both excitotoxic and oxidative stress mechanisms have been speculated to be responsible for its neurotoxic effects. However, occurrence and the involvement of oxidative stress mechanisms in experimental animals following Khesari dhal consumption in vivo is less well understood.

Accordingly in the present study attempts have been made to obtain evidences to demonstrate the neuroprotective properties of *Centella asiatica* (Leaf powder and standardized aqueous extract) and *Bacopa monnie*ri (leaf powder and standardized ethanolic extract) employing prepubertal male mice as the experimental model.

The basic objectives envisaged at the beginning of the investigations were:

1) To elucidate the protective role of selected phytochemicals in modulating oxidative damage in experimentally- induced neuronal dysfunctions.

2) To understand the impact of selected phytochemicals on various neuronal dysfunctions

3) To establish the nature and extent of mitochondrial dysfunctions in specific regions of the brain under experimentally –induced neuronal dysfunctions.

4) To understand the vital biochemical pathways likely to mediate the specific pathophysiological alterations responsible for the neuronal dysfunctions.

SECTION A

MATERIALS

Chemicals

1,1,3,3-tetramethoxypropane, 1-chloro-2,4-dinitro benzene, 2,4-dinitro phenyl hydrazine, 5,5-dithio-bis (2-nitrobenzoic acid), adenosine tri phosphate, ascorbic acid, bovine serum albumin, caffeic acid, cetyl trimethylammonium chloride, collagenase, diphenyl-β-picryl hydrazyl (DPPH), 2', 7'-dichloro-fluorescein (DCF), DL-isocitrate, ethidium bromide, ferricytochrome – C, glutathione (GSH and GSSG), glutathione reductase, hydrogen peroxide, EGTA sodium salt, oxaloacetate, ouabain, collagenase, triton –X-100, proteinase K, mercaptoethanol, bromophenol blue, NADH, NADP,NADPH, quercetin, t-butyl hydroperoxide, (70% aqueous CAS # 75-91-2), thiobarbituric acid (TBA), trypan blue, trypsin, xanthine and xanthine oxidase were from M/s. Sigma chemicals Co.,(St. Louis, MO, USA). Ferric chloride, ferrous sulphate, Folin ciocalteus reagent, O-pthalaldehyde, potassium ferricyanide, sodium dodecyl sulphate, trichloro acetic acid, tris, acrylamide, coomassie brilliant blue were from M/s. Sisco Research Laboratories, Mumbai India. All other chemicals used were of analytical grade.

Neurotoxicants

3-nitropropionic acid (3-NPA)

3-NPA (N5636; lot no: 073K3448; FW-119.08; ≥97%) was obtained from M/s. Sigma chemicals Co., (St. Louis, MO, USA).

Rotenone

Rotenone (R8875; lot no: 082K1294; FW-394.4; ≥95-98%) was obtained from M/s. Sigma chemicals Co., (St. Louis, MO, USA).

Khesari dhal (KD)

Khesari dhal was procured from the Regional Centre, Central Food Technological Research Institute (CFTRI), Mumbai, India. The samples were purchased from the local market of Nagpur, Maharashtra, India. It was powdered without producing much heat in a dhal mill.

Detoxified Khesari dhal

The detoxification process was carried out at the Department of Grain Science Technology, CFTRI, Mysore. It involved essentially a two-stage water leaching method as described elsewhere (CFTRI Annual report, 1994). Briefly, the method consisted of soaking of KD for 2 h in 3 volumes of water followed by boiling, draining and re-soaking in 4 volumes of fresh water at room temperature for 2 h. Finally, the water was drained and the grains were sun dried. The β-ODAP levels in KD and DKD samples used for the study were quantified by a previously described method (Rao, 1978). The mean β-ODAP levels were in the following range: KD, 1056 ±50 mg/100 g; DKD, 150±15 mg/100 g.

Nutrient Composition of Control, KD and DKD* diet

Components	Control	KD
Protein (g %)	17	24.0
Fat (g %)	3.5	3.61
Fiber (g %)	7	7
Ash (g %)	8	8
Carbohydrates (g %)	34.5	40.08
Caicium (mg %)	1.30	1.75
Phosphorus (mg %)	0.80	1.13
Sodium chloride(mg %)	0.70	0.70
Vit A (IU/100g)	9,00	9,00
Vit E (IU/100g)	6	6
Lysine (mg %)	0.85	0.85
Methionine (mg %)	0.35	0.35
Threonine (mg %)	0.64	0.64
Selenium (mg/100g)	0.02	1.7
Digestable energy(g %)	11.80	11.80
Moisture (g %)	14	14

KD diet also contained the following : Thiamine (mg%)-0.12; Riboflavin (mg%)-0.06; Niacin (mg%)-0.87and macronutrients such as Iron (mg%)-1.89 ; Magnesium(mg%)-2.8; Sodium(mg%)-12.

*The composition of DKD diet was essentially similar to that of KD diet except that the detoxified khesari dhal was used .

Phytochemicals

Centella asiatica (CA) powder and aqueous extract

Centella asiatica plant was collected during early summer from the state of Kerala and authenticated by Prof. C.M Joy, Department of Botany, Sacred Heart College Thevara, Mahatma Gandhi University, Kerala, India. Fresh leaves of CA were shade dried, powdered and used for dietary study.

For the preparation of aqueous extract, a method of Veerendra Kumar and Gupta, 2002 with minor modifications was followed. Briefly, to a known quantity of CA leaf powder (5g) was added 50 ml of double distilled water and kept in a boiling water bath for 5 hrs with occasional mixing. Later the extract was cooled, sieved through a 400 micron sieve, rotary evaporated at 40^0C, followed by lyophilization to get a greenish brown powder (1.5g). The total yield was 30% of the initial material.

The extract was standardized for asiaticoside and triterpene content using HPLC method (Inamdar et al., 1996). Chromatographic separation was performed with SS C_{18} column (Suppelco, 30x 0.39 cm) using water: acetonitrile as mobile phase, with UV detection at 220 nm and attenuation of 0.1 AUFS. 20 µl sample was injected and the flow rate was adjusted to 1ml/min. The retention time of asiaticoside was found to be 12.69 min. Further the antioxidant potential of the extract was evaluated using various chemical and biological systems.

Bacopa monnieri (BM) powder and ethanolic extract

Bacopa monnieri plant was collected during early summer from the state of Kerala and authenticated by Prof. C.M Joy, Department of Botany, Sacred Heart College Thevara, Mahatma Gandhi University, Kerala, India. Fresh BM plant was shade dried and the leaves were separated from the stem and powdered (using a mill) without the production of much heat and it was then sieved through a 400 micron sieve and used for dietary studies. The ethanolic extract was prepared according to the method of Jyoti and Sharma, 2006 with minor modifications. Powdered leaves (20g) were added to 150 ml of 90% ethanol, kept over a magnetic stirrer at 4-8^0C for 48 hrs. It is then filtered through a 400 micron sieve and stored at -20^0C. The filtrate was flash evaporated and the semi liquid pellet was lyophilized to get a greenish brown powder (4 g). The percent total yield of the ethanolic extract was 20%.

Five hundred milligrams of the BM ethanolic extract was dissolved in a medium of sodium sulphate buffer (0.05 M; pH 2.3) and acetonitrile (50: 50), sonicated for 10 min, diluted to 100 ml with the same buffered acetonitrile and filtered through 0.45mm membrane filter. The extract was standardized to bacosides content by HPLC. HPLC experiments were performed with C18, 5mm (4.6x250 mm) column. The mobile phase was a mixture of 0.05 M sodium sulphate buffer pH 2.3 and acetonitrile (68.5: 31.5, v/v) at flow rate of 1 ml/min and the column temperature was maintained at 30 °C. The detection wave length was set at 205 nm. The injection volume was 20µl. The total run time was 75 min. and standards were also run simultaneously (Murthy et al., 2006).

Tables showing the proximate composition of CA and BM leaves

Proximate composition of *Centella asiatica* leaves	
Component	**Analytical Results**
Moisture	80.4g/100g
Protein	17.25g/100g
Fat	0.75 g/100g
Crude Fiber	9.10g/100g
Ash	9.07g/100g
Calcium	146mg/100g
Phosphorus	104mg/100g
Iron	11.0mg/100g
Reducing sugars	4.55g/100g
Non-reducing sugars	1.97g/100g
Energy	40cal/100g

Proximate composition of *Bacopa monnieri* leaves	
Component	**Analytical Results**
Moisture	88.4g/100g
Protein	2.1g/100g
Fat	0.6g/100g
Carbohydrate	5.0g/100g
Crude Fiber	1.05g/100g
Ash	1.9g/100g
Calcium	202mg/100g
Phosphorus	16mg/100g
Iron	7.8mg/100g
Ascorbic acid	63mg/100g
Nicotinic acid	0.3mg/100g
Energy	38cal/100g

Animals and care

Experiments were carried out using prepubertal, 4 wk old (22-25 g) and adult male mice (30-35g), CFT-Swiss strain .They were drawn from the stock colony of the 'Institute animal house facility' and were housed in rectangular polypropylene cages (27" long, 20" wide and 14" high, n= 3/cage) kept on racks built of slotted angles and the cages were provided with dust free paddy husk as a bedding material. The animals were housed in a controlled atmosphere with a 12h light

/ dark cycle. They were acclimatized for one week prior to the start of the experiment and were maintained on a powdered diet and tap water *ad libitum*.

Ethical considerations: 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.

Autopsy and isolation of tissues

At the end of experimental period, animals were sacrificed by decapitation under mild anesthesia. Brain, liver, kidney, spleen, thymus and heart were isolated, rinsed in ice cold saline and processed immediately.

Preparation of samples

Tissue homogenates

Brain was excised and frozen immediately. The brain regions, cerebral cortex, cerebellum hippocampus and striatum were subsequently dissected over ice. The tissues were immediately homogenized in phosphate buffer (0.1 M, pH 7.4) using a glass-Teflon grinder and the homogenate was centrifuged at 1000xg for 10 min. and the supernatant was used for the quantification of malondialdehyde (MDA), reactive oxygen species (ROS), protein carbonyls, total thiols and non-protein thiols levels. For GSH, GSSG and enzyme assays, the homogenate was centrifuged at 10,000x g to obtain a cytosol fraction.

Mitochondrial Fractions (*Moreadith and Fiskum,1984; trounce et al., 1986)*

Mitochondria were prepared by differential centrifugation according to the method of Moreadith and Fiskum (1984) and Trounce et al., (1986) with minor modifications. Briefly, 10% homogenates of the brain regions were prepared in ice-cold Tris-sucrose buffer (Tris-2mM; Sucrose-0.25 M, pH 7.4) using a glass–teflon grinder at 4^0 C. The homogenates were centrifuged at 1000xg for 10 min at 4^0 C to obtain the nuclear pellet. Mitochondria were obtained by centrifuging the post-nuclear supernatant at 10,000xg for 20 min at 4^0 C. The pellet was washed three times in Mannitol-Sucrose-HEPES buffer (Mannitol-200mM; Sucrose-70mM; EDTA-0.1mM; HEPES-10mM, pH 7.4), resuspended in the buffer and stored at -80^0 C until use. Protein was estimated according to the method of Lowry et al., 1951.

Microsomal Fractions *(Shimoji and Aniya, 1994)*

Microsomes were prepared from brain regions according to the modified method of Shimoji and Aniya, 1994). 10% homogenate of the tissues were made in ice-cold Tris-sucrose buffer (Tris-2mM; Sucrose-0.25 M, pH 7.4) using a glass–teflon grinder at 4^0 C. The homogenates were centrifuged at 1000xg for 10 min at 4^0 C to obtain the nuclear pellet. The pellet was discarded and the supernatant was centrifuged at 100,000xg for 45 min. at 4^0 C. The pellet so obtained is resuspended in Mannitol-Sucrose-HEPES buffer (Mannitol-200mM; Sucrose-70mM; EDTA-0.1mM; HEPES-10mM, pH 7.4) and stored at -80^0 C till use.

Preparation of synaptosomes (Gil et al ., 2001)

Crude synaptosomal fraction was prepared as per the method of Gil et al., 2001.Whole brain or cerebral cortex was homogenized in 40 volume (w/v) phosphate buffer, pH 7.4 supplemented with 0.32M sucrose. The homogenization was performed with twelve strokes (900 rev/min) of a glass–teflon grinder at 4^0 C. The homogenate is centrifuged at 1000xg for 10min a t4^0 C. The supernatant obtained was subjected to further centrifugation at 12,000Xg for 20 minutes. The crude synaptosomes was resuspended in 10ml of HEPES-Sodium buffer (NaCl-140mM; KCl-5mM; $NaHCO_3$-5mM; $MgCl_2$-1mM; HEPES/NaOH-20mM and Glucose-10mM, pH 7.4).

Preparation of tissue slices/punches

Brain was dissected out and the parts were separated over ice. Equal weight punches were made using a hollow glass cylinder with 2mm diameter. The punches were stored in Krebs ringer buffer solution containing NaCl-120mM, KCl-4.8mM, $NaHCO_3$-25.2mM, KH_2PO_4 -1.2mM, $CaCl_2$-1.3 mM, $MgSO_4$ -1.2mM and glucose-11mM/L, pH 7.2 and used within 3 hrs of separation.

SECTION B

ASSAY METHODS

Determination of Protein (Lowry et al., 1951)

Protein concentrations in tissue homogenates, mitochondria, microsomes, cytosol and synaptosomes were determined according to the method of Lowry *et al* (1951), using bovine serum albumin as the standard. Briefly to 990 μl of distilled water, 10 μl of sample and 1 ml of Lowry's reagent (2% Na_2CO3 in 0.1N NaOH, 1% Cu_2SO4, 2% Na^+K^+ tartarate) was added and incubated for

another 5 minutes at room temperature. Further 100ul of Folin and Ceocalteus reagent was then added and incubated at for 30 minutes and absorbance was read at 750 nm against a reagent blank.

Lipid peroxidation (LPO) (Ohkawa et al., 1979).

Induction of oxidative damage was ascertained by measuring the extent of LPO in brain (cortex, cerebellum, hippocampus and striatum) homogenates. The extent of LPO was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Briefly, the reaction mixture contained 0.2 ml of brain regions homogenate/ mitochondria (1mg protein), 1.5 ml of acetic acid (pH 3.5, 20%), 1.5 ml of 0.8 % thiobarbituric acid (0.8% w/v) and 0.2 ml SDS (8% w/v). The mixture was heated to boiling for 45 min and TBARS adducts were extracted into 3 ml of 1-butanol and absorbance was measured in a UV-Visible spectrophotometer at 532 nm and expressed as malondialdehyde (MDA) equivalents using 1, 1, 3, 3-tetramethoxypropane as the standard.

Reactive oxygen species generation (Driver et al., 2000)

ROS generation in brain regions 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 (Driver et al., 2000). Briefly, the homogenate was diluted 1:20 times with ice-cold Locke's buffer to obtain a concentration of 5 mg tissue/ml. The reaction mixture (1ml) containing Locke's buffer (pH 7.4), 0.2 ml homogenate or mitochondria (0.5mg protein) and 10 μl of DCFH-DA (5 μM) was incubated for 15 min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group to be cleaved by esterases. After 30 min of further incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured in a spectrofluorimeter with excitation wave length of 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DA in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS formation was quantified from a DCF- standard curve and data are expressed as p mol DCF formed /min /mg protein.

Measurement of hydroperoxide levels (Wolff, 1994).

Water soluble hydroperoxides were measured according to the original method using FOX 1 recipe. An aliquot of tissue homogenate or mitochondria (100 μg protein) was added to 1ml FOX

reagent (100μM xylenol orange; 250μM ammonium ferrous sulphate; 100 μM sorbitol; 25mM H_2SO_4/L) and incubated for 30 min at room temperature. The mixture was centrifuged at 600xg and the supernatant was read at 560nm in a spectrophotometer. The concentration of hydroperoxides were calculated using the MEC of 1.5 X 10^{-4} mM and expressed as μmoles hydroperoxide /mg protein (Wolff, 1994).

Determination of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Mokrasch and Teschke, 1984)

GSH was measured according to the fluorimetric method of Mokrasch and Teschke, 1984. Briefly, 100 μl of 10% homogenates (prepared in phosphate buffer pH 7.4) was added to 2 ml formic acid (0.1M) and centrifuged at 10,000xg for 20 minutes. The supernatant (100 μl) was mixed with 0.1ml buffered formaldehyde (1:4 (v/v), 37% formalin: 0.1M Na_2HPO_4). After 5 min, 1.0 ml of sodium phosphate buffer (0.1M, EDTA 5mM, pH 8.0) was added to each tube followed by 0.10 ml of *o*-phthalaldehyde. After 45 minutes at ambient temperature, the fluorescence was measured in a spectrofluorimeter with excitation wavelength at 345nm and emission at 425 nm. Concentration of GSH was calculated from the standard curve and the values were expressed as μg GSH /mg protein.

For the estimation of GSSG, to the supernatant, 0.1 ml buffered formaldehyde (1:4 (v/v) 37% formalin: 0.1M Na_2HPO_4). After 5 min, 1.0 ml, NaOH (0.1M) was added to each tube followed by 0.10 ml of *o*-pthalaldehyde. After 45 minutes at ambient temperature, the fluorescence was measured in a spectrofluorimeter with excitation wavelength of 345nm and emission 425 nm. Concentration of GSSG was calculated from standard curve and values were expressed as μg GSSG/mg protein.

Determination of reduced glutathione (GSH) (Hissin and Hilf, 1976)

Measurement of GSH was done according to the fluorimetric method of Hissin and Hilf (1976). Briefly, tissue was homogenized (5%w/v) in 25% HPO_3 and centrifuged at 10,000 g for 15 min. An aliquot of the supernatant was added to the reaction mixture containing phosphate buffer, (0.1M, pH 8.0, 5 mM EDTA) and 100μl of O-phthalaldehyde (50μg/ml) and incubated at room temperature for 15 min. The fluorescence was measured at an excitation wavelength 350 nm and emission 420 nm. Concentration of GSH was calculated from the standard curve and expressed as μg GSH/mg tissue.

Total thiols (Ellman, 1959)

Estimation of total thiols and non-protein thiols was done according to the method of Ellman, (1959).To estimate total thiols, 125 µl of the homogenate was added to 375 µl of tris buffer (0.2M, pH.8.2), 25 µl of DTNB (10mM in absolute methanol) and 1.975ml of methanol and allowed to stand for 30 min at room temperature with occasional shaking. Following centrifugation at 3000xg for 15 min, the supernatant was read at 412 nm against distilled water blank and calculated using MEC 13.6 $mM^{-1}cm^{-1}$ and expressed as η mol oxidized DTNB formed/mg protein.

Non protein thiols (Ellman, 1959)

For non-protein thiols, 125 µl of homogenate (5% in 20 mM EDTA) was added to 1ml of distilled water and 250 µl 5% TCA. The mixture was shaken for 10 min and centrifuged at 3000xg for 15 min. 0.5 ml of the supernatant was added to 1 ml of tris buffer (0.4M, pH.8.9) and 25 µl of DTNB (10mM in absolute methanol) was added and held at room temperature for 15 min followed by centrifugation at 3000xg for 5min.The supernatant obtained was read at 412nm against a distilled water blank and calculated using MEC 13.6$mM^{-1}cm^{-1}$ and data expressed as η mol oxidized DTNB formed/mg protein.

Ascorbic acid (Omaya et al., 1979)

Vitamin C/Ascorbic acid levels in brain regions were analyzed by 2,4-dinitrophenyl-hydrazine method. 1ml tissue homogenate (25%, homogenized in phosphate buffer (0.1M, pH 7.4) was mixed with an equal amount of trichloroacetic acid (0.62mol/L) and then centrifuged for 10 min at 3500X g. Supernatant (0.25ml) and DTC solution (3g 2,4-dinitrophenyl-hydrazine in 4.5 mol/L H_2SO_4, 0.4g thio urea and 0.05g $CuSO_4$ $5H_2O$) were mixed and incubated in a water bath at 37ºC for 3hrs. After incubation period, 0.75 ml ice-cold H_2SO_4 (8.17mol/L) was added and allowed to stand at room temperature for 30 min. The absorbance was measured at 520 nm. The concentration of ascorbic acid was calculated using a reference standard and expressed as ηg/mg tissue.

Iron estimation (Peters et al., 1956)

Iron content tissues was determined by bathophenanthroline method with minor modifications and expressed as OD/mg protein. The bathophenanthroline method is a sensitive colorimetric method for determining both the intrinsic and added iron. The bathophenanthroline method measures only the nonheme iron as compared to atomic absorption spectroscopy which

determines both heme and nonheme iron. To 1ml homogenate (10% in PB, 7.4), 500ul of protein precipitating reagent (100g TCA, 30 ml of thioglycolic acid and 2ml HCl/L) was added, centrifuged at 7500Xg for 10 min. To the supernatant 1 mL of bathophenanthroline color reagent [62.5 mg bathophenanthroline-disulfonic acid and 0.25 mL thioglycolic acid in distilled water and diluting to 25 ml], saturated sodium acetate (4.5 M) and distilled water (1:20:20 by volume] was added and kept at room temperature for 10 minutes. Absorbance was determined spectrophotometrically at 535 nm, and results are expressed as OD/mg protein.

Determination of Protein carbonyls (Levine et al., 1990)

Protein carbonyl content was determined according to the method of Levine *et al.,* (1990). Briefly a 10% homogenate in 20mM Tris-HCl- 0.14 M NaCl (pH 7.4) was made and centrifuged at 10,000x g for 10 min. at 4^0C.To 100 μl of the supernatant, 100 μl of 20% TCA was added and centrifuged at 10,000xg for 10 minutes at 4^0C .The supernatant was discarded and the pellet was re-suspended in 1ml of DNPH (10mM in 2N HCl) and kept at dark for 1 hour with occasional mixing. 500 μl of 20% TCA was added to precipitate protein and the pellet was washed in 1ml acetone and dissolved in 1ml of 2% SDS prepared in 20mM tris HCl. The absorbance was read at 360nm and the results were expressed as ηmoles carbonyls/mg protein using MEC-22.0$mM^{-1}cm^{-1}$.

Antioxidant enzymes

Brain regions (cortex, cerebellum, hippocampus and striatum) were homogenized in phosphate buffer (50mM, pH 7.4) and sonicated at 4°C. The activities of enzymes *viz*., catalase, glutathione–S-transferase, glutathione peroxidase, glutathione reductase and Superoxide dismutase were measured in cytosolic fractions which were obtained after centrifugation of the tissue homogenate at 10,000x g.

Catalase (Aebi, 1984)

Catalase activity was assayed by the method of Aebi, (1984).The enzyme activity was expressed as μmol H_2O_2 consumed /min/mg protein (MEC = 43.6 mM^{-1} cm^{-1}). Briefly, the 1ml of the reaction mixture containing 50 μl sample, 900 μl phosphate buffer (0.1M, pH 7.0) and 50 μL of H_2O_2 (8.8mM).The decrease in absorbance (at 240nm) was followed for 5 min at room temperature using a UV-Visible spectrophotometrically.

Glutathione peroxidase (Flohe and Gunzler, 1984)

The activity of glutathione peroxidase was determined using t-butyl hydroperoxide as the substrate according to the method of Flohe and Gunzler, (1984). Briefly, the reaction mixture containing 50 µl sample, phosphate buffer (0.1M containing 0.5mM EDTA), 100 µl Glutathione reductase (0.24 U) ,100 µl GSH (1mM),100 µl NADPH (0.15mM) was incubated at 37ºC for 3 min and the reaction was initiated by the addition of 100 µl tbHP (0.12mM). The change in absorbance at 340 nm was followed for 5 min spectrophotometrically. The activity was expressed as η moles of NADPH oxidized /min/mg protein (MEC =6.22$mM^{-1}cm^{-1}$)

Glutathione –S-transferase (Guthenberg et al., 1985)

Glutathione –S-transferase was assayed by measuring the rate of enzyme catalyzed conjugation of GSH with 1-chloro 2-4-dinitro benzene (CDNB) according to the method of (Guthenberg *et al.,* 1985). Briefly, to 1 ml reaction mixture containing phosphate buffer (0.1M, pH 6.5 containing 0.5mM EDTA), CDNB (1.5mM) and 50 µl GSH (1mM), 50 µl sample was added and the increase in absorbance at 340nm was monitored for 5 min. The enzyme activity was expressed as η moles of S- 2, 4, dinitrophenyl glutathione formed /min/mg protein (MEC- 9.6$mM^{-1}cm^{-1}$).

Glutathione Reductase *(Carlberg and Mannervick,1985)*

The enzyme activity was measured by NADPH coupled assay.1ml of reaction volume consisted of 900 µl of phosphate buffer (0.2M, pH 7.0, 2mM EDTA), enzyme sample (~150 µg protein), 50 ul of NADPH (2mM) and 50 ul of 20mM oxidized glutathione. The rate of decrease in absorbance was monitored at 340 nm at 37ºC.The enzyme activity was expressed as η mol of NADPH oxidized/min/mg protein (MEC =6.22$mM^{-1}cm^{-1}$).

Superoxide dismutase *(McCord and Fridovich, 1969).*

Superoxide dismutase activity was measured by monitoring the inhibition of ferricytochrome–c reduction using xanthine-xanthine oxidase as the source of $O_2^{\cdot-}$. To a semi micro cuvette were added 2.9 ml of solution A (5 ul xanthine in 0.01N NaOH + 2ul Cytochrome C+ 50 mM phosphate buffer with 0.1mM EDTA) and 0.1 ml solution B (an equal volume of xanthine oxidase in 0.1mM EDTA).The reaction mixture with out the enzyme served as blank. After adding various volumes of enzyme sample, inhibition of cytochrome C reduction was monitored for 5 min

at 560 nm. One unit was defined as the amount of enzyme that decreases the initial rate of cytochrome –C reduction to 50% of maximal value for the particular sample being analyzed.

Thioredoxin reductase (TRR) (Luthman and Holmgren, 1982)

Thioredoxin reductase activity in mitochondrial preparations were measured according to the method of Luthman and Holmgren, 1982. To 100mM potassium phosphate buffer (pH 7.0 containing 10mM EDTA, 50 µl of 0.2mM NADPH,50 µl of 0.2 mg/ml BSA, 50 µl of 1% ethanol), 50 µl of 5mM DTNB and 50µl of sample was added. The absorbance at 412 nm was followed for 3 minutes. The activity was calculated as micromoles of NADPH oxidized/min/mg protein according to ΔA_{412} x 0.5/13.6x 2, since one mol of NADPH yields 2 mol of thionitrobenzoate.

Cholinergic enzymes

Acetylcholinesterase *(AChE) activity (Ellmann et al., 1961)*

AChE activity was determined according to the method of Ellmann *et al*., (1961). To the reaction mixture containing 2.85 ml phosphate buffer (0.1 M, pH 8.0), 50 µl of DTNB (10 mM), 50 µl sample and 20 µl acetylthiocholine iodide (150mM) were added and the change in absorbance was monitored at 412 nm for 5 min in a spectrophotometer. The enzyme activity was expressed as ηmoles of substrate hydrolyzed /min/mg protein (MEC=$1.36X10^{-6}$ $mM^{-1}cm^{-1}$)

Butyrylcholinesterase *(BChE) activity (Ellmann et al., 1961).*

BuChE activity was determined according to the method of Ellmann *et al*., (1961). To the reaction mixture containing 2.85 ml phosphate buffer (0.1 M, pH 8.0), 50 µl of DTNB (10 mM), 50 µl sample and 20 µl butyrylthiocholine iodide (78mM) were added and the change in absorbance was monitored at 412 nm for 5 min in a spectrophotometer. The enzyme activity was expressed as ηmoles of substrate hydrolyzed /min/mg protein. (MEC=$1.36X10^{-6}$ $mM^{-1}cm^{-1}$).

Mitochondrial electron transport chain enzymes

NADH:ubiquinone oxidoreductase (complex-I) (Ragan et al., 1987).

Complex-I activity was assayed according to Shultz et al. (1995) with minor modifications following the decrease in the absorbance due to oxidation of NADH at 340 nm. The reaction mixture contained 10 mM potassium phosphate pH 7.2; 5 mM sodium azide; 50 mM coenzymeQ_{10} and 70–120 µg mitochondrial protein in a final reaction volume of 1 ml. After preincubation for 3

min at 32^0C, the reaction was initiated by the addition of NADH (120 mM) and the rate of decrease in the absorbance was monitored at 340 nm for 2 min. The enzyme activity was expressed as η moles of NADH oxidized/min/mg protein (MEC= 6.2/ $mM^{-1}cm^{-1}mM$)

NADH:cytochrome c reductase (complex-I - III) (Navarro et a., 2004)

100 µg of thawed brain mitochondria was added to 1 ml of 100mM potassium phosphate buffer, pH 7.4 containing 0.2mM NADH and 1mM KCN. The reaction was started by adding 0.1mM Cytochrome C and the absorbance at 550 nm was followed for 3 minutes. The activity of enzyme was expressed as η mol/min/mg protein (MEC=19.6mM/cm) (Navarro et al, 2004).

Succinate-ubiquinone oxidoreductase (Complex II) (Trounce et al.,1996).

Activity was measured by following the decrease in absorbance due to the coupled reduction of 2,6-dichlorophenolindophenol (DCPIP) at 610 nm. The reaction mixture consisted of 10 mM potassium phosphate (pH 7.8), 10 mM succinate, 2 mM EDTA, 0.1% BSA, 3 µM rotenone, 1 µM antimycin A, 0.3 mM KCN, 80 µM DCPIP and ~ 20 µg of mitochondrial protein. The reaction was initiated by the addition of CoQ10 (final concentration 50 µM) and was monitored for 2 min.

Succinate –cytochrome C reductase (Complex II - III) (Navarro et al., 2004).

To 1 ml of 100mM potassium phosphate buffer, pH 7.4 containing 20mM sodium succinate and 1mM KCN, 100 µg of mitochondrial protein was added. The reaction was started by adding 0.1mM Cytochrome C and the absorbance at 550nm was followed for 3 minutes. The activity of enzyme was expressed as η mol/min/mg protein (MEC=19.6$mM^{-1}cm^{-1}$).

Cytochrome c oxidase (Complex IV) (Rustin et al., 1994).

Activity was measured by following the decrease in absorbance due to the oxidation of reduced cytochrome c at 550 nm. The reaction mixture contained 40 mM potassium phosphate (pH 7.0), 0.1% fatty acid-free BSA, 2.5 mM lauryl maltoside and 20 µg of mitochondrial protein. The reaction was initiated with the addition of reduced cytochrome c (40 µM) (Rustin et al., 1994).

Mitochondrial swelling measurement (Rigobello et al., 2005)

Mitochondrial swelling, which indicates the occurrence of membrane permeability transition, was followed spectrophotometrically by the decrease in absorbance at 540 nm. Brain

mitochondria (0.25mg/ml) were incubated at 25 ^{0}C in medium containing 0.22M mannitol, 71mM sucrose, 5mM HEPES-Tris (pH 7.4), 5mM succinate, 5 µM rotenone and 3 µM oligomycin.

Mitochondrial function enzymes

Citrate synthase activity (CS) (Srere, 1969).

Citrate synthase activity was determined as the rate of color change of 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) at 412 nm. The reaction mixture contained 100 mM Tris–HCl (pH 8.1), 0.2 mM DTNB, 0.1% Triton X-100, 0.1 mM acetyl-CoA and 20 µg of mitochondrial protein. The reaction was initiated by the addition of 20 µl of 10 mM oxaloacetate (final concentration 0.2 mM) (Srere, 1969). The results are expressed as n mol DTNB /min/mg protein (MEC=13.6 $mM^{-1}cm^{-1}$).

*Isocitrate dehydrogenase (ICDH) (*Bai et al., 1999)

Isocitrate dehydrogenase activity was done according to the method of Bai et al., 1999. The samples for ICDH was homogenized in Tris (1mM,pH 7.0) buffer with CTAB (0.1%) and later centrifuged at 10,000xg at 4^{o}C for 30 minutes. To 1ml cuvette 835 µl of triethanolamine buffer (100mM, pH 7 .3), 15 µl $MnCl_2$, 25 µl sample (150 µg protein) and 100 µl DL-isocitrate substrate (67mM) was added. The change in absorbance at 340 nm was followed for 5 minutes and calculated as n mol NADP reduced/min/mg protein (MEC-6.22 $mM^{-1}cm^{-1}$).

Succinate dehydrogenase (SDH), (Pennington, 1961)

Succinate dehydrogenase (SDH) activity was determined using a modification of the method of Pennington, 1961. A volume of 50 µL of mitochondria was added to a 2-mL reaction vial with 300 µL of a 0.01 mol/L sodium succinate solution in a 0.05 mol/L phosphate buffer, pH 7.5. After 15 min of incubation at 37°C, 100 µL of 2.5 g/L solution of *p*-iodonitrotetrazolium violet was added to the reaction vial. The reaction vials were kept in a water bath at 37°C for an additional 10 min. The reaction was stopped by the addition of 1 ml of a 5:5:1 (v:v:w) solution of ethyl acetate:ethanol:trichloroacetic acid. Absorbance was measured at 490 nm. The values are expressed as OD/mg protein.

Fumarase (Hill and Bradshaw, 1969)

Fumarase activity was measured by following the change in fumarate concentration spectrophotometrically at 250 nm in 200 µl reaction mixture (pH 7.3) containing malate (50 mM),

sodium phosphate (50mM) buffer, and 0.1% bovine serum albumin (BSA). The increase in absorbance was measured for 5 min and activity expressed as ηmoles/min/mg protein.

Malic dehydrogenase (MDH) *(Kitto,1969)*

Malate dehydrogenase was done according to the method of Kitto,1969. To 920 µl of potassium phosphate buffer (0.1M, pH 7.5) ,0.03 ml NADH(14.3mM) and 0.05 ml oxaloacetate (20mM) was added and the decrease in absorbance at 340nm was followed for 3 minutes in a spectrophotometer. Amount of enzyme used is adjusted to give a decrease in OD ~0.04/min.The enzyme activity is expressed as ηmoles NADH /min/mg protein (MEC-6.22 x 10^6 cm^2mol^{-1}).

Lactate dehydrogenase (LDH) *(Kornberg, 1974)*

Lactate dehydrogenase was measured as per the method of Kornberg, 1974. Tissue was homogenized in 10%w/v, Tris-HCl buffer (82.4 mM,pH 7.2 containing 210mM NaCl) and centrifuged at 10,000xg for 10 minutes at 4^0C. To 10ul of the supernatant 40 ul distilled water was added and 0.8 ml NADH (0.25mM , in Tris –HCl, pH 7.2)and 0.15 ml sodium pyruvate (10.66mM) was added. The absorbance was followed for 5 minutes at 340nm.Results are expressed as n mol NADH oxidized/min/mg protein.

Na^+,K^+ ATP ase (*Borges et al., 2005)*

Mitochondrial protein equal to 100 µg protein was added to a reaction mixture containing 3 mM $MgCl_2$; 125 mM NaCl; 20 mM KCl; 50 mM Tris –HCl, PH 7.4 in a final volume of 500 µl. The reaction was initiated by adding ATP (final concentration-3.0 mM). Controls were carried out under same conditions with the addition of 0.1 mM Ouabain. The $Na^+ - K^+$ ATP-ase activity was calculated by the difference between the two assays (with and without ouabain). The released inorganic phosphate was measured by the method of Fiske and Subbarrow (1925) (Borges et al., 2005).

Dopamine levels in striatum-HPLC method *(Dalpiaz et al., 2007)*

Dopamine levels were measured in striatal region of brain of prepubertal mice injected (i.p.) rotenone for a period of 7 days using HPLC-UVD-280 nm (C-18 SS column ;150mm X 4.6mm). The mobile phase as 0.2% trifluoroacetic acid and methanol (70/30, v/v) with a flow rate of 0.8 ml/min. The tissue was homogenized in phosphate buffered saline, centrifuged at 7500x g for 10minutes and 20 µl of the sample was injected (Dalpiaz et al., 2007).The dopamine levels are expressed as ηmoles /mg protein.

Histopathological examination

Brains were kept in 10% formalin solution for 24 hrs using Hartz technique (1974). The fixed tissues were washed with double distilled water, dehydrated in a series of alcohol, cleared in xylene and then embedded in paraffin blocks and stained by haematoxylin and eosin (Carelton et al., 1967). The tissue sections were then examined by light microscope. Histopathological examinations of striatal sections were done blind to the group identity of the animal.

Determination of antioxidant activity

DPPH radical scavenging activity (Cotelle et al., 1996)

The hydrogen atoms or electrons donation ability of the corresponding extract was measured from the bleaching of purple colored methanolic solution of DPPH. This spectrophotometric assay uses stable radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH) as a reagent . 50 µl of various concentrations of the extracts were added to 3 mL of (100µM in MeOH) of DPPH solution. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition of free radical DPPH in percent was calculated according the formula:

Inhibition % = $(A_{blank} \_ A_{sample}) / A_{blank}$ X 100

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound)and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC50) was calculated form the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate. The synthetic antioxidant buthylated hydroxy toluene (BHT) was included in experiments as a positive control (Cotelle et al., 1996).

Determination of Reducing power (Hsieh and Yan, 2000)

Reducing power of test compounds were determined according to the method of Hsieh and Yan, 2000, with some minor modifications. Briefly samples were mixed with potassium ferricyanide ($K_3Fe(CN)_6$, 2.5 ml,1%) in sodium phosphate buffer (2.5ml,0.2mol/L, pH6.6) and the mixture was incubated at 50^oC for 20 minutes. At the end of the incubation period, trichloroacetic acid (2.5 ml,10%) was added to the mixture and then centrifuged at 3000xg for 10 min. 2.5 ml of the supernatant was taken and mixed with aqueous $FeCl_3$ (0.5 ml, 0.1%).The absorbance of the final

mixture was measured at 700nm.Increased absorbance of the reaction mixture indicated a higher reducing power of the test compound.

Determination of Total polyphenol content (Singleton and Rossi, 1965)

Phenolic compounds were assayed, according to the Folin–Ciocalteu method (Singleton and Rossi, 1965). Samples (150µl, three replicates) were added to test tubes containing 750µL of Folin-Ciocalteu's reagent and 600 µL of sodium carbonate (7.5%). The reagents were mixed and incubated at 50°C for 10 min. Absorbance was measured at 760 nm in a spectrophotometer. The total phenolic content was expressed as quercetin equivalents (QE).

Super oxide scavenging activity (Robak et al., 1998)

Superoxide scavenging activity of test compounds were measures according to the method of Robak et al., 1998 with minor modifications .All the solutions were prepared n 100mM phosphate buffer, pH 7.4.1 ml of Nitroblue tetrazolium chloride (156µM), 1 ml of NADH (468µM) and 3 ml of test solution to give final concentrations of 1 to 100µg/ml were mixed. The reaction was started by adding 100µl of phenazine methosulphate (PMS-60µM) and the mixture wasthen incubated at 25°C for 5 minutes followed by measurement of absorbance at 560 nm, The percentage inhibition was calculated from the formula given below

Percentage inhibition=

$$\frac{\text{(Absorbance of control-Absorbance of Test)}}{\text{Absorbance of control}} \times 100$$

IC_{50} (concentrations, required to inhibit NBT reduction by 50%) values were calculated from dose-inhibition curves.

Inhibition of hydroxyl radical (Chung et al., 1997).

Hydroxyl radical scavenging activity was determined according to the method of Chung et al., 1997). The Fenton reaction mixture consisted of 200 µl of $FeSO_4$ $7H_2O$ (10 mM), EDTA (10 mM) and 2-deoxyribose (10 mM). Then, 200µl of the extract and 1 ml of 0.1M phosphate buffer (pH 7.4) were mixed together and made the total volume of 1.8 ml. Thereafter, 200 µl of 10mM H_2O_2 was added and the reaction mixture was incubated at 37°C for 4 h. After incubation, 1 ml of 2.8% TCA and 1 ml of 1% TBA were mixed and placed in a boiling water bath for 10 min. After

cooling, the mixture was centrifuged (5 min, 395xg) and the absorbance was measured at 532 nm with a UV–visible spectrophotometer. Percentage inhibition was calculated using the formula

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of Test})}{\text{Absorbance of control}} \times 100$$

Measurement of chelating activity on ferrous ions (Carter, 1971)

The chelating activity of BM extract and CA ex on Fe^{2+} were estimated based on the decrease in the maximal absorbance of the iron (Fe2+)-Ferrozine complex according to the method of Carter P, 1971. Briefly ,0.2 ml test compounds dissolved in deionized water were incubated with 0.5ml of $FeCl_2.4H_2O$(2mmol/L).The reaction was initiated by the addition of 0.2ml of ferrozine (5 mmol/L) and then the total reaction volume was made upto to 0.8 ml with methanol. After the mixture has reached equilibrium (10 minutes), the absorbance was read at 562 nm. The negative control was prepared with out the test compounds. EDTA served as the positive control. Chelating activity of test compound on Fe^{2+} was calculated as follows

$$\text{Chelating activity (\%)} = [A_{562(\text{control})} - A_{562(\text{sample})}] / A_{562(\text{control})} \times 100$$

Determination of oxidative damage to Deoxyribose (Halliwell et al., 1987)

The deoxyribose assay was carried out essentially, as described by Halliwell et al., 1987. The reaction mixture (3.5ml), which contained test compounds (0-1mg), deoxyribose (6mmol/L), H_2O_2 (3mmol/L), KH_2PO_4-K_2HPO_4 buffer (20mmol/L,pH 7.4), $FeCl_3$ (400 µmol/L),EDTA (400µmol/L) and ascorbic acid (400µmol/L) was incubated at 37^0C for 1 hour. To examine the extent of deoxyribose degradation, TBA (1ml, 1%) and Trichloroacetic acid (1ml, 2.8%) were added to the mixture which was then heated in a water bath at 100^oC for 20 minutes. The absorbance of the final mixture was read at 532 nm against a blank control, which contained all reagents except deoxyribose. The negative controls didn't have the test compounds. The results are expressed as percent inhibition.

SECTION C

Cell culture methodology

N27 (rat dopaminergic cell line 1RB3AN27 (N27) cells were derived from embryonic rat mesencephalic neurons via SV40 large T antigen immortalization (Bharath and Andersen, 2005). Cells were subcultured once a week via trypsin treatment. N27 cells possess all the physiological and biochemical properties of dopaminergic neurons and represent those cells that are lost during PD. N27 cells have been successfully tested for cell-transplantation in the rodent model of PD and also have been tested in mitochondrial experiments associated with PD. Intact Complex I has been immunopurified from N27 mitochondria followed by successful mass spectrometric identification of all subunits suggesting the presence of healthy mitochondria. Mitochondria prepared from N27 cells did not possess any detectable ultrastructural damage as examined by electron microscopy .Based on these features, N27 cell line has been considered as a superior cell model of PD compared with other dopaminergic cells such as rat adrenal pheochromocytoma (PC12) cell line. As per the culture conditions recommended by the authors of the original paper (Prasad et al., 1994) and others (Clarkson et al., 1998), we have grown N27 cultures in RPMI medium 1640 containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) and maintained them at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were subcultured once a week *via* trypsin treatment.

Preparation of homogenates

The cells were harvested by trypsin treatment and homogenates were prepared using a glass homogenizer in phosphate buffered saline (pH 7.4). Later the homogenates were sonicated for two cycles of 5 seconds each, centrifuged at 10,000xg for 10 minutes and the supernatant was used for the GSH, GSSG, ROS and HP assays as described in earlier sections of materials and methods.

Preparation of Mitochondria (Trounce et al., 1996)

The cells were harvested by trypsin treatment and prepared homogenates using a glass homogenizer in ice-cold isolation buffer [320 m*M* sucrose, 5 m*M* TES [tris (hydroxymethyl) methylaminoethanesulfonic acid, 1 mM EGTA, pH 7.2]. The homogenate was centrifuged at 1,000x *g* for 5 min at 4°C, and then the supernatant was centrifuged at 8,500 x*g* for 10 min at 4°C. The

mitochondrial pellet was re-suspended in reconstitution buffer (250 m*M* sucrose, 10 m*M* TES, pH 7.2) and stored as aliquots at –80°C (Trounce et al., 1996).

MTT assay for viability of cells *(Vali et al., 2007.*

For cell survival studies, cells were seeded in 96-wellplates at a density of 5,000 cells per well. Following treatment at various concentrations of BM extract, Rotenone,3-NPA, BM extract +Rotenone and BM extract +3-NPA, viable cells were measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). In brief, 20 µl of 5 mg/ml MTT was added to the cells and incubated at 37°C for 2 h. The medium was discarded, the dark blue formazan crystalline product was dissolved in dimethyl sulfoxide, and the absorbance was analyzed in a Spectramax plate reader (Molecular Devices) at 570 nm (Vali et al., 2007).

LDH assay in medium *(Wroblewski and LaDue, 1955)*

LDH was measured at room temperature using the method of Wroblewski and LaDue (1955). Samples of media (0.2 ml) were added to 2.3 µM of sodium pyruvate in 0.1 M KH_2PO_4 buffer (pH 7.5 at 25°C) containing 0.2 mg of NADH (total volume, 3 ml). The absorbance of the reaction mixture at 340 nm, was followed for a period of 3 minutes using a spectrophotometer. The ΔA/min was calculated and the results are expressed as percent increase or decrease.

Quantification of proteins *(Bradford, 1976)*

The Bradford method depends on quantitation of the binding of a dye, Coomassie brilliant blue, to an unknown protein and comparing this binding to that of different amounts of a standard protein, bovine serum albumin. It is designed to quantify 1-10ug protein. Into 8 microcentrifuge tubes aliquot duplicate amounts of 0.5mg/ml BSA and with NaCl bring the volume in each to 100µl. Into 2 microcentrifuge tubes,aliquot 100µl of 0.15NaCl as blank. 1 ml Coomassie brilliant blue solution (In a 1L volumetric flask, dissolve 100mg coomassie brilliant blue G-250 in 50 ml of 95% ethanol. Add 100ml of 85% phosphoric acid. Bring to volume with water. Filter through wattmann No.1 filter paper, store at 4ºC) was added and vortexed. Allow to stand for 2 minutes at room temperature. Determine the absorbance at 595nm using a 1cm path length cuvette (1ml) and make a standard curve plotting absorbance at 595 nm versus protein concentration. Determine the absorbance of the unknown and use the standard curve to determine the concentration of the unknown.

SDS-PAGE for total cell lysates and mitochondrial proteins

Mitochondrial protein extracts (35 µg/lane) were run on 12% SDS PAGE at 100 V for ~2.5 hours. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma). The cells were harvested by trypsin treatment and prepared homogenates using a glass homogenizer in phosphate buffered saline (pH 7.4).Later the homogenates were sonicated for two cycles of 5 seconds each, centrifuged at 800Xg for 5minutes and the supernatant was used to obtain the protein profile in N27 cells on treatment with BME, 3-NPA and rotenone or their modulation. The samples were mixed with equal volume of dye (50mM Tris-Cl, pH, 6.8; 100mM Dithiothreitol (DTT); 2% SDS, 0.1% bromophenol blue, 10% glycerol) and kept for boiling for 10 min at 90ºC. After boiling spin at 800Xg for 1min and load equal amounts of protein and subject to SDS –PAGE (Laemmli, 1970) using 12% acrylamide gels. A constant current of 50mA was employed. After the run the proteins were stained with 0.25% Coomassie brilliant blue for 1 hr and later transfer to destaining solution (Methanol: acetic acid, 6:4v/v).

Comet assay (Singh et al., 1988)

Comet assay/single cell gel electrophoresis is a simple, rapid and sensitive technique for detection of DNA single strand breaks and alkali labile damage at the level of individual cells. For this assay N27 cell lines exposed to various neurotoxicants and BME extracts were trypsinized, washed with PBS and counted using a haemocytometer.

Preparation of slides and cell lysis: Three layers of agarose were prepared on the frosted slides. For the first layer, 150µl of 0.75% normal melting agarose (NMPA) in PBS (prepared at 40ºC) was dispensed into fully frosted slides and covered with a cover slip (24X 50mm) to solidify agarose and the slides were kept at 4ºC for 10 min and later cover slip were removed. An aliquot of cells were suspended in 75µl of 0.5% low melting agarose (LMPA) in PBS (Prepared at 37ºC) and was plated into the first layer of agarose, covered with cover slip and was kept at 4ºC to solidify. Finally a third layer of 0.5% LMPA was applied over the second layer. After solidification of the third layer, the cover slip was removed and the cells were lysed for 60min at 4ºC by immersing the slides in lysing solution (2.5M NaCl, 100mM Na_2EDTA and 10 mM Tris), pH adjusted to 10 with NaOH/HCl. The solution was stored at 4ºC and just prior to use Triton X 100 and 10% dimethylsulfoxide was added.)

Electrophoresis of microgel slides: After the slides were removed from the lysing solution, they were placed in an electrophoresis tank horizontally, side by side. Slides were covered with fresh electrophoresis buffer (1mM Na_2EDTA, 300mM NaOH) to a depth of 2-3 mm above the slides and kept at 4ºC for 30 min. To prevent additional DNA damage, slides were processed in dim yellow light and electrophoresis was carried out at 25V and 300 mA for 20 min at 4ºC in a dark chamber.

Neutralization and staining: The slides were gently lifted from the buffer and placed on a staining tray. The slides were carefully flooded with neutralization buffer (0.4M tris, pH 7.5) at 4ºC in dark for 10 min and the buffer was drained off. The process was repeated twice. Finally the slides were stained by ethidium bromide (2µg/ml) directly onto the slides and covered with cover slip.

Evaluation of DNA damage: Observations were made of ethidium bromide stained DNA using 10X and 40X objectives on a fluorescent microscope.(Olympus optical Co Ltd. Tokyo) equipped with an excitation filter of 515-550 nm. DNA damage was quantified by measuring the incidence of comets.

SECTION D

Motor behavioral studies

Rotarod (Rondi-Reig et al., 1999).

Animals were placed on a stationary rod (diameter= 70 mm) and were trained to stay on it as it rotated at 5 rpm. Usually 4-6 training sessions on consecutive days (each constituted by a maximum of 10 trials) were needed to achieve the maximal performance established at 120 s . After the end of the training period, all the animals reached the criterion of 120 s on their first run. The variable studied after intoxication was the total time spent on the rod during a 10-trial session. The total number of trials needed to reach the maximal performance (120 s) was also taken into account. Rotarod testing was performed at baseline and further at all the 7 days of rotenone intoxication. The tests were done daily early morning (8-10 am) before rotenone intoxication.

Pole test (Matsuura et al., 1997)

The pole test was performed according to *Matsuura et al.,1997* with minor modifications. The mouse was placed head upward on the top of a vertical wooden rough-surfaced pole (diameter 1 cm, height 50 cm). Each mouse was habituated to the pole on the day prior testing,

then allowed to descend five times. The total time until the mouse reached the floor with its four paws was recorded (T-total) as well as the time needed for the mouse to turn completely head downward (T-turn). For each session of five descents, the best performances were kept for the T-turn and T-total. If the mouse was unable to turn completely downwards, fell or slipped down, the default value of 120 s was taken into account. The pole test was performed at baseline, then at all the 7days of rotenone intoxication.

Stride length (D'Hooge et al., 1999)

Stride length was analyzed with the method adapted from D'Hooge et al., (1999) by wetting the animals' fore- then hind-paws with black/red ink and letting them trot on a strip of paper (4.5 cm wide, 42 cm long) down a brightly lit runway towards a dark goal box. Forelimb stride lengths and hind limb stride length were done simultaneously by inking the hind and forelimbs with different color inks. Mice were habituated to the experimenter and to the apparatus (total of three runs) for 3 days prior to starting the experiments. The measurements were made after three training runs. Stride length was measured manually as the distance between two paw prints. The three longest stride lengths (corresponding to maximal velocity) were measured from each run. Paw prints made at the beginning (7 cm) and end (7 cm) of the run were excluded because of velocity changes. Runs in which the mice made stops or obvious deceleration observed by the experimenter were excluded from analysis. Stride lengths were measured at baseline and at all 7days of rotenone administration. Measurements were made for each limb, then the mean length for both forelimbs and for both hind limbs were used as the variables

Statistical analysis

Experimental data obtained were expressed as mean ± standard deviation (SD) and analysed 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 in all chapters 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. P value less than 0.05 was taken as significant through out the studies.

1.0 INTRODUCTION

Brain development begins very early in human gestation and continues well after birth through adolescence. During development, brain cells proliferate, migrate to the appropriate location in the brain architecture, differentiate into the correct cell type and establish connections (synapses) with nearby and distant cells in complex neuronal circuits. Myelin sheathing on the neurons begins to develop after cell proliferation and migration and continues through adolescence. Experimental data show that extremely short-term exposures to developmental neurotoxicants can have permanent impacts on later brain function and that the abnormalities observed after exposure to a single toxic agent may vary with the timing of the exposure (Rice and Barone, 2000).

The brain and nervous system are prone to oxidative stress and are inadequately equipped with antioxidant defense systems to prevent ongoing oxidative damage. Indeed increased oxidative damage, mitochondrial dysfunction, accumulation of oxidized proteins, inflammation and defects in protein clearance constitute complex intertwined pathologies that conspire to kill the neurons (Halliwell, 2006). Mitochondria are critical regulators of cell death, a key feature of neurodegeneration. In all major examples of neurodegenerative diseases there is strong evidence that mitochondrial dysfunction occurs early and acts causally in disease pathogenesis. Moreover, an impressive number of disease-specific proteins interact with mitochondria. Thus, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria, hold great promise (Lin and Beal, 2006).

3-Nitropropionic acid (3-NPA) is a natural environmental toxin synthesized by plants (e.g. *Indigofera endecaphylla*) and fungi (e.g. *Aspergillus flavus*). Human exposure to 3-NPA occurs *via* ingestion of fungal contaminated peanuts, corn and sugar cane (Ludolph et al., 1991). The toxicity of 3-NPA is thought to be due to its irreversible covalent binding to the 70 kDa subunit of succinate dehydrogenase (SDH), inhibiting the enzyme resulting in ATP depletion (Huang et al., 2006). The serial cascades induced by 3-NPA intoxication are: impaired mitochondrial Ca^{2+} homeostasis, elevated intracellular Ca^{2+} and an impaired buffering capacity of intracellular Ca^{2+} in astrocytes and neurons (Nasr et al., 2003). Moreover, 3-NPA is known to activate calpain and caspase-9, which

are involved in neuronal cell death (Fu et al., 1995; Bizat et al., 2003). 3-NPA increases production of lipid peroxides and oxidized protein levels both in mitochondria and the cytosol with a corresponding increase in the activities of SOD and glutathione peroxidase (Fu et al., 1995; Tunez et al., 2004; Pocernich et al., 2005) while, acute systemic administration of 3-NPA does not replicate Huntington's disease (HD), while neurotoxicity was observed after either one or several i.p. injections given over a short period of time (1-5 days). Further 3-NPA toxicity is shown to be age dependent (Beal et al., 1993; Bossi et al., 1993). The acute effects of 3-NPA on prepubertal mice in terms of early oxidative damage and associated mitochondrial dysfunctions have not been studied earlier.

Parkinson's disease (PD) is a multifactorial disease in which both genetic and environmental factors are known to play a role in its aetiology. Among environmental factors, the vital role of pesticide exposures which cause selective mitochondrial complex I inhibition (eg: Rotenone) is receiving wide attention. The occurrence of PD in the rural population involved in gardening and agriculture is higher (Gorell et al., 1998). Rotenone, a naturally occurring compound derived from the roots of *Derris* and *Lonchocarpus* plant species, is used worldwide as a natural pesticide and insecticide (Jenner, 2001). Due to its extreme lipophilicity, rotenone easily crosses biological membranes and does not depend on membrane transporters (Greenamyre et al., 2001). Inside nerve cells, rotenone acts with high affinity as a specific inhibitor of mitochondrial NADH dehydrogenase within complex I of the respiratory chain (Thiffault et al., 2000). The precise mechanism/s whereby systemic complex I dysfunction leads to selective dopaminergic neurotoxicity is not clear. In this direction, a new model of PD based on subchronic, continuous infusion of rotenone in rats is reported (Betarbet et al., 2000). While acute doses of rotenone increases Dopamine (DA) turnover (Thiffault et al., 2000), sub-chronic, intermittent treatment (by i.p. injection) only moderately lowers DA content in the striatum (Alam et al., 2002). Rotenone is demonstrated to selectively damage dopaminergic neurons in experimental animals and cell cultures (Dukes et al., 2005). Dopaminergic cell death due to rotenone treatment is attributed to oxidative stress resulting from the release of free radicals and superoxides following complex I inhibition (Sherer et al., 2003). Further down stream mitochondrial damage by rotenone leads to caspase-3-dependent apoptosis in primary dopaminergic neurons (Moon et al., 2005). Although rotenone has been used as a model compound in adult rodents, the susceptibility of prepubertal mice to short term exposure to rotenone has not been investigated.

The need and the importance of assessment of adolescent neurotoxicity have been critically reviewed recently (Spear, 2007). In general experimental studies have focused on neural and behavioral consequences of neurotoxicant exposure during prenatal and early postnatal periods with little emphasis on exposure periods that subsume adolescence. Based on the increasing recognition that adolescence period is a time of considerable neural structuring and sculpting of the brain (Spear, 2000), there has been a growing interest in understanding whether this developmental transition is a vulnerable period for neurotoxicity. It is in this context for our studies we chose two model neurotoxicants (*viz.,* 3-nitropropionic acid and Rotenone) to characterize the pattern of induction of early oxidative stress and mitochondrial dysfunctions following short term exposure in a prepubertal mice model.

In humans, prolonged consumption of the seeds of *Lathyrus sativus* (common name: Khesari Dhal) as a staple diet has been reported to result in a progressive neurodegenerative condition known as Neurolathyrism (NL), a form of motor neuron disease clinically characterized by spastic paraparesis in the hind legs that mainly targets the Betz cells and corticospinal tracts (Haimanot and Kidane, 1990). Khesari dhal seeds are a cheap source of food containing high protein content and thus constitute one of the main food sources of third world countries in Asia and Africa mainly during the draught season (Getahun, 1999). Highly prevalent among young males after a continuous dietary intake (up to 2-4 months), the neurotoxicity of *L. sativus* is believed to be due to the neuro-excitatory non-protein amino acid 3-N-oxalyl-L-2, 3-diaminopropionic acid (β-ODAP) (Ross et al., 1985). The neurotoxic effects of β-ODAP have been demonstrated in several species of animals such as mice, rats, guinea pigs and macaques (Rao, 1977; Spencer et al., 1986). It is postulated that this natural toxin may act through multiple mechanisms and/or sites of action in the CNS beyond those that are directly linked with its activity as a non–NMDA receptor agonist (Ravindranath, 2002). In particular, β-ODAP is reported to inhibit the cysteine–glutamate exchanger system leading to decreased levels of glutathione and a corresponding increase in oxidative damage (Warren et al., 2004).

Since the toxin responsible for the neurotoxicty is identified as β-ODAP, numerous attempts have been made to detoxify the *L. sativus*. Differences in mode of cooking can alter the toxicity of KD through isomerisation of β-ODAP to the α- isomer which is considered non -toxic. In addition, other methods such as overnight soaking, steeping and boiling have also been employed

to detoxify the seeds (Lambein, 2000). At our institute, a process for detoxification of KD has been developed by which 85-90% of the neurotoxin can be removed (CFTRI Annual report, 1994). Numerous attempts have been made to develop animal models for NL by administration (oral or i.p) of larger doses of β-ODAP to mice, rats and monkeys (Spencer et al., 1986). While multiple mechanisms have been proposed, the occurrence of oxidative damage *in vivo* following dietary consumption of KD in mice has been less investigated. We hypothesize that dietary consumption of KD (at doses equivalent to human consumption levels) may cause significant oxidative stress in brain regions of mice which is likely to be progressive and likely to participate in the development of neuronal degeneration (Shinomol and Muralidhara, 2007).

Accordingly in this chapter, the neurotoxicant induced oxidative dysfunctions were characterized in different brain regions of prepubertal male mice. These data were a prerequisite for our further studies in which we have assessed the neuroprotective properties of two well known medicinal plants *viz., Centella asiatica* and *Bacopa monnieri.* Further, in growing male mice, the occurrence and progression of oxidative stress response in brain of male mice following dietary intake of KD along with perturbations in the activities of various antioxidant enzymes and protein carbonyl content (a measure of protein oxidation) was investigated. Further, we have addressed the question of the potential beneficial effects of feeding the detoxified KD in terms of diminution or abrogation of oxidative stress induction under similar conditions of exposure. The results obtained have been presented separately under three subsections. Subsection (i) describes studies pertaining to the neurotoxicant, 3-NPA with emphasis on induction of oxidative stress response *in vitro* in different brain regions and *in vivo* oxidative stress and associated mitochondrial dysfunctions in striatum and cortex of prepubertal male mice. Subsection (ii) describes studies related to Rotenone induced oxidative dysfunctions in various brain regions (Cytosol and mitochondria) of prepubertal mice. Subsection (iii) describes comparative dietary studies related to Khesari dhal induced oxidative stress in brain regions and the comparative effects of detoxified khesari dhal.

2.0 OBJECTIVE

The primary objective of these investigations was to obtain evidences to demonstrate that cytosol and mitochondria in brain of prepubertal mice administered neurotoxicants (3-NPA and rotenone) and that of adult mice fed khesari dhal (containing the neurotoxicant: β-N Oxalyl-L-2,3-diaminopropionic acid) are subjected to severe oxidative stress. Further the nature of functional alterations in mitochondria and cytosol associated with these neurotoxicants was also investigated.

3.0 EXPERIMENTAL DESIGN

(i) 3-nitropropionic acid (3-NPA) model: prepubertal mice

Induction of lipid peroxidation in vitro

In order to determine the extent of oxidative stress induction caused by 3-NPA *in vitro,* synaptosomal preparations of whole brain/cortex and mitochondria isolated from different brain regions were incubated with 3-NPA at various concentrations (0.5,1.0,1.5 and 2.0mM) for 1 hr under standard conditions (as described in Materials and Methods section). Induction of lipid peroxidation was monitored by quantification of malondialdehyde (MDA) content, reactive oxygen species (ROS) generation and hydroperoxide (HP) formation. Similar studies were also conducted in tissue slices of various brain regions.

Induction of oxidative stress in vivo

Preparation of 3-NPA solution: 3-NPA was dissolved in freshly prepared phosphate buffered saline, pH 7.4. The doses employed were 50, 75 and 100mg/kg bw.

Preliminary study

With an objective of determining the optimum dosage of 3-NPA that would induce significant oxidative stress and low mortality, preliminary studies were conducted. Groups of prepubertal mice (n=6) were administered (i.p.) with 3-NPA at dosages of 0, 50, 75 and 100mg/kg bw/day for 2 days. Recording the symptoms and mortality, the survivors were sacrificed 24 hr after the second dose and biochemical investigations were conducted. The investigations comprised of

quantification of SDH activity in brain, heart and liver mitochondria and determination of markers of oxidative stress in brain, heart, liver and status of glutathione (GSH) and antioxidant enzymes.

Determinative study: Oxidative response to 3-NPA (75 mg/kg bw /d for 2 days).

Based on the results from the preliminary study, 75mg/kg bw was selected as the optimum dosage for prepubertal mice. In this study, prepubertal male mice were randomly assigned to control and treatment groups (n=6). While the controls received saline, mice of the treatment groups were injected with 2 doses of 3-NPA (75mg/kg bw) at 24 hrs intervals. Food intake and body weights of the animals were recorded during the experimental period. Both control and treated mice were sacrificed 24 hr after the last dose; brain, heart, liver and thymus were excised and weighed. Cytosolic and mitochondrial fractions prepared from brain regions (striatum and cortex) were subjected to the following biochemical analysis.

Oxidative stress induction: Quantification of oxidative stress was done in freshly prepared mitochondria and cytosolic fractions of the brain by measuring generation of ROS (using dihydrodichlorofluorescein dye), lipid peroxidation (quantified as malondialdehyde levels) and hydroperoxide levels (quantified using FOX reagent).

SDH activity in Cortex and Striatum: SDH activity was determined in freshly prepared mitochondria obtained from brain regions (cortex and striatum) of control and treated mice.

Protein carbonyls, Glutathione levels: To quantify the extent of protein oxidative damage induced the protein carbonyl content was quantified in cytosol and mitochondria of brain regions. The levels of GSH and GSSG were determined by fluorimentric method.

Perturbations in antioxidant enzymes: Alterations in the status of antioxidant enzymes like catalase (cytosol only), GSH-Px, GST and SOD (cytosol and mitochondria) were measured.

(ii) Rotenone model: prepubertal mice

Induction of oxidative stress in vivo

Preparation of Rotenone (Rot) solution: Rotenone was dissolved in DMSO (1mg/ml), with DMSO volume kept as low as 50µl in the injected volume.

Preliminary study

Acute doses of rotenone: With an objective of determining the optimum dosage of rotenone that would induce significant oxidative stress in brain and low mortality, preliminary studies were conducted where in groups of prepubertal mice (n=6) were administered rotenone (i.p.) at acute dosages of 0, 0.5,1,2, 4 and 5 mg/ kg bw. The incidence of mortality was recorded.

Repeated doses of rotenone: Since the highest dose of rotenone (5mg/kg bw) induced significant mortality among prepubertal mice, sub-lethal doses of 0.5, 1.0, 2.0 mg/kg bw/day for 7 days were employed for repeated dosing regime. Daily body weight and food intake were monitored and mortality was recorded as and when it occurred.

Determinative study: repeated doses

Since the highest dose of rotenone (2mg/kgbw) in the 'repeated dosing regime' induced 100% mortality after 3 days, for the determinative study only two dosages *viz.,* 0.5 and 1.0 mg/kg bw were employed. In this study, prepubertal male mice were randomly assigned to control and treatment groups (n=6). While the control mice received DMSO alone, mice of the treatment groups were injected with rotenone at dosages of 0.5 and 1mg /kg bw/d for 7 consecutive days (9.00-10.00 hr am). Food intake and body weights were recorded during the experimental period. Both control and treated mice were sacrificed 24 hrs after last dose and brain and liver were excised and weighed. Cytosolic and mitochondrial fractions prepared from the brain regions *viz*., cortex, cerebellum, hippocampus and striatum were subjected to the following biochemical analysis.

Quantification of oxidative damage: Generation of ROS (using dihydrodichlorofluorescein dye) lipid peroxidation (quantified as malondialdehyde levels), hydroperoxide levels (quantified using FOX reagent) and protein oxidation (quantified as protein carbonyls) were measured in freshly prepared mitochondria and cytosol of striatum and cortex.

Antioxidant status of brain regions: The activities of antioxidant enzymes like GSH-Px, GST and SOD were measured in both cytosol and mitochondria and catalase measured only in cytosol. The status of GSH was also determined in freshly prepared mitochondria and cytosol of brain regions.

Effect on cholinergic enzymes: Studies were carried out to determine the effect of rotenone administration on cholinergic enzymes *viz.,* AChE and BChE in cytosol of brain regions.

Effect on Electron Transport Chain (ETC) enzymes: Since rotenone is demonstrated to cause inhibition of complex I activity, the activity of NADH-ubiquinone oxidoreductase and NADH–cytochrome c reductase activity was measured in freshly prepared mitochondrial samples of different brain regions.

Effect on MTT reduction: To determine the status of mitochondrial dehydrogenase activity and viability of mitochondria after rotenone administration, MTT assay was conducted.

(iii) Khesari dhal model: Adult mice

Rationale of dose selection of Khesari dhal (KD)

The doses were selected primarily based on human consumption levels reported in literature to cause Neurolathyrism (NL) (Shinomol and Muralidhara, 2007). It is estimated that 300g of KD must be consumed daily at least 3 months to produce typical disease symptoms in human males. The β-ODAP content in KD is 1056 ± 50 mg/100 g. Hence an average male (50 kg) consuming 300g of KD will be ingesting 300 mg of β-ODAP/day. In our study, the average daily intake of β-ODAP among mice fed KD diet would not exceed 15 mg of β-ODAP/day/mouse (taking an average diet intake of 5 gm /day /mouse weighing 30 g). Accordingly, the daily intake of β–ODAP would correspond to 1/10 of the human consumption levels required to induce typical symptoms of NL

The preparation of detoxified khesari dhal (DKD) was carried out at the Grain Science Technology department of CFTRI, Mysore. It involved essentially a two-stage water leaching method as described in Materials and Methods section. DKD was fed to mice at 30% in the diet.

Determinative study

Khesari Dhal was powdered and mixed with the commercial diet at 30% level. Adult mice (n=6) were fed with either normal, KD or DKD incorporated commercial powdered diet for a period of 4 or 12 wks. Mice fed with only commercial diet served as the normal controls. Diet was prepared twice every week. During the experimental period, food intake was monitored daily and individual body weights were recorded once a week. Mice from control, KD and DKD groups were sacrificed (Interim- 4 weeks; terminal- 12 weeks) and the following biochemical investigations were carried out. For the biochemical investigations, either homogenate or sub-cellular (mitochondria and microsomes) fractions were used.

Quantification of oxidative damage: Generation of ROS, lipid peroxidation and protein carbonyls were measured in freshly prepared homogenates of cortex and cerebellum of KD and DKD fed mice.

Antioxidant status of brain regions: The status of GSH in KD and DKD mice was determined in freshly prepared mitochondria and cytosol. The activities of antioxidant enzymes were measured in both cytosol and mitochondria (catalase, GSH-Px, GST and SOD in cytosol and GSH-Px, GST and SOD in mitochondria) of various brain regions.

Effect on cholinergic enzyme: Studies were carried out to determine the effect of KD and DKD administration on cholinergic enzyme- AChE in cytosol of brain regions.

Oxidative damage in sub-cellular fractions: To determine the extent of oxidative damage caused by KD and DKD in various brain regions, mitochondria and microsomes were isolated from different brain regions (cortex, cerebellum, hippocampus and striatum). Status of lipid peroxidation (MDA levels), ROS generation and protein carbonyls content were determined (at 4 and 12 wks). GSH level was quantified along with determination of the activity of the antioxidant enzymes in mitochondria and microsomes.

4.0 RESULTS

(i) 3-nitropropionic acid (3-NPA) model: prepubertal mice

Oxidative stress induction in mice brain *in vitro*

Response in whole brain and cortical synaptosomes: Synaptosomes on exposure to 3-NPA showed a significant induction in oxidative stress as indicated by elevated levels of ROS, MDA and HP (Fig.1.1). The increase in MDA levels was concentration dependent (beyond 1mM) in synaptosomes of both whole brain (20-61%) and cortex (10-54%). Generation of ROS was markedly enhanced (48-98%) even at the lowest concentration (0.5mM), beyond which the response was concentration dependent in whole brain synaptosomes. The cortical synaptosomes showed more robust elevations in ROS levels (60-117%), while the increase in HP levels were more robust in both cortex (58-178%) and whole brain (108-288%).

Response in miitochondria of brain regions: Mitochondria on exposure to 3-NPA showed significant induction of oxidative stress as demonstrated by elevated levels of MDA, ROS and HP (Fig.1.2). The increase in MDA levels was significant and concentration dependent in all brain regions (Ct: 25-78%; Cb: 38-109%; Hc: 21-81%; St: 43-113%). Further the levels of ROS were also markedly elevated in a concentration dependent manner in mitochondria (Ct: 25-78%; Cb: 38-109%; Hc: 21-81%) in all brain regions, while striatal mitochondria showed more robust induction (43-113%). On the other hand, the increase in HP levels were more robust in hippocampus (70-184%) than in other brain regions (Ct: 70-171%; Cb: 65-135%; St: 72-126%).

Induction of lipid peroxidation in brain slices: Exposure of brain slices to 3-NPA resulted in robust elevation in LPO. At similar doses a dramatic increase in MDA levels was evident in all brain regions (Ct: 32-261%; Cb: 16-371%; Hc: 35-413%; St: 42-562%) (Data not shown).

Oxidative induction pattern *in vivo*

Mortality data

To assess the incidence of 3-NPA- induced mortality, a preliminary study was conducted using three dosages viz., 50, 75 and 100mg/kg bw, administered for two days. All the animals administered the higher dosage died after the second injection (data not shown).

Preliminary dose finding study

General effects of 3-NPA in prepubertal mice: Significant reduction (13-20%) in food intake was evident at both doses during post administration (Data not shown). There was a marginal decrease in body weights (21%) only at the higher dose (75 mg/kg bw/d for 2 days), which was accompanied by decrease in organ weights (Liver-16%; Heart-23% and Thymus-39%). Although no decrease in body weight was evident at the lower dose, significant decrease in vital organ weights (Liver-13%; Heart-19% and Thymus-22%) was evident (Table 1.1).

Effect of 3-NPA on brain SDH activity

Among 3-NPA administered mice, the activity of succinate dehydrogenase was significantly reduced in brain at both the doses (Fig.1.3). While the lower dose of 3-NPA caused 33% reduction, the higher dose resulted in 57% reduction. Similar reduction in SDH activity was also demonstrable in heart (26-65%) and liver (17-25%).

Evidence of oxidative stress induction in brain

In general, there was a dose related increase in MDA, ROS and PC levels in both cytosol and mitochondria of heart, liver (data not shown) and brain of 3-NPA administered mice (Table1.2). The MDA levels were significantly elevated in whole brain cytosol (43-85%), heart (52-109%) and liver (29-57%). Likewise the ROS levels were also elevated in brain cytosol (37-77%). The elevations in mitochondrial MDA levels in brain were more robust (35-171%) compared to those in heart (33-50%) and liver (26-56%). Concomitantly, the ROS levels were also elevated in mitochondria (27-54%). Markedly elevated protein carbonyls content were observed in both cytosol (40-60%) and mitochondria (41-73%) among mice administered 3-NPA.

Effect on 3-NPA on glutathione and antioxidant enzymes in brain

The reduced glutathione (GSH) levels in brain were diminished significantly among 3-NPA administered mice (cytosol: 12- 37%; mitochondria: 21-30%) (Table1.2). In contrast there was a significant increase in GSSG (cytosol: 11-24%; mitochondria: 17-55%) levels (Data not shown). The activities of antioxidant enzymes *viz.,* GST, GSH-Px and SOD were decreased in both cytosol and mitochondria while, the activity of catalase was significantly decreased (27-37%) in cytosol. The elevation in activity of GST was consistent in cytosol (30-45%) and mitochondria (22-38%), whereas the degree of reduction in GSH-Px was higher in mitochondria (20-31%). However the activity of SOD was marginally decreased in both mitochondria and cytosol.

Determinative study: Response to 3-NPA (75 mg/kg bw /d for 2 days)

Body weight and organ weights

A general decrease in food intake and body weights (10-27%) was observed among 3-NPA administered mice. The animals were hypoactive. Marginal but significant decrease was evident in the weights of liver- (10%) and heart (15%) among 3-NPA administered mice.

Evidence of Oxidative stress in cortex and striatum

3-NPA caused a marked increase in all oxidative stress markers measured in both cytosol and mitochondria of brain cortex and striatum (Fig 1.4). While the elevation in cytosolic MDA levels in striatal region (84%) was more robust than cortex (61%), the induction in mitochondria was more uniform (Striatum-70%; Cortex-65%). Further the elevation in cytosolic ROS levels in striatum (80%) and cortex (73%) were consistent, while the increase in mitochondria was more robust in striatum (100%) than in cortex (76%). In cortex, significant increase in HP levels was observed in both cytosol (58%) and mitochondria (41%). A similar trend of increase was observed in striatal mitochondria (78%), while the cortex showed less robust elevation (55%).

SDH activity in Cortex and Striatum

In general there was marked decrease in the activity of SDH both in striatum and cortex. The percent decrease in striatum was relatively higher than the cortex (74% *vs* 62%) (Fig.1.4).

Protein carbonyls (PC), Glutathione levels (GSH) and antioxidant enzymes

There was a significant increase in protein carbonyls in cytosol and mitochondria of both striatum and cortex (Fig 1.4). While the elevation in cytosolic PC in striatum was 62%, the increase was relatively lower in cortex (32%). A similar increase was evident in mitochondria of striatum (53%) and cortex (44%).

3-NPA caused significant depletion in the levels of reduced glutathione in both cytosol and mitochondria of brain regions (Fig.1.5). While there was more robust decrease in cytosolic GSH in striatum (47%) than cortex (33%), the mitochondria showed more uniform depletion (Striatum-30%; Cortex-24%) (Fig.1.6). However, 3-NPA caused a uniform increase in GSSG levels in cytosol (Striatum-27%; Cortex-36%) and mitochondria (Striatum-28%; Cortex-32%) (Data not shown).

3-NPA administration resulted in significant perturbations in the activity of antioxidant enzymes (Fig 1.5). The activity of catalase was markedly reduced in cytosol of both brain regions

(Cortex- 41%; Striatum- 52%). The activity of glutathione related antioxidant enzymes *viz.*, GSH-Px and SOD levels were also reduced in both cytosol and mitochondrial fractions. The activity of GSH-Px was consistently decreased in cortex (Cytosol-20%; Mitochondria-34%) and striatum (Cytosol-32%; Mitochondria-39%). GST activity was uniformly enhanced in cortex and striatum. Reduction in SOD activity was less robust in both regions.

(ii) Rotenone model: prepubertal mice

Mortality profile

Administration of acute doses of rotenone (0.5, 1.0, 2.0, 4.0, 5.0 mg/kg body weight) resulted in significant mortality at 2mg/kgbw and beyond. While the lowest dose (1mg/kg bw) failed to induce mortality, a dose dependent mortality was observed at higher doses (2mg- 16.33%; 4mg-66.66%; 5mg- 100%) (Data not shown). Hence for short term rotenone exposure, dosages of 0.5 and 1.0 mg/kg bw were selected.

Body weights, organ weights and food intake

There was a general decrease in body weight gain, organ weights and food intake in rotenone administered mice. The decrease in body weight ranged between 7-28%. A general decrease (10-17%) in daily food intake was evident (Data not shown).

Evidence of Oxidative impairments in brain

Biochemical data has been provided only for doses *viz.,* 0.5 and 1.0 mg/kg bw. In general Rotenone administration caused a marked increase in all oxidative stress markers measured in both cytosol (Table 1.3) and mitochondria (Table 1.4) of brain regions .The elevation in cytosolic MDA levels in brain regions were comparable to that of mitochondria except in hippocampus at the higher dose. Enhanced ROS levels were also evident in both cytosol and mitochondria of brain regions. While the elevation in cytosolic ROS levels in brain regions was consistent there was less robust increase in mitochondria.

Antioxidant enzyme activity in cytosol and mitochondria

Rotenone administration resulted in significant perturbations in the activity of antioxidant enzymes (Table 1.5). The activity of catalase was markedly reduced (55-61%) in all the brain regions, while there was significant decrease in GSH-Px activity in cytosol (25-41%) and mitochondria (25-33%). The activity of GST was consistently increased in both cytosol (41-64%)

and mitochondria (24-45%), while there was an increase in SOD activity in cytosol (55-76%) and a decrease in mitochondria (17-26%) in all brain regions.

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Reduced glutathione levels and protein carbonyls

Rotenone administration resulted in significant reduction in GSH levels in both cytosol (12-14%) and mitochondria (34-38%) only at the higher dose (Fig.1.6). There was significant increase in protein carbonyls in cytosol of all brain regions (60-144%) and mitochondria (72-120%).

Cholinergic enzymes

The activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were significantly enhanced in all brain regions of mice administered rotenone (Fig.1.7). There was significant dose dependent increase in AChE activity in all regions of brain except in striatum (Ct: 28-34%; Cb: 18-21%, Hc: 35-45%, St: 12-17%). In contrast, BChE activity was markedly decreased in all brain regions (Ct: 38-42%; Cb: 26-30%, Hc: 48-47%; St: 26-42%).

Electron transport chain (ETC) enzymes and MTT assay

Rotenone administration resulted in significant decrease in the activity of mitochondrial ETC enzymes and MTT reduction in all brain regions. The degree of reduction in the activity of complex I was in the following order: Ct>St>Cb>HC. (26-72%) (Fig.1.8A) and that of Complex I-III was: St>Ct=Hc>Cb (13-47%) (Fig.1.8B). Marginal decrease in reduction of MTT was observed in all brain regions (Fig.1.8C).

(iii) Khesari dhal model: Adult mice

Growth Characteristics, food intake and organ weights

There were no significant changes in food intake among KD and DKD fed mice. The body weight gain among mice fed either KD or DKD incorporated diet was comparable to those of normal controls. Among KD mice, the liver weights were decreased at both sampling times (4 wks-21%; 12 wks-17%), while in DKD mice no significant alterations were evident (Data not shown).

Oxidative damage: Lipid peroxidation (LPO) and Reactive oxygen species (ROS)

In general, consumption of KD induced a significant increase in MDA levels in cortex and cerebellum at both sampling times. While cortex showed markedly enhanced MDA levels (80%

increase over the basal levels) at wk 4, the increase was relatively less (35%) at wk 12. On the other hand, cerebellum showed a marginal (21%) increase at wk 4 and a dramatic enhancement (120 %) at wk 12. Interestingly, the MDA levels among DKD mice were comparable to that of normal controls at both sampling times suggesting absence of any oxidative damage (Fig.1.9A). Among KD fed mice, the ROS levels were marginally increased in cerebellum (by 15 %), while cortex showed a moderate increase (by 35%) (Fig. 1.9 B). However, the brain regions of DKD mice did not show any significant increases in ROS levels.

Reduced Glutathione status protein carbonyl content

The GSH levels in brain cortex of KD mice measured at wk 4 and 12 revealed significant (16- 21%) depletion over the endogenous levels, while no changes were evident in DKD mice (Fig.1.10A). Consumption of KD diet resulted in a significant elevation in protein carbonyl content in both regions of the brain (Ct-26%; Cb- 20%) (Fig: 1.10B). However, no alterations in protein carbonyl content were observed in the brain and liver of DKD mice.

Activities of antioxidant enzymes

Significant changes in the activities of antioxidant enzymes were discernible of KD mice. (Table1.7) suggesting induction of oxidative stress. Varying degree of decrease in CAT activity was observed in cortex (KD- 25%; DKD- 15%) and cerebellum (KD-16%; DKD-10%). Significant decrease in GSH-PX activity was also observed in the cortex (KD-35%). Further, a marginal decrease in GST activity could be seen in KD fed mice, while no such alteration was evident among DKD mice. Interestingly, the activity of SOD was markedly enhanced (48%) in the cortex of KD mice. In general, measurement of enzyme activities at wk 4 showed only marginal changes in the KD mice (data not shown).

Brain AChE activity

The activity of AChE enzyme was significantly reduced in brain regions of KD fed mice at wk 12. While the cortex showed a relatively higher and consistent reduction (38-43%) in enzyme activity, cerebellum showed a lower degree of reduction (22%) initially, but a higher degree of reduction at wk 12 (41%). However, among DKD mice only a marginal reduction in the activity of AChE was observed at both sampling times (Fig.1.11**)**.

Oxidative damage in sub-cellular fractions

Significant increases in oxidative stress markers were observed in brain mitochondria and microsomes of brain in mice fed KD for 4 weeks (Data not shown). There were no perceptible changes among DKD fed mice. Marked increase in MDA (22-54%) and ROS levels (17-26%) were evident in all brain regions. The protein carbonyl levels were elevated uniformly in mitochondria (27-34%) of KD fed mice, while the induction was less robust in microsomal fractions. However, at 12 wk the elevations in oxidative markers were more robust among KD mice. Interestingly the oxidative stress marker levels were comparable to that of controls in DKD fed mice suggesting absence of oxidative damage. Significant elevations in MDA levels were observed both in mitochondria (15-34%) and microsomes (23-26%) of brain regions of KD fed mice. There was significant increase in ROS levels in all brain region mitochondria (42-56%) and microsomes (18-32%) of KD fed mice. Significant increase in protein carbonyl levels were also discernible in the mitochondria (27-45%) and microsomes (16-29%) (Table 1.8).

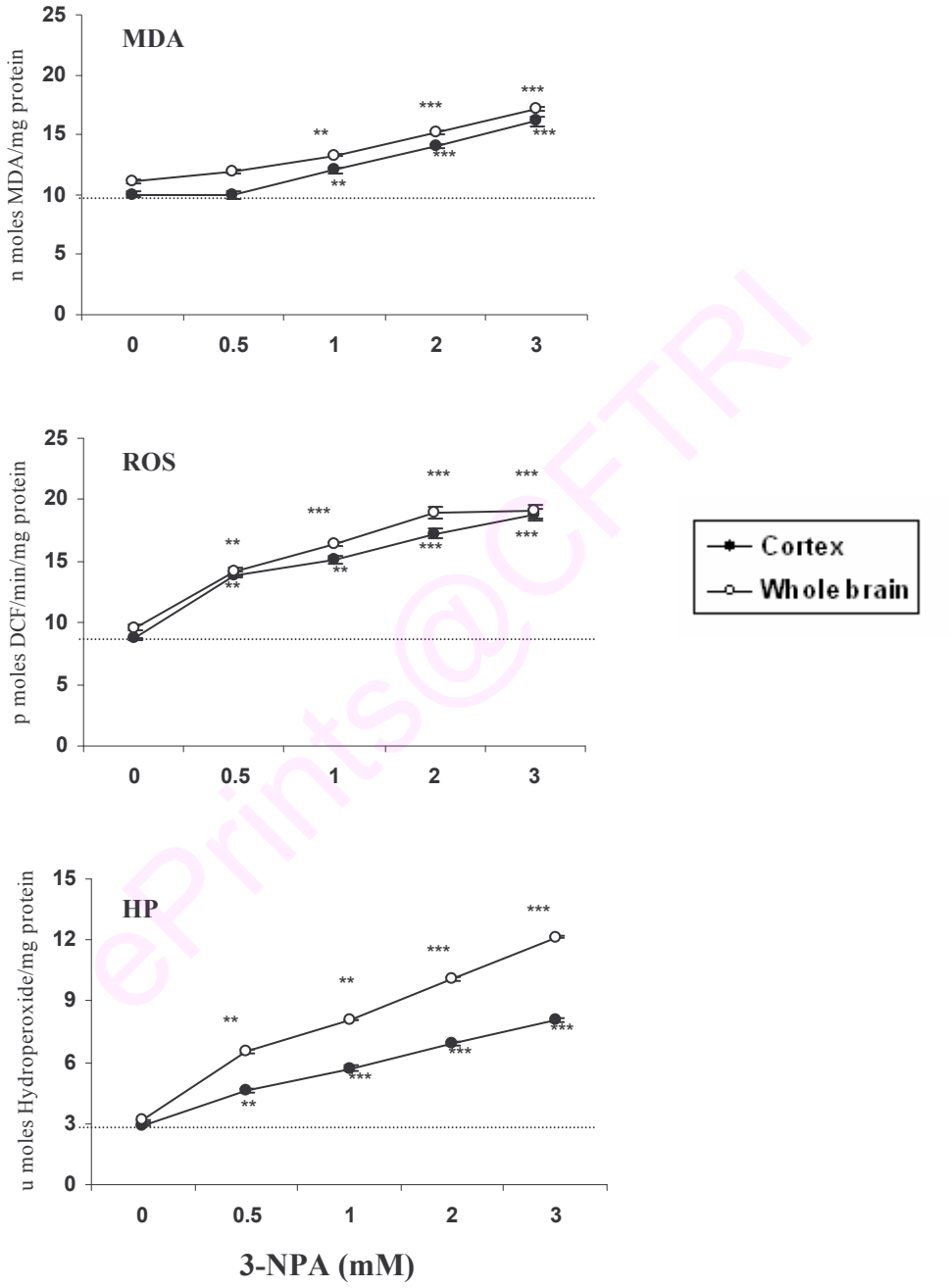
Glutathione levels and antioxidant enzyme activity

Marginal decrease in GSH levels in mitochondria (20%) and microsomes (15%) were observed in brain regions of mice fed KD for 4 wks, while no significant changes were evident in DKD fed mice. Among KD fed mice, marginal decrease in mitochondrial GSH-Px (20%) activity, increase in GST (15%) and SOD (20%) activities were accompanied by decrease in CAT activity (32%) in microsomes. There were no such alterations among DKD group (Data not shown).

However, terminally significant decrease in GSH levels in mitochondria (10-27%) and microsomes (11-32%) were notable in brain regions of mice fed KD for 12 weeks, while there was no noticeable change in DKD group (Data not shown). Significant decrease was also evident in the activities of antioxidant enzymes in mitochondria (GSH-Px: 23-35%, GST: 23--43%, CAT: 26-56%). No major alterations were observed in DKD group (Table1.9). Interestingly in DKD group varying degree of increase (Ct- 16%; Cb-30%; Hc-8%; St-44%) in catalase activity was observed (Data not shown).

Fig: 1.1

Oxidative stress induction in synaptosomes of cortex and whole brain of prepubertal male mice exposed to 3-nitropropionic acid *in vitro*

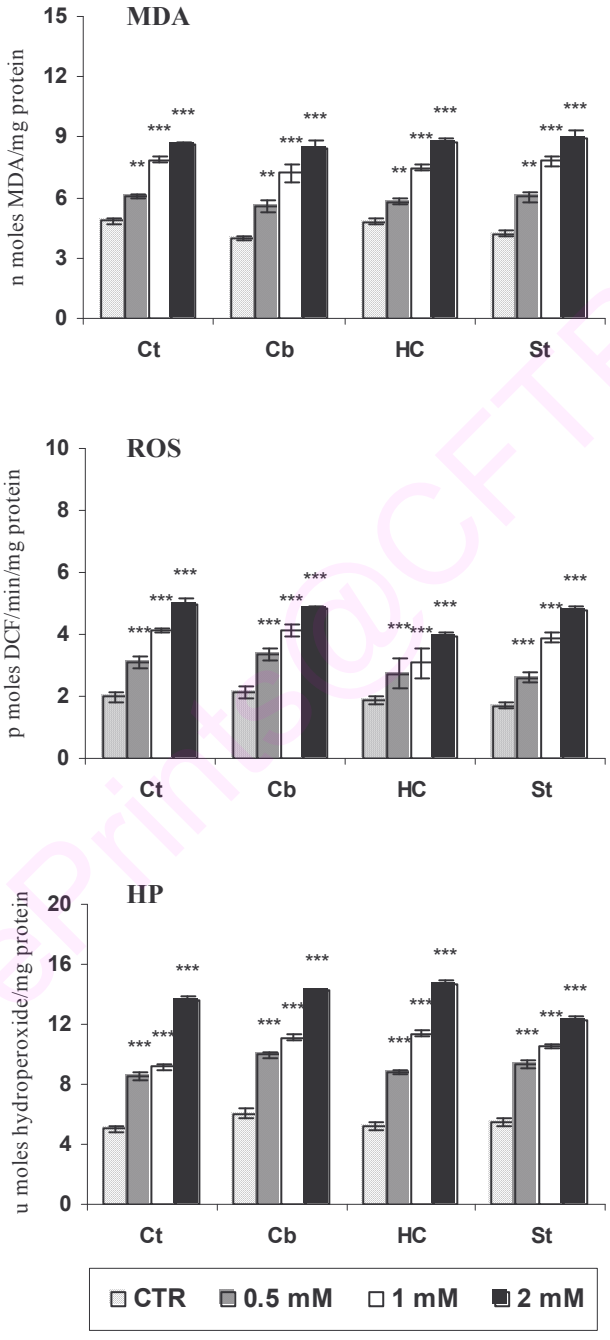


Values are mean ±SD (n=6). Data analyzed by one way ANOVA (**P<0.01; ***P<0.001)

MDA-Malondialdehyde; ROS- Reactive oxygen species; HP- hydroperoxides

Fig: 1.2

Oxidative stress induction in mitochondria of brain regions of prepubertal male mice exposed to 3-nitropropionic acid *in vitro*



Values are mean ±SD (n=6). Data analyzed by one way ANOVA (**P<0.01; ***P<0.001).

Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum

MDA-Malondialdehyde; ROS- Reactive oxygen species; HP- hydroperoxides

Table 1.1

Effect of 3-nitropropionic acid administration (i.p) on body and organ weights of prepubertal male mice

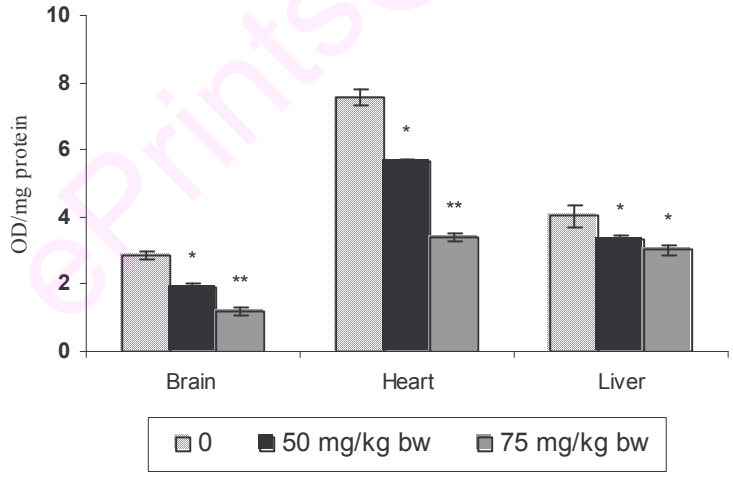
3-NPA (mg/kgbw)	Body weight (g)		Organ weights (g)		
	Day 0	Day 3	Liver	Heart	Thymus
0	23.50±0.01	24.75±0.48	1.79±0.09	0.180±0.01	0.090±0.004
50	23.66±0.28	23.66±0.28	1.56±0.05	0.146±0.005*	0.071±0.005*
75	23.40±0.58	19.06±0.28*	1.50±0.04*	0.139±0.009*	0.055±0.003*

Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05)

3-NPA administered for 2 days and mice sacrificed after 24 hrs of last dose

Fig: 1.3

SDH activity in mitochondria of brain, heart and liver of prepubertal mice administered 3-nitropropionic acid.



Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05 **P<0.01).

OD was measured at 490 nm

Table 1.2

Oxidative impairments in cytosol and mitochondria of brain of prepubertal mice administered 3-nitropropionic acid.

Parameters	3-NPA (mg/kg bw)		
	0	50	75
Cytosol			
MDA[1]	10.18±0.78	14.60±0.60**	18.90±0.70**
ROS[2]	12.57±0.50	17.23±1.02**	22.23±1.92**
PC[3]	5.26±0.24	7.35±0.55**	8.35±0.75**
GSH[4]	7.85±0.15	6.91±0.34**	4.91±0.34**
CAT[5]	1.98±0.05	1.45±0.07*	1.25±0.07**
GPx[6]	19.56±0.98	17.21±1.02*	15.21±1.02*
GST[7]	20.23±1.01	26.34±1.23*	29.34±1.23**
SOD[8]	40.25±1.50	34.36±2.23*	30.50±2.10**
Mitochondria			
MDA[1]	10.91±0.17	14.75±0.18**	18.70±0.28**
ROS[2]	14.36±0.06	18.19±0.10**	22.09±0.10**
PC[3]	3.21±0.10	4.54±0.45*	5.54±0.45**
GSH[4]	12.82±0.05	10.12±0.09*	9.02±0.59**
GPx[6]	17.02±0.78	13.78±0.97*	11.78±0.97**
GST[7]	21.89±1.02	26.67±1.22*	30.17±1.22**
SOD[8]	48.90±1.20	42.35±1.01*	39.21±2.10*

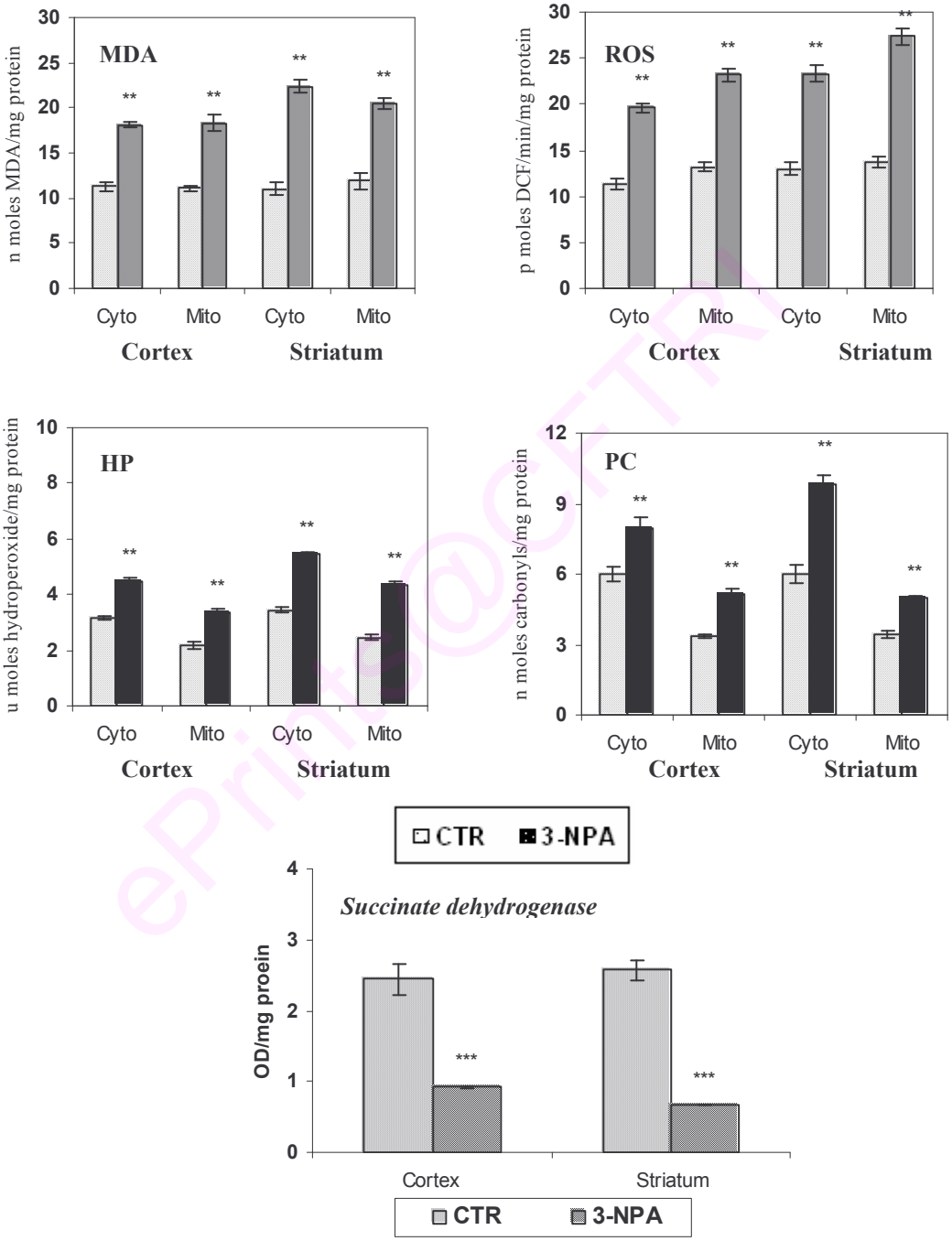
Values are ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01)

MDA- malondialdehyde; ROS- Reactive oxygen species; PC-Protein carbonyls;
CAT- Catalase; GSH- Reduced glutathione; GPx- Glutathione peroxidase;
GST- Glutathione-s-transferase; SOD- superoxide dismutase.

1-ηmoles MDA/mg protein; 2-ρmoles DCF/min/mg protein
3-ηmoles carbonyls/mg protein; 4-µg GSH/mg protein
5-µ moles H_2O_2degraded /min/mg protein; 6-η moles NADPH/min/mg protein
7-ηmoles conjugate/min/mg protein; 8-Units/mg protein

Fig: 1.4

Effect of 3-NPA on oxidative stress markers and SDH activity in cerebral cortex and striatum of prepubertal male mice.

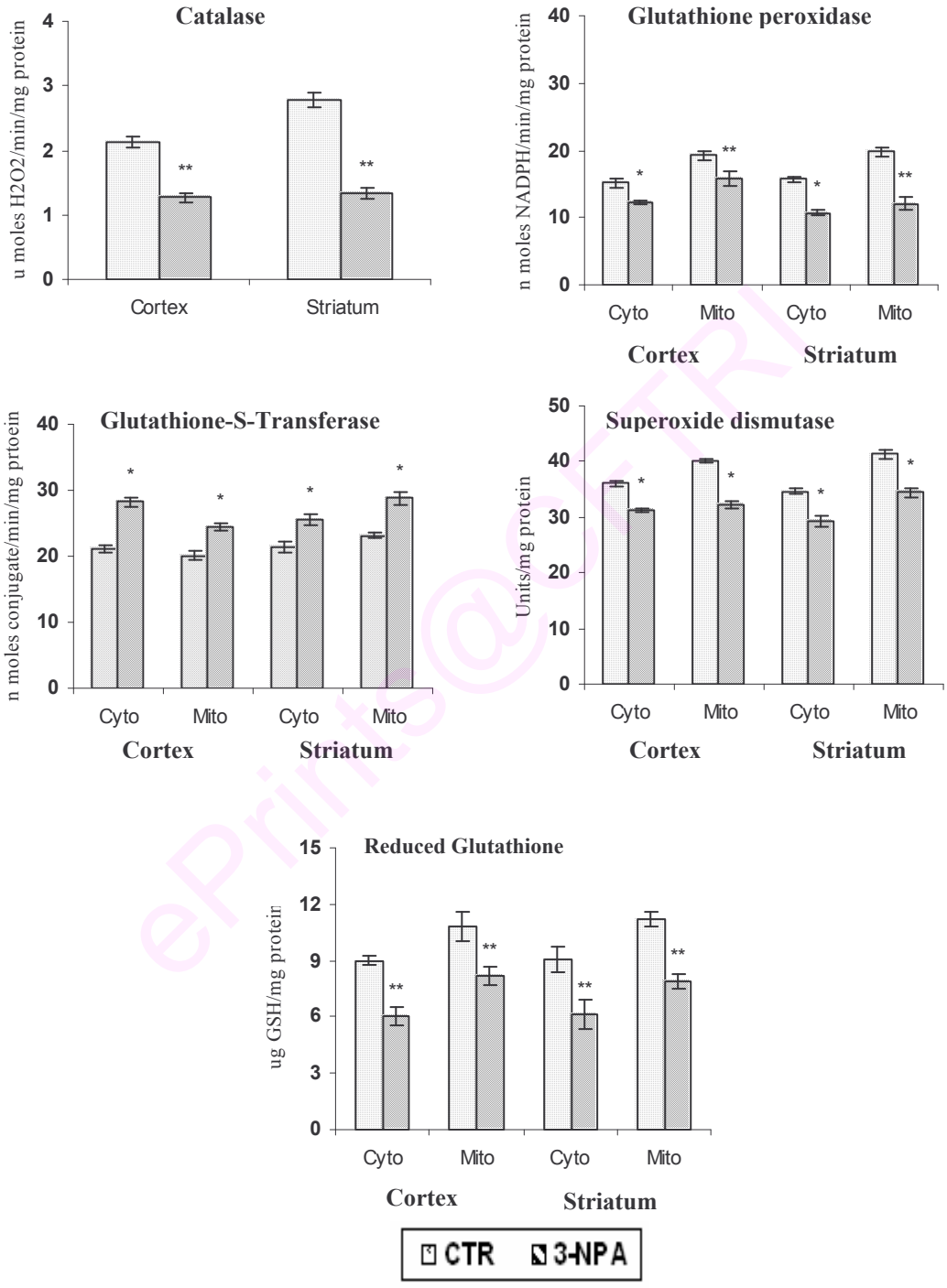


Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.0; ***P<0.001)

3-NPA- 75mg/kg bw; MDA-Malondialdehyde; ROS- Reactive oxygen species;
HP- Hydroperoxides;PC- Protein carbonyls; Cyto- Cytosol; Mito- Mitochondria.
OD measured at 490nm

Fig: 1.5

Effect of 3-NPA on the activity of antioxidant enzymes and reduced glutathione levels in cortex and striatum of prepubertal mice.



Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01).
3-NPA- 75mg/kg bw/ 2 days; Cyto-cytosol; Mito-mitochondria

Table 1.3

Effect of Rotenone administration on oxidative stress markers in cytosol of brain regions of prepubertal male mice.

Rotenone (mg/kgbw)	**Malondialdehyde[1]**				**Reactive oxygen species [2]**			
	Ct	Cb	Hc	St	Ct	Cb	Hc	St
0	10.0± 0.38	10.3± 0.54	10.3± 0.60	9.9± 0.63	11.2± 0.50	12.1± 0.79	11.8± 0.78	11.4± 0.70
0.5	11.2± 0.30** (13%)	12.8± 0.70** (24%)	12.5± 0.32** (21%)	11.5± 0.52** (16%)	16.5± 1.02** (48%)	16.2± 1.34** (34%)	17.0± 2.06** (44%)	18.0± 2.06** (59%)
1.0	12.9± 0.40** (30%)	14.8± 0.75** (43%)	14.5± 0.50** (41%)	14.5± 0.62** (47%)	19.8± 1.92** (78%)	21.2± 1.54** (75%)	23.0± 1.96** (95%)	25.0± 1.96** (121%)

Table 1.4

Effect of Rotenone administration on oxidative stress markers in mitochondria of brain regions of prepubertal male mice.

Rotenone (mg/kgbw)	**Malondialdehyde[1]**				**Reactive oxygen species[2]**			
	Ct	Cb	Hc	St	Ct	Cb	Hc	St
0	13.6± 0.38	14.3± 0.54	12.1± 0.60	13.8± 0.63	15.5± 0.50	15.2± 0.79	14.5± 0.78	13.0± 0.70
0.5	15.1± 0.30* (11%)	16.6± 0.70* (16%)	13.6± 0.32* (13%)	16.5± 0.82* (20%)	19.6± 1.02* (27%)	18.0± 1.34* (19%)	16.6± 2.06* (15%)	18.6± 2.06* (43%)
1.0	19.6± 0.40** (43%)	19.9± 0.75** (40%)	22.8± 0.50** (89%)	21.2± 1.02** (53%)	23.5± 1.92** (52%)	20.2± 1.54** (33%)	21.2± 1.96** (47%)	21.0± 1.06** (61%)

Common foot note: Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01)

1-ηmoles MDA/mg protein; 2-ρ moles DCF/min/mg protein
Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum.

The values in parenthesis denote percent increase over the controls

Table.1.5

Effect of Rotenone administration on activities of antioxidant enzyme in cytosol and mitochondria of brain regions of prepubertal male mice.

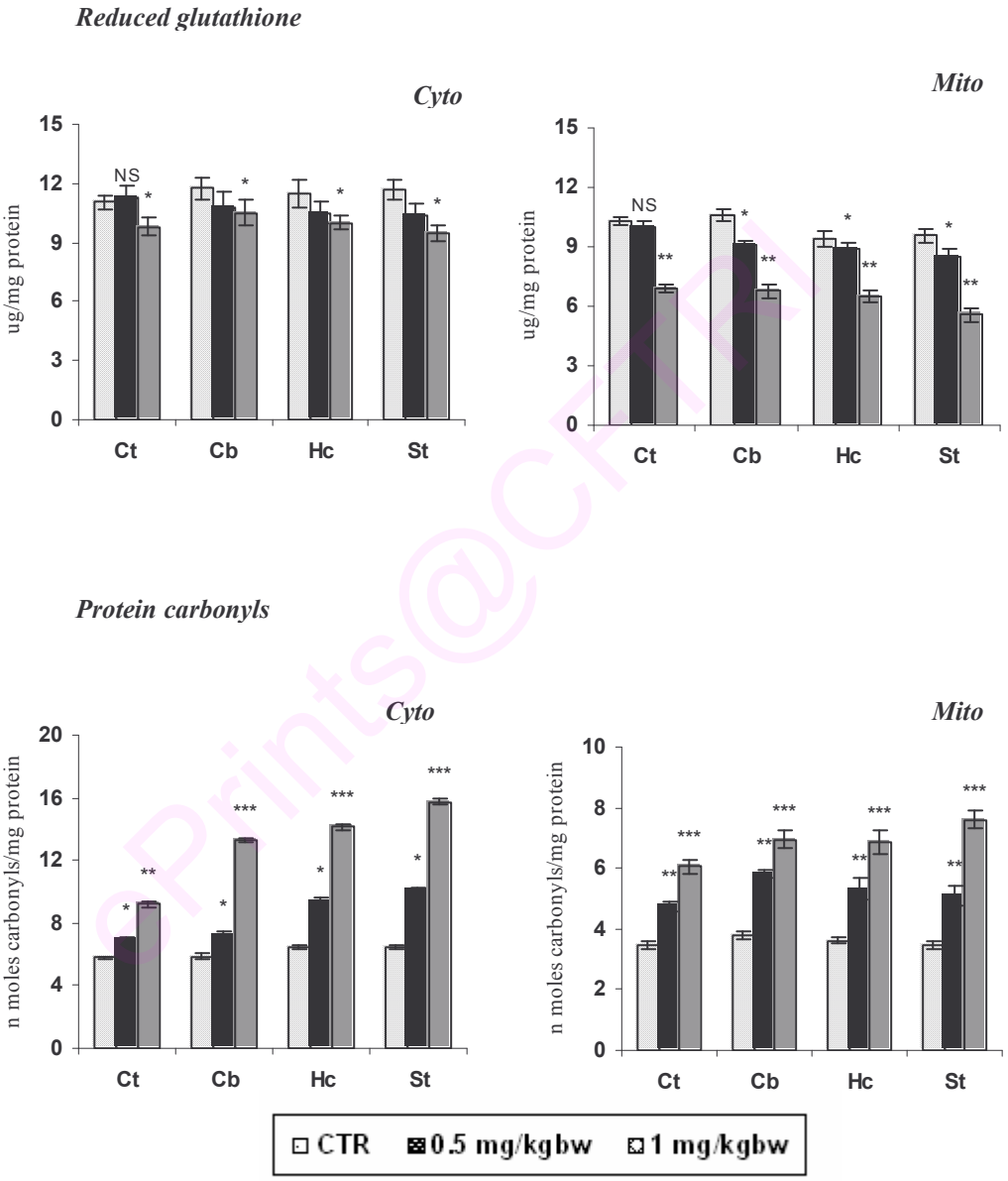
Rotenone (mg/kgbw)	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Cytosol				
Catalase [1]				
0	1.98±0.18	1.82±0.10	1.90±0.10	1.91±0.08
0.5	1.23±0.10*	1.36±0.08*	1.52±0.12*	1.22±0.12*
1.0	0.90±0.40**	0.96±0.05**	0.82±0.10**	0.76±0.08**
Glutathione S transferase [2]				
0	21.17±0.50	22.11±0.79	21.79±0.78	21.35±0.70
0.5	26.53±1.02*	26.21±1.34*	27.03±2.06*	28.03±2.06*
1.0	29.83±1.92**	31.21±1.54**	33.03±1.96**	35.03±1.96**
Glutathione peroxidase [3]				
0	25.76±0.94	25.86±0.13	26.47±1.02	26.43±1.06
0.5	22.94±1.55*	21.26±1.51*	21.37±0.76*	20.08±0.76*
1.0	19.20±0.75**	18.32±0.59**	17.15±0.96**	15.71±0.96**
Superoxide **dismutase** [4]				
0	34.24±2.24	35.15±1.67	35.56±1.32	34.15±1.24
0.5	50.22±2.01*	48.45±2.16*	50.56±2.13*	48.90±1.87*
1.0	58.90±3.34**	54.33±2.24**	59.05±2.54**	60.23±2.37**
Mitochondria				
Glutathione S-transferase [2]				
0	21.90±0.50	23.20 ±0.79	22.95±0.78	20.36±0.70
0.5	23.07±1.02	27.66±1.34**	26.03±2.06*	26.20±2.06*
1.0	27.17±1.92**	32.21±1.54**	30.03±1.96**	29.48±1.96*
Glutathione peroxidase [3]				
0	25.76±0.24	25.86±0.13	26.47±0.26	26.43±0.26
0.5	22.94±0.55*	23.36±0.51*	23.37±0.76*	20.08±0.76*
1.0	19.20±0.75**	19.32±0.59**	18.15±0.96**	17.71±0.97**
Superoxide dismutase [4]				
0	43.23±1.02	40.34±1.12	42.34±1.01	42.13±1.11
0.5	40.13±1.12*	36.78±1.15*	39.02±1.09*	38.56±1.10*
1.0	35.61±0.98**	30.17±1.03**	35.12±1.12**	31.05±1.08**

Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01).

1-μ moles H_2O_2 degraded/min/mg protein; 2-ηmoles conjugate/min/mg protein;
3- ηmoles NADPH oxidized /min/mg protein; 4-Units/mg protein

Fig.1.6

Reduced glutathione and protein carbonyl levels in cytosol and mitochondria of brain regions of mice administered rotenone for 7 days

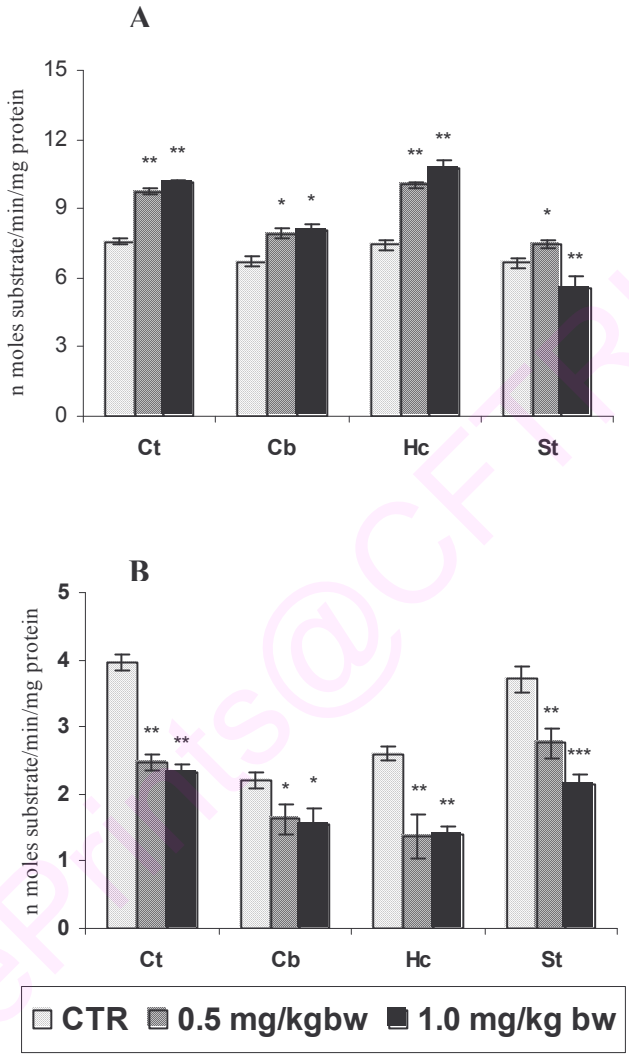


Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01; ***P<0.001; NS-not significant).

Cyto- Cytosol; Mito- Mitochondria
Ct- Cortex; Cb- Cerebellum; Hc- Hippocampus; St- Striatum

Fig 1.7

Effect of Rotenone on the activities of Acetylcholinesterase (A) and Butyrylcholinesterase (B) in brain regions of prepubertal mice.

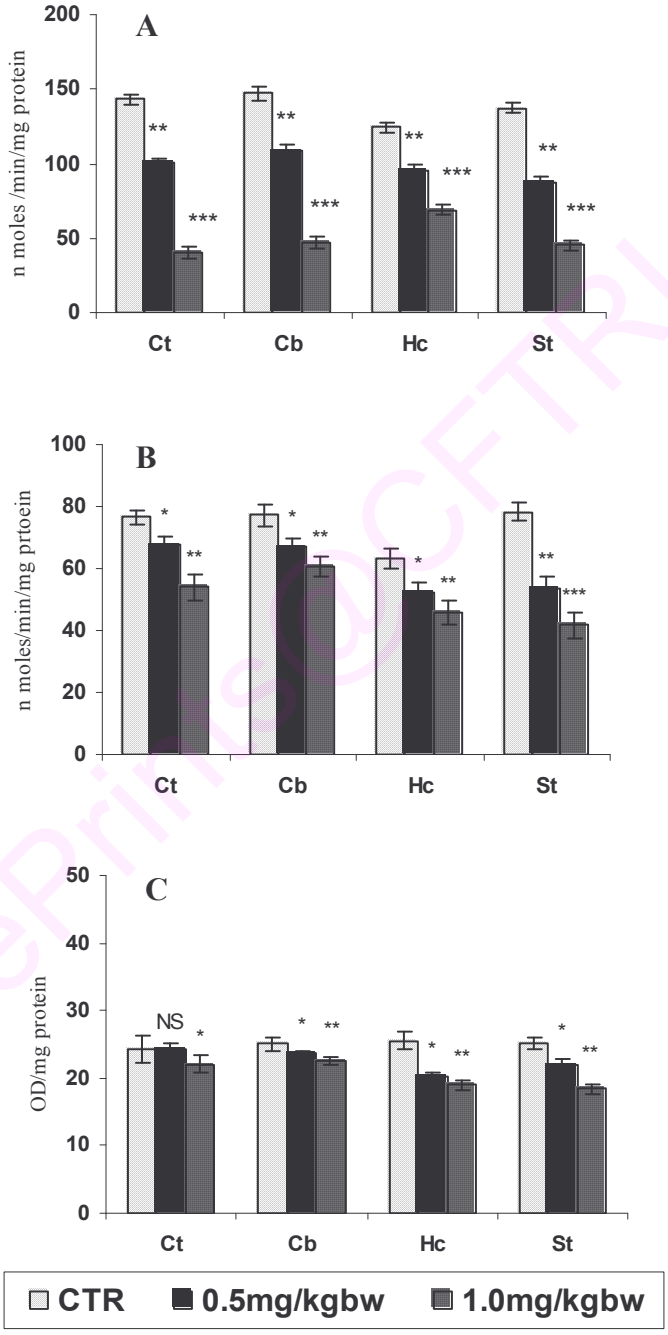


Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01; ***P<0.001)

Ct- Cortex; Cb- Cerebellum; Hc- Hippocampus; St- Striatum

Fig.1.8

Effect of rotenone administration on the activities of NADH-Ubiquinone oxidoreductase (A), NADH-Cytochrome C reductase (B) activity and MTT assay (C) in brain regions of prepubertal mice

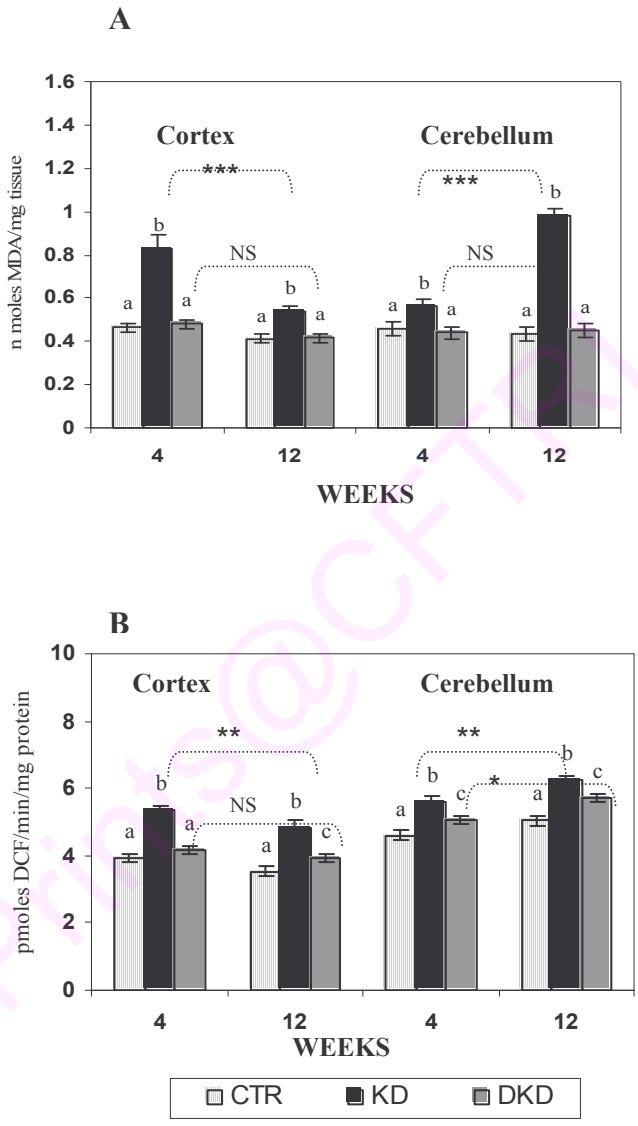


Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05; **P<0.01; ***P<0.001).

Ct- Cortex; Cb- Cerebellum; Hc- Hippocampus; St- Striatum

Fig.1.9

Status of oxidative markers in brain regions of adult mice fed KD and DKD for 4 and 12 weeks in diet.



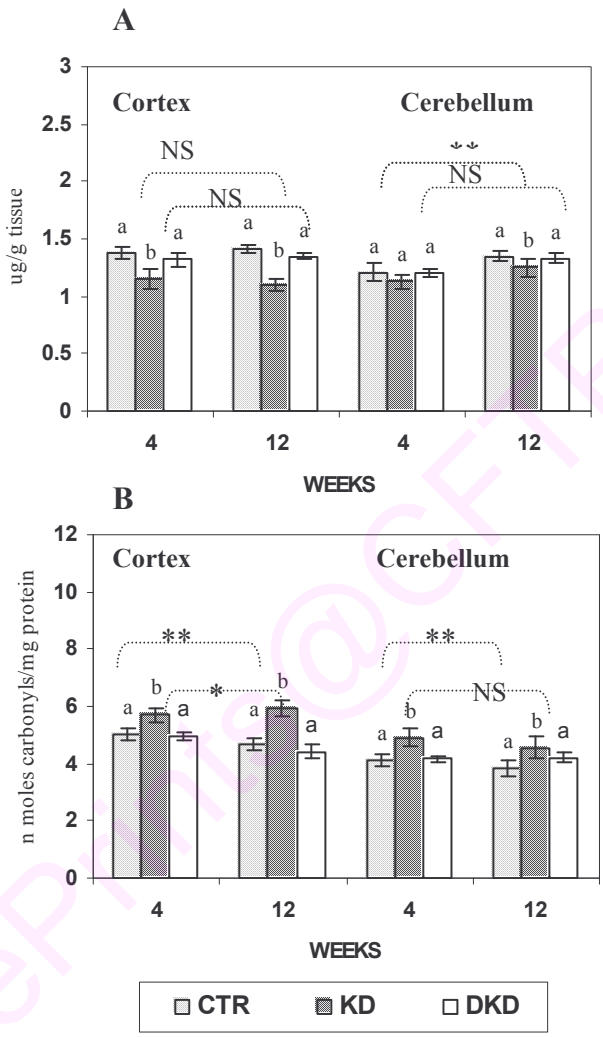
Values are mean ± SD of 6 determinations each; Data analyzed by ANOVA (P<0.05) appropriate to completely randomized design with six replicates. Means followed by different letters differ significantly according to DMRT; Comparison of parameters at different sampling times (wk 4 vs wk 12) are denoted with * (P<0.05), **(P<0.01), ***(P<0.001) and NS=no significance using nested lines above the bars.

MDA- malondialdehyde levels (**A),** ROS –Reactive oxygen species **(B**),

KD- Khesari dhal ; DKD-detoxified Khesari dhal.

Fig.1.10

Glutathione levels and protein carbonyls in brain regions of adult mice fed Khesari dhal (KD) and Detoxified Khesari Dhal (DKD) for 4 and 12 weeks in diet.



Values are mean ± SD of 6 determinations each. Data analyzed by ANOVA appropriate to completely randomized design with six replicates. Means followed by different letters differ significantly according to DMRT ($P<0.05$). Comparison of parameters at different sampling times (week 4 vs week 12) are denoted with **($P<0.01$) and NS= no significance using nested lines above the bars .

Table 1.6

Activities of antioxidant enzymes in cortex and cerebellum of male mice fed Khesari dhal (KD) and Detoxified Khesari dhal (DKD) in the diet for 12 weeks.

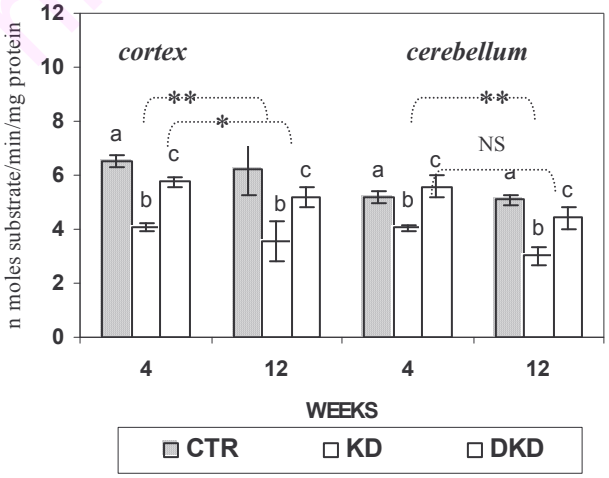
Parameters	Group		
	CTR	KD	DKD
Cortex			
CAT[1]	1.41± 0.35 [a]	1.05±0.08[b]	1.20±0.36 [c]
GST[2]	20.76±1.46[a]	18.99±1.60[b]	21.36±3.12 [c]
GPX[3]	12.85±0.85[a]	8.28±0.92[b]	11.92±0.58 [a]
SOD[4]	38.80±2.15[a]	57.63±5.79[b]	42.50±4.03 [c]
Cerebellum			
CAT[1]	1.21± 0.18[a]	1.02±0.29[b]	1.19±0.27 [a]
GST[2]	23.80±4.20[a]	21.34±0.27 [b]	23.41±4.03 [a]
GPX[3]	10.11±0.39[a]	9.01±0.76 [a]	10.07±0.39 [a]
SOD[4]	37.33±2.32 [a]	41.55±2.15 [b]	38.45±3.23 [a]

Values are mean ± SD (n=6); Data analyzed by ANOVA. Means followed by different letters differ significantly according to DMRT, (P<0.05).

1-µmoles H_2O_2 degraded/min/mg protein; 2-µmoles conjugate formed /min/mg protein
3-µmoles NADPH oxidized /min/mg protein ; 4-Units/mg protein.

Fig.1.11

Acetylcholinesterase activity in brain regions of mice fed KD and DKD incorporated diet for 4 and 12 weeks.



Values are mean ± SD of 6 determinations each; Data analyzed by ANOVA appropriate to completely randomized design with six replicates. Means followed by different letters differ significantly according to DMRT (P<0.05). Comparison of parameters at different sampling times (week 4 vs week 12) are denoted with **(P<0.01) and NS= no significance using nested lines above the bars .

Table1.7

Effect of dietary Khesari dhal on the oxidative stress markers in mitochondria and microsomes of different brain regions of male mice fed KD and DKD for 12 weeks.

Group	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Mitochondria				
MDA[1]				
CTR	12.3±0.50[a]	12.7±0.77[a]	13.7±0.50[a]	13.2±0.70[a]
KD	15.0±0.35[b]	17.0±1.02[b]	15.8±0.39[b]	16.1±0.90[b]
DKD	12.8±0.40[a]	12.0±0.78[a]	13.9±0.60[a]	13.2±0.80[a]
ROS [2]				
CTR	17.1±0.50[a]	17.3±0.69[a]	17.9±0.90[a]	17.3±0.80[a]
KD	25.5±0.50[b]	25.0±1.12[b]	27.9±1.10[b]	24.6±0.98[b]
DKD	18.1±0.36[c]	17.5±0.90[a]	18.1±0.67[a]	17.4±0.65[a]
PC [3]				
CTR	3.21±0.06[a]	3.34±0.08[a]	3.64±0.07[a]	3.34±0.09[a]
KD	4.12±0.09[b]	4.82±0.08[b]	4.87±0.08[b]	4.23±0.07[b]
DKD	3.35±0.10[a]	3.63±0.09[a]	3.81±0.08[a]	3.51±0.09[a]
Microsomes				
MDA[1]				
CTR	12.3±0.67[a]	13.45±0.60[a]	12.5±0.45[a]	13.3±0.50[a]
KD	15.6±0.70[b]	16.56±0.90[b]	15.7±0.49[b]	16.7±0.60[b]
DKD	12.1±0.50[a]	13.14±0.38[a]	13.5±0.50[c]	13.7±0.50[a]
ROS [2]				
CTR	13.5±0.45[a]	13.7±0.55[a]	13.5±0.45[a]	12.9±0.49[a]
KD	16.0±0.50[b]	17.8±0.56[b]	16.8±0.59[b]	15.8±0.66[b]
DKD	13.7±0.69[a]	13.0±0.80[a]	13.1±0.75[a]	13.1±0.70[a]
PC [3]				
CTR	2.80±0.04[a]	2.85±0.05[a]	3.15±0.05[a]	3.03±0.05[a]
KD	3.52±0.06[b]	3.61±0.05[b]	3.77±0.07[b]	3.52±0.06[b]
DKD	3.01±0.06[a]	2.95±0.04[a]	3.11±0.05[a]	3.16±0.06[a]

Values are mean ±SD (n=6). Data analyzed by one way ANOVA (*p<0.05).
Means followed by different letters differ significantly according to DMRT, (P<0.05).

MDA-Malondialdehyde; ROS-Reactive oxygen species
KD-30%; DKD-30%
1-η moles MDA/mg protein; 2-ρ moles DCF/min/mg protein
3-η moles carbonyls/mg protein

Table 1.8

Antioxidant enzyme activity in Mitochondria of different brain regions of male mice fed dietary KD for 12 weeks.

	Brain Regions			
Group	**Cortex**	**Cerebellum**	**Hippocampus**	**Striatum**
GPx[1]				
CTR	24.56±1.02[a]	26.78±0.90[a]	25.67±0.86[a]	26.23±0.89[a]
KD	18.78±1.12[b]	17.45±0.87[b]	18.90±0.79[b]	20.23±0.76[b]
DKD	23.95±1.25[a]	25.67±1.05[a]	26.57±1.10[a]	27.89±1.10[a]
GST[2]				
CTR	28.98±1.23[a]	26.70±1.12[a]	25.01±1.13[a]	26.70±2.13[a]
KD	34.45±1.02[b]	36.80±2.31[b]	35.67±2.13[b]	35.90±1.23[b]
DKD	28.90±1.35[a]	28.50±2.15[a]	28.65±2.10[a]	29.30±1.14[a]
SOD[3]				
CTR	45.67±1.80[a]	44.50±2.13[a]	44.76±2.67[a]	44.80±2.35[a]
KD	65.67±3.15[b]	59.80±3.45[b]	56.34±3.45[b]	58.90±3.15[b]
DKD	44.50±2.56[a]	46.35±3.14[a]	45.12±3.15[a]	46.35±2.33[a]

Values are mean ±SD (n=6). Data analyzed by one way ANOVA. (*p<0.05).
Means followed by different letters differ significantly according to DMRT, (P<0.05).

GPx-Glutathione peroxidase; GST-Glutathione S-Transferase; SOD-Superoxide dismutase

1-η moles NADPH/min/mg protein; 2-η moles conjugate/min/mg protein
3-Units/mg protein

5.0 DISCUSSION

With the increasing recognition that adolescence/prepubertal stage is a time of considerable neural restructuring and sculpting of the brain, there has been a growing interest in assessing whether this developmental transition is a vulnerable period for neurotoxicity. Recent evidences suggest that adolescence is the time of enhanced neurobehavioral toxic risk associated with exposure to drugs of abuse, therapeutic drugs, hormones and environmental toxicants (Spear, 2000). The brain of the adolescent undergoes pronounced sculpting and modification and whether this remodeling reflects a window of opportunity for unusual plasticity and recovery is still unexplored. The remodeling of adolescent brain can be a time of increased or decreased vulnerability to drugs/toxicant exposures and the sculpting of the adolescent brain disrupted by exposure to neurotoxicants, resulting in the production of a different brain may result in long-lasting alterations in neural functioning. This highly sensitive window of altered vulnerability or resilience varies with neural region/test substance (Spear, 2007). It is in this context, we chose to investigate the vulnerability of prepubertal mice to neurotoxicants with special focus on early oxidative stress.

3-NPA model in prepubertal mice: 3-nitropropionic acid (3-NPA) is a major mitochondrial toxin that effectively induces specific behavioral changes and selective striatal lesions in rats and non-human primates (Lee and Chang, 2005). Brain lesions produced by a systemic administration of 3-NPA are more or less specific of the striatum, although hippocampus, thalamus and brain cortex are also affected (Borlongan et al., 1997). For this reason, 3-NPA has been widely used as a suitable model for disruption of energy metabolism, and more specifically as a model of HD (Beal et al. 1993; Brouillet et al., 1999). The primary mechanism of action of this toxin involves the inhibition of complex II (succinate dehydrogenase (SDH) and this irreversible blocking decreases the levels of ATP leading to neuronal cell death (Tunez et al., 2004). The impaired energy metabolism can produce oxidative stress through the formation of reactive oxygen and nitrogen species (Schulz et al., 1995; Andreassen et al., 2001; Kim and Chan, 2001), which are suspected to be critically involved in neuronal cell death. However studies describing the susceptibility of prepubertal brain to 3-NPA-induced early oxidative impairments and associated mitochondrial dysfunctions are scarce.

In the present study, 3-NPA at the administered dose caused significant oxidative damage in heart, liver and brain of prepubertal mice, a finding consistent with earlier observations in adult

animals (Fu et al., 1995; Binienda and Kim, 1997; Gabrielson et al., 2001). 3-NPA administration resulted in mitochondrial complex II inhibition as evident from SDH inhibition. Marked enhancement in the levels of lipid peroxidation, ROS generation and hydroperoxide levels in both mitochondria and cytosol of striatum clearly demonstrates the induction of oxidative stress. A similar response was observed in cortex although less robust than striatum suggesting the differential vulnerability of striatal tissue. These responses are consistent with the *in vitro* results obtained in synaptosomes, mitochondria and slices of various brain regions in our study.

Growing body of evidences suggest that brain protein oxidation is an important event in AD (Butterfield and Lauderback, 2002). The notion that oxidative modification of proteins might precede neurodegeneration has been demonstrated after injections of 3-NPA in rat brain (La Fontaine et al., 2000), indicating a role for protein alterations participating in the processes of neuronal defects associated with NDD. In the present study, a clear increase in protein carbonyls was evident indicating enhanced protein oxidation which may lead to changes in protein function, chemical fragmentation or increased susceptibility to proteolytic attack (Droge, 2002).

The disruption in mitochondrial electron transport can result in reduction in GSH levels (Saravanan et al., 2005) which is evident in 3-NPA administered mice brain regions. Enzymatic defense against ROS is performed by antioxidant enzymes such as CAT, GSH-PX, GST and SOD. In the present study depletion in GSH levels was accompanied by significantly decreased activities of catalase, GSH-Px and SOD in mitochondria and cytosol of brain regions. Earlier reports have described reduction in SOD activity (Herrera-Mundo, 2006) and decreased GSH levels (Tadros et al., 2005) following 3-NPA administration, while certain others have reported an increase in various antioxidant enzymes in different brain regions (Binienda et al., 1998). This can be attributed to the differences in the dosage, route of administration and differences in the exposure window period. In our study, administration of 3-NPA resulted in significant reduction in the activities of antioxidant enzymes, while GST activity was increased significantly in both striatum and cortex mitochondria/ cytosol suggesting the participation of this enzyme under 3-NPA intoxication. Collectively these data suggest that 3-NPA administration to prepubertal mice resulted in the induction of significant early oxidative damage as evidenced from both *in vitro* and *in vivo* results.

Rotenone model in prepubertal mice: Rotenone is a lipophilic compound and is a specific inhibitor of mitochondrial complex I. Inhibitors of complex I increase ROS formation and this may

affect vital mitochondrial parameters like ATP production (Sherer et al., 2003). In all the models used to reproduce PD symptoms including rotenone, oxidative stress was one of the major factors that contributed to dopaminergic neuronal lesions (Maharaj et al., 2004). The rotenone model suggests that dopaminergic neurons may be more vulnerable than other cell populations to the effects of complex I inhibition, and several observations are consistent with a higher sensitivity of dopaminergic neurons to oxidative stress (Di Monte et al., 2002). The mechanisms of selective damage may be inter-related, because inhibition of mitochondrial complex I is likely to result in augmented production of ROS (Mc Lennan, 2000). Evidence of an association between mitochondrial impairment and selective degeneration of dopaminergic neurons has prompted the suggestion that inhibitors of mitochondrial complex I should be screened as potential environmental factors in PD (Betarbet et al., 2000).

Though extensively studied, the effects of rotenone on the induction of early oxidative damage in prepubertal mice brain regions are not well known.In the present study, administration of rotenone to prepubertal mice resulted in significant increase in oxidative stress measured in terms of elevated MDA, ROS and hydroperoxide levels. Further there was a clear decrease in the levels of GSH which is in accordance with the earlier studies (Tada-Oikawa et al., 2003; Mao et al., 2007). Moreover the depletion of GSH in the model was accompanied by decrease in catalase (cytosol) and GSH-Px (cytosol and mitochondria) of brain regions. Further, while an increase in the activity of SOD was observed in cytosol, a decrease was observed in mitochondria. These changes may be due to the increased hydroperoxides which needs to be removed from the system. There was a concomitant increase in GST activity indicative of the perturbations in antioxidant defense systems which may probably be a response towards increased ROS as GST catalyses the reaction of the thiol group of GSH with a wide variety of second substrates to form thioesters (Keen and Jakoby, 1987). The decrease in GSH in the model group could also reflect the increase in GST activity.

Further there was a decreased capacity of mitochondria to reduce MTT, suggesting early oxidative stress as well as a metabolic compromise as a result of mitochondrial dysfunctions. A decrease in complex I enzyme activity observed demonstrates mitochondrial dysfunctions induced by rotenone. Interestingly, increase in the activity of cholinergic enzymes (AChE and BChE) demonstrates the effect of rotenone on cholinergic functions of brain. There have been reports that

chronic rotenone administration in rats causes a significant decrease in the number of striatal neuronal subtypes, including cholinergic inter neurons (Hoglinger et al., 2003). The observations by Bonsi et al., (2004) provide the first description of the early consequences of mitochondrial complex I inhibition occurring at cellular level in striatal cholinergic inter neurons and have been suggested as a possible initial and sufficient trigger for neuronal death. Interestingly, recent studies demonstrate that the levels of the striatal vesicular acetylcholine transporter, a reliable marker of cholinergic activity, is severely reduced in brain tissue from PSP patients, a finding consistent with the loss of cholinergic inter neurons (Suzuki et al., 2002). Our data provide comprehensive evidences suggesting induction of early oxidative stress and mitochondrial dysfunctions in brain regions of prepubertal mice.

Dietary Khesari dhal model in growing mice: Khesari dhal (KD) which provides a nourishing and inexpensive component of daily diet among poor people in certain areas of Asia and Africa is reported to cause Neurolathyrism (NL) predominantly among males (Getahun et al., 1999; Roy, 1988). The criteria for selection of mice was based on earlier reports of their increased susceptibility to β-ODPA induced neurotoxicty (Rao et al., 1967; Vardhan et al., 1997; Sriram et al., 1998; Ravindranath, 2002). Interim sampling (wk 4) was included to ascertain any early oxidative damage and follow its progression.

Our major finding that dietary intake of KD in mice induces a significant increase in MDA and enhanced ROS levels in cortex and cerebellar regions clearly suggest induction of oxidative stress *in vivo.* In contrast, the status of LPO was normal in mice fed detoxified KD which is indicative of a clear absence of oxidative stress induction. In the cortex of KD brain oxidative impairments were evident in cytosol, mitochondria and microsomal fractions suggesting the propensity of KD to induce general oxidative stress. Further, the persistent nature of oxidative impairments among KD fed mice, allow us to logically speculate that oxidative stress mechanism/s may at least in part contribute significantly towards the expression of neurotoxicty subsequently. Our data also indicate that the process used for detoxification of KD has substantially removed the neurotoxin (β-ODAP) shown to be chiefly responsible for the induction of neurotoxic effects by earlier workers (Rao, 1977; Spencer et al., 1986; Ravindranath, 2002). This observation is consistent with the chemical analysis data which showed nearly 85-90% removal of the neurotoxin.

In KD fed mice, significantly enhanced MDA levels in brain regions at week 4, suggested the occurrence of oxidative impairments during early phase. While both cortex and cerebellum appeared to be equally susceptible to oxidative damage initially, markedly enhanced MDA levels at wk 12 in cerebellum is suggestive of its increased vulnerability to KD. The reason for the differential susceptibility of brain regions to KD induced oxidative stress, is however not clear and merits further study. Nevertheless, it is reasonable to speculate that the increased susceptibility of cerebellum to oxidative damage may play a vital role in the development of paraparesis of legs and dragging movement during severe NL conditions (Marieb, 1997). Since cerebellum is the center of co-ordination of voluntary motor movements, balance and muscle tone, specific cerebellar injury could result in a gradual deterioration.

In the present study, in KD fed mice, enhanced lipid peroxidation in brain regions were accompanied by concomitant alterations in the activities of antioxidant enzymes and reduced GSH levels. In cortex, decrease in activities of CAT and GSH-Px was coupled with an elevated SOD activity suggesting the possible increase in the production of H_2O_2. In contrast, KD induced only marginal decrease in the activities of CAT, GSH-PX and marginal increase in SOD activity in cerebellum. Further studies are essential to understand the underlying reasons for the differential induction of oxidative impairments in brain regions and the increased susceptibility of cerebellum. We also observed enhanced oxidative damage in mitochondria and microsomes as evident from increased LPO and ROS generation. This increased oxidative stress was associated with perturbations in antioxidant enzymes and reduced glutathione levels suggesting the possible role of mitochondrial oxidative stress in the development of NL in humans.

GSH is among the most abundant soluble antioxidant molecule in the brain which plays an important role in the counteracting of ROS (Dringen, 2000). GSH reacts directly with radicals in non- enzymatic reactions and *in vitro* evidences have shown that depletion of GSH in brain cells damages mitochondria and increases lipid peroxidation (Sen, 1997). In the present study, KD consumption caused a significant depletion in GSH in cortex, although there was only a marginal decrease in cerebellum. There was no change in GSH levels in both cortex and cerebellum in DKD fed mice. The decrease in GSH availability in the brain is believed to promote mitochondrial damage *via* increased ROS (Mytilineou et al., 2002). This line of thinking is consistent with the evidences of mitochondrial dysfunction in brain as a consequence of oxidation of protein thiol

groups as a result of elevated ROS levels (Ross et al., 1989; Ravindranath, 2002). Recent findings such as selective loss of mitochondrial complex I activity in the fronto-parietal cortex regions in mice following β-ODAP exposure at early time points have led to our hypothesis that mitochondrial dysfunctions may be one of the primary event in the manifestation of its toxicity (Ravindranath, 2002). Interestingly, β- ODAP was also shown to inhibit X_c^- system, which leads to the blocking of the import of L-cystine and a resultant decrease in intracellular concentrations of L-cysteine. As L-Cysteine is the rate limiting precursor in the biosynthesis of the antioxidant glutathione, the inhibition of transporter ultimately leads to decreased levels of glutathione and thus a corresponding increase in oxidative damage (Patel et al., 2004). Further it has also been reported that presence of reducing equivalents of GSH is critical for recovery of complex I function following exposure to β- ODAP, since Glutaredoxin which regenerates protein thiols from glutathione mixed disulfides requires reducing equivalents of GSH (Kencheppa et al, 2003). Hence the diminution in reduced GSH levels in the brain of KD fed mice would certainly favor the oxidative stress condition.

In the present study, further evidence of KD induced oxidative stress was obtained in terms of enhanced levels of protein carbonyls in brain which reflects a high rate of protein oxidation consistent with high LPO state. Among the various oxidative modifications of amino acids in proteins, protein carbonyl formation is extensively employed as an early marker for protein oxidation (Levine et al., 1990: Grune et al., 2004). It also reflects a very low rate of oxidized protein degradation and or low repair activity since oxidized forms of some proteins and proteins modified by LPO products are not only resistant to proteolysis, but also can inhibit the ability of proteases which degrade the oxidized forms of other proteins and form aggregates (Dalle-Donne, 2003). Our observation of enhanced protein carbonyls in the cortex and cerebellum of KD mice is consistent with the reports of accumulation of protein carbonyls in several human diseases including NDD (Smith et al., 2000). However, normal protein carbonyl levels in the brain of DKD fed mice suggested the absence of oxidative damage to proteins.

In the current study, altered cholinergic function was clearly evident in KD fed mice, since we observed substantial reduction of AChE activity in both cortex and cerebellum. However, lesser degree of AChE reduction was also seen in DKD mice. While there have been no data on the AChE inhibitory activity of β-ODAP *in vivo*, a recent study in rats and guinea pigs have reported alterations in brain neurotransmitter levels following chronic consumption of Khesari Dhal (Amba et

al., 2002). AChE, the enzyme found in the synaptic clefts of the cholinergic synapses, cleaves the neurotransmitter acetylcholine into its constituent acetate and choline thus limiting the size and duration of the post synaptic potential. AChE has been implicated in cholinergic actions as well as in neuronal death. The enzyme is also shown to affect cell proliferation, differentiation and response to various insults including stress (Amba et al., 2002). Recent evidences implicate that oxygen free radicals or ROS can alter the structure of AChE (Calderon et al., 1998), and also inhibit its activity (Tsakiris et al., 2000). Interestingly, in humans, reduction of cerebral AChE activity is demonstrated in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Weiner et al., 1994). Since AChE is a marker of cholinergic function and cognitive deficits, the cognitive impairments reported earlier among NL patients (Paleacue et al., 1999) may be attributed to AChE inhibition in brain. However, further studies are necessary to establish the correlation between oxidative stress, AChE inhibition and NL related cognitive deficits.

6.0 SUMMARY

1. 3-nitropropionic acid exposure *in vitro* caused a marked concentration-dependent induction of oxidative stress in mitochondria and synaptosomes of various brain regions of prepubertal male mice while, more robust response was evident in brain slices.

2. Administration of 3-nitropropionic acid (i.p., 50 and 75 mg/kg bw/d for 2d) to prepubertal male mice resulted in significant reduction in the activity of SDH in organs *viz..,* brain, heart and liver accompanied with dose-dependent enhancement in various markers of oxidative stress, compromised antioxidant enzyme activities and depleted levels of glutathione in cytosol and mitochondria suggesting a state of oxidative stress *in vivo.*

3. 3-NPA caused a more robust induction of oxidative stress in striatum compared to the cortex as evidenced by elevated lipid peroxidation, protein oxidation, activities of antioxidant enzymes and reduced glutathione. Higher degree of inhibition of SDH activity was evident in striatal region.

4. Rotenone administration (i.p., 0.5 and 1.0 mg/kg bw/d for 7 d) resulted in a marked dose dependent elevation in lipid peroxidation, ROS generation and protein carbonyls in brain regions of prepubertal mice. The effect of rotenone on enzymic antioxidants comprised of

decreased catalase/GSH-Px and elevated SOD/GST activities in cytosol and mitochondria of all brain regions. Depletion of glutathione levels was a common feature in both cytosol and mitochondria of all brain regions.

5. Mitochondrial dysfunctions in cortex and striatum among rotenone administered mice comprised of: decreased mitochondrial viability as measured by MTT assay, reduction in the activities of complex I and complex I-III.

6. Significant increase in the activities of cholinergic enzymes, AChE and BChE were also evident among brain regions of rotenone exposed mice.

7. Growing male mice fed Khesari dhal (30% in diet) exhibited significant elevation in markers of oxidative stress as suggested by enhanced MDA levels and ROS generation. Concomitantly significant alterations in the activity of various antioxidant enzymes (CAT, GST, GSH-PX and SOD) were also discernible along with increased protein carbonyl levels in brain regions. However no such alterations were noticeable in mice fed detoxified KD.

8. KD fed mice showed marginal depletion of GSH levels in all brain regions while detoxified KD fed mice showed normal levels.

9. Among KD fed mice, both mitochondria and microsomal fractions of brain regions were also subjected to significant oxidative stress induced dysfunctions as determined by elevated levels of oxidative markers, diminution of GSH and alterations in activity of antioxidant enzymes.

10. KD fed mice showed significant inhibition of AChE activity in brain regions, clearly suggesting cholinergic dysfunctions, while no significant alterations were evident in DKD fed mice brain regions.

1.0 INTRODUCTION

Centella asiatica (L) Urban (Umbelliferae) a plant native to countries like India, Srilanka, Madagascar, South Africa and Malasia (Kartnig, 1988) is used in the Ayurvedic system of medicine to treat various ailments like headache, body ache, insanity, asthma, leprosy, ulcers, eczemas and wound healing (Suguna et al., 1996; Shukla et al., 1999). Recent studies have shown various neuropharmacological effects with *Centella asiatica* (CA) and the major effects described in experimental animals comprise of memory enhancement and cognitive function (Veerendra kumar and Gupta, 2002; Wijeweera et al., 2006), increased neurite elongation *in vitro* and acceleration of nerve regeneration *in vivo* (Soumyanath et al., 2005). CA has also been shown to have immunostimulatory activity *in vitro* (Wang et al., 2005) as well as adjuvant properties to antiepileptic drugs along with an added advantage of prevention of cognitive impairment. Further, oral administration of an aqueous extract of CA is demonstrated to reduce brain malondialdehyde levels, increase glutathione levels in whole brain of adult rats (Veerendrakumar and Gupta, 2002) and improve learning and memory (Gupta et al., 2003).

Notable bioactive compounds of CA are the triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins *viz..,* madecassic and asiatic acid (Mangas et al., 2006). CA contains numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempferol, catechin, rutin, and naringin which are known antioxidants (Zainol et al., 2003; Hussin et al., 2007). The whole plant has been shown to improve general mental ability of mentally retarded children (Kakkar et al., 1984). Treatment with CA extract during post natal period influenced neuronal morphology and promote higher brain functions of juvenile and young adult mice resulting in enhanced learning and memory (Rao et al., 2005). Oxidative stress due to increase in free radical generation or impaired endogenous antioxidant mechanism is an important factor that has been implicated in various neurodegenerative diseases (Halliwell, 2006). The brain is highly susceptible to free radical damage because of its high utilization of oxygen and the presence of relatively low concentration of antioxidant enzymes and free radical scavengers. There have been efforts to find various therapeutic agents (both natural and synthetic) that could reduce oxidative stress and improve memory (Cho, 2006). It has been postulated that the mechanistic basis of the neuroprotective activity of antioxidants does not rely only on the general free radical

trapping or antioxidant activity *per se* in neurons, but also on the suppression of genes induced by pro-inflammatory cytokines and other mediators released by glial cells (Wang et al., 2006).

3-Nitropropionic acid (3-NPA), a mitochondrial toxin, causes preferential neuronal degeneration in the striatum and produces anatomical changes similar to Huntington's disease in experimental animals (Beal et al., 1993). Enhanced ROS generation and MDA levels have been demonstrated in brain regions of rats challenged with 3-NPA indicating the vital role of oxidative stress in the manifestation of neurotoxicity (Fu et al., 1995). Earlier, 3-NPA induced neurotoxicity has been shown to be attenuated by taurine and S-allylcysteine (Tadros et al., 2005; Herrera-Mundo et al., 2006). However, attempts to modulate 3-NPA- induced oxidative stress either *in vivo* or *in vitro* by specific phytochemicals have not been attempted.

Consumption of the Khesari dhal (KD) for prolonged periods leads to a neurodegenerative disorder called as NL in humans (Selye, 1957) which is manifested by damage of upper motor neurons, degeneration of anterior horn cells and loss of axons in pyramidal tracts of lumbar spinal cord (Streifler et al., 1977). KD contains the neurotoxin β-ODAP and it exerts neurotoxicity through the AMPA subclass of glutamate receptors (Ross et al., 1989) and causes inhibition of mitochondrial complex I (NADH-ubiquinone oxidoreductase) in motor cortex and lumbosacral cord (Sriram et al., 1998) of mice. More over it has been postulated that oxidative stress plays a key role in the development of NL (Shinomol and Muralidhara, 2007).

Accordingly the modulatory effects of Centella asiatica against 3-NPA induced oxidative dysfunctions in brain regions of prepubertal mice were characterized *in vitro* and *in vivo.* The results are presented separately under two sections. In **section A**, there are two subsections. In *subsection (i*) Studies on the propensity of dietary *CA* leaf powder to modulate endogenous levels of oxidative markers and response of antioxidant defenses is presented, where as in *subsection (ii) Results on the* modulatory effect of CA leaf powder on Khesari dhal induced oxidative dysfunctions are elaborated. In **Section B** there are two sub sections where in *subsection (i)* Evidences on the *in vitro* antioxidant potential of an aqueous extract of CA both in chemical and biological systems are presented and *subsection (ii)* describes the prophylactic efficacy of CA extract against 3-NPA induced early oxidative stress and mitochondrial dysfunctions.

2.0 OBJECTIVE

The primary focus of these investigations was to obtain evidences to show that *Centella asiatica* leaf powder *per se* can reduce the basal levels of oxidative markers in mitochondria and cytosol of brain regions of normal prepubertal mice as well as protection against Khesari dhal induced oxidative alterations in mitochondria and cytosol of brain regions. Further the prophylactic propensity of CA extract against 3-NPA induced oxidative alterations in mitochondria was also investigated.

3.0 EXPERIMENTAL DESIGN

SECTION A

(i) Propensity of *Centella asiatica* leaf powder to modulate endogenous oxidative markers in brain of prepubertal mice

Preparation of Centella asiatica supplemented diet

Centella asiatica leaves along with the petiole were shade dried and powdered in a mill and sieved through a mesh (400 micron). Commercially available pellet diet for mice was powdered in a mill to coarse powder and CA leaf powder was mixed at two dietary levels (0.5 and 1.0 %).

Experimental protocol

Prepubertal male mice were randomly assigned to control and treatment groups. While the control mice received normal powder diet, mice of the treatment groups received CA (0.5 and 1.0) incorporated diet for 30 days. Daily food intake was recorded by weighing the residual diet. Body weight gain was recorded every week. In order to assess whether CA administration has any measurable effect in short time, an interim autopsy and sampling was conducted after 15 days. Terminally, mice of both control and treatment groups were sacrificed by cervical dislocation and vital organs *viz,* brain, liver and kidney were excised, blotted and weighed. Brain regions [cortex (Ct), cerebellum (Cb), hippocampus (Hc) and striatum (St)] were dissected out

and processed to obtain cytosolic and mitochondrial fractions. The following biochemical investigations were conducted in order to determine the modulatory effect of CA.

Modulation of oxidative markers in brain regions

The status of oxidative markers *viz.,* lipid peroxidation (quantified as malondialdehyde levels), ROS generation (using a fluorescent dye-dihydrodichlorofluorescein) and hydroperoxide levels (quantified using FOX reagent) were assessed in all brain regions. Further, protein carbonyl content was determined as a marker of protein oxidation in the brain regions.

Perturbations in GSH and thiols

The levels of GSH, total thiols and non-protein thiols were determined in freshly prepared samples of cytosol and mitochondria of all the brain regions.

Perturbations in antioxidant enzymes

The effect of CA on the status of antioxidant enzymes status was assessed by measuring the activities of selected enzymes *viz.,* catalase, GSH-Px, GST and SOD in cytosol and mitochondria. The activity of Thioredoxin reductase (TRR) was also measured in mitochondria.

Effect on cholinergic systems

The modulatory effect of CA on cholinergic function in brain was assessed by measuring the activity of AChE in different brain regions.

(ii) Efficacy of CA leaf powder to ameliorate Khesari dhal (KD) induced oxidative dysfunctions

Preparation of KD –incorporated diet

Khesari Dhal was powdered and mixed with the commercial diet at 30% level.

Experimental protocol

Adult male mice (CFT-Swiss strain, 8 weeks old) were assigned to four groups (n=6). Group I mice were fed with normal diet (control group). Group II mice were given CA (1%) incorporated diet, group III mice were provided with Khesari dhal (30%) incorporated diet and group IV mice were fed KD (30%) incorporated diet supplemented with CA (1%) for 30 days.

Body weight gain and food intake were monitored through out the experimental period. Terminally, mice of all groups were sacrificed and biochemical estimations were carried out in mitochondria and cytosol of different brain regions.

Modulatory effect of CA on oxidative damage

The protective efficacy of CA on KD induced oxidative damage was assessed by determination of selected oxidative markers *viz.,* Lipid peroxidation, ROS generation and hydroperoxides levels. Further the protein oxidation was quantified as protein carbonyls in mitochondria and cytosol of all brain regions.

Antioxidant status: enzymic and non-enzymic

The modulatory effect of CA on KD induced perturbations in antioxidant status was ascertained by measuring both reduced and oxidized glutathione levels, total thiols, non–protein thiols and activity of antioxidant enzymes *viz*., catalase, GSH-Px, GST and SOD in cytosol and mitochondria.

Amelioration of mitochondrial dysfunctions and LDH activity

In order to determine the viability of mitochondria, MTT assay was conducted in freshly isolated mitochondrial samples. Further the activity of Lactate dehydrogenase activity was measured in cytosol of all brain regions. The activity of Na^+ K^+ ATP-ase was estimated in freshly prepared mitochondria. Since β-ODAP toxin present in KD is a known inhibitor of complex I enzyme, the activity of this enzyme complex was also determined in all brain regions.

Acetylcholinesterase activity

Studies were carried out to determine the effect of CA supplementation on KD induced inhibition of the cholinergic enzyme, AChE in cytosol of brain regions.

SECTION B

(iii) Antioxidant potential of CA aqueous extract *in vitro*

Preparation of CA aqueous extract

CA leaves were shade dried and later extracted using boiling water (8 parts W/V) and lyophilized to green brown powder.

Chemical systems

To determine the antioxidant potential of *Centella asiatica* aqueous extract, various chemical assay systems were employed such as DPPH radical scavenging, nitric oxide scavenging, hydroxyl radical scavenging and superoxide scavenging, reducing power and protection against deoxyribose oxidation. Total polyphenol content in extract was also estimated.

Biological systems

In vitro exposure of synaptosomes/mitochondria: Synaptosomes (whole brain and cortex) or mitochondria (cortex, cerebellum, hippocampus, striatum) were pre incubated with CA extract (0.5 and 1µg/ml) for 30 minutes. Further untreated and CA pretreated synaptosomes and mitochondria were exposed to 3-NPA (2mM) for 1hr. The extent of oxidative stress induction was quantified in terms of malondialdehyde formation, reactive oxygen species generation and hydroperoxide levels as per the methods described in Materials and Methods. Similar experiments were also conducted in slices obtained from various brain regions.

(iv) Prophylactic efficacy of CA extract: 3-NPA induced oxidative stress in brain regions of prepubertal mice

Experimental protocol

Preliminary study: The effect of CA prophylaxis on endogenous oxidative stress markers in brain regions was investigated employing three doses *viz..,* 1.25, 2.5 and 5.0, mg/kg bw/day for 10 consecutive days to prepubertal male mice. For determinative studies only the highest dose was selected.

Determinative study: Dosages of 3-NPA and CA aqueous extract were selected on the basis of previous study conducted in our laboratory. Prepubertal male mice (4 wk old) were orally administered with an aqueous extract of *Centella asiatica (* 5 mg /kg bw) for a period of 10 days (Prophylaxis group). Both normal and mice given CA prophylaxis were injected 3- NPA (i.p. 75mg/kg bw/d) on days 9 and 10 and sacrificed on day 11. Mice given physiological saline served as the normal controls. During the experimental period, food intake and individual body weights were monitored daily. The induction of mitochondrial oxidative damage and associated dysfunctions were determined in striatum and other brain regions.

Effect of CA extract on 3-NPA-induced oxidative damage

In order to determine the protective efficacy of CA on 3-NPA induced oxidative damage in brain regions, the extent of ROS generation, levels of lipid peroxidation, hydroperoxides and protein carbonyls were measured in mitochondria and cytosol of different brain regions.

Activity of antioxidant enzymes *viz.,* catalase, GSH-Px, GST and SOD were measured in cytosol while GSH-Px, GST and SOD were measured in mitochondria obtained from the brain regions. GSH and GSSG levels, total thiols and non–protein thiols were also determined.

Amelioration of mitochondrial dysfunctions

MTT assay: To determine the modulatory effect of CA prophylaxis on 3-NPA induced alterations on mitochondrial viability, MTT assay was carried out in fresh mitochondrial samples of different brain regions.

Na^+ K^+ ATP-ase: To determine the ameliorative potential of CA prophylaxis on 3-NPA-induced membrane damage, Na^+ , K^+ ATP-ase activity was measured.

Membrane potential: In order to determine the effect of 3-NPA on brain mitochondrial voltage gaited channels, mPT was measured in terms of mitochondrial swelling in fresh mitochondria.

Activity of mitochondrial enzymes

The activities of selected marker enzymes *viz*., citrate synthase, malate dehydrogenase and succinate dehydrogenase were measured in mitochondria from brain regions of control and 3-NPA administered animals.

Electron transport chain enzymes

To assess the ameliorative effect of CA prophylaxis against 3-NPA induced alterations in the activity of ETC enzymes, the activity levels of complexes *viz.,* Complex II (succinate ubiquinone oxidoreductase), Complex II-III (succinate-Cytochrome C reductase), Complex I-III (NADH-cytochrome C reductase) were determined in different brain regions.

Cholinergic activity

In order to determine the effect of CA prophylaxis on 3-NPA induced alterations in the cholinergic enzymes, the activities of AChE and BChE were measured in cytosol of brain regions.

4.0 RESULTS

SECTION A

(i) Propensity of *Centella asiatica* leaf powder to modulate endogenous oxidative markers in brain of prepubertal mice

Growth characteristics food intake and organ weight

There was no significant alteration in body weights among mice of CA group except for a marginal decrease at week 4. Neither consumption of food nor organ weights were affected among mice fed both the doses of CA (Data not shown).

Effect of CA on oxidative markers in brain regions

Interim sampling data: Analysis of brain samples of prepubertal mice fed CA at 15 days showed significant decrease in various oxidative stress markers. Varying degrees of reduction in MDA levels were evident in all brain regions (Ct-14%; Cb-26%, Hc-13%; St-16%). There was a similar trend of decrease in ROS generation (Ct-22%; Cb-24%; Hc-22%; St-19%) (Data not shown).

Terminal sampling data: In general, brain regions of mice fed CA diet showed markedly reduced MDA levels in both cytosol and mitochondria (Fig.2.1). In cytosol, the reduction was dose related, and at higher dose the percent reduction varied between 23-27%. Similar trends of reduced MDA levels were evident in mitochondrial fractions of brain regions. The ROS levels in brain regions of mice fed CA were also significantly diminished in both cytosol and mitochondria (Fig.2.1). At the

higher dose, the decrease in ROS levels was more consistent (Ct-26%; Cb-22%; Hc-33%; St-25%) in brain cytosol while, significant decrease in ROS levels was evident (Ct-30%; Cb-31%; Hc-35%; St-28%) in mitochondria. There was a general reduction in basal HP levels in brain regions of mice fed CA and the reduction varied between 42-46%. A similar trend of decrease (Ct-24%; Cb-36%: Hc-21%: St-20%) was also evident in mitochondria, even though the decrease was less robust (Fig.2.1).

Protein oxidation : Protein carbonyls levels

There was a significant decrease in basal levels of protein carbonyls in both cytosol and mitochondria of all the brain regions of mice fed CA (Fig.2.2). At the higher dose, the basal levels of protein carbonyls were significantly diminished in cytosol (Ct-36%; Cb-41%; Hc-41%; St- 41%) and a similar trend of decrease (22-28%) was also evident in mitochondria.

Reduced glutathione (GSH) and thiol levels

In general, a moderate increase in GSH levels were observed in the brain regions of CA fed mice (Fig.2.3). Although the increase was marginal at the lower dose, significant elevation was evident at the higher dose in both cytosol (21-28%) and mitochondria (22%). A marginal increase of total thiols as well as non- protein thiols was observed in brain regions at the higher dose of CA. The increase varied between 13-25% in all brain regions (Fig.2.3). A similar trend of increase in total thiols (19-28%) and non-protein thiols (23-36%) were also observed in mitochondria.

Antioxidant enzyme activities

CA consumption caused varying degree of enhancement in the activities of antioxidant enzymes in all brain regions (Fig.2.4). In cytosol (at higher dose), the activity of CAT significantly increased (33-37%) while the GSH-Px activity was increased significantly only at the higher dose (24-37%). While, SOD activity at the high dose was uniformly elevated (39-49%) in all brain regions, the activity of GST showed no significant changes. In mitochondria, the activities of both GSH-Px and SOD were uniformly elevated (by 25%) in all brain regions, while the activity of GST was unaffected. The activity of TRR was also elevated significantly in mitochondria in the brain regions (Fig.2.4).

Acetylcholinesterase (AChE) activity

CA consumption significantly enhanced the AChE activity in all brain regions. While the low dose caused only a marginal elevation in cerebellum and hippocampus (12%), the elevation was higher in cortex (20%). However at the higher dose, a consistent increase was evident irrespective of the brain region (Ct-40%; Cb-36%; Hc-38%; St-22%) (Fig.2.5).

(ii) Efficacy of CA leaf powder to ameliorate Khesari dhal induced oxidative dysfunctions

Food intake, growth and organ weights

No significant changes were observed in food intake in any of the treatment groups throughout the experiment period (Data not shown). A marginal decrease (7%) in body weight, reduction in liver weight (23%) and kidney weights (29%) was observed among mice fed KD. No significant change in organ weights were observed in mice fed CA *per se* while, CA supplementation conferred significant protection against these perturbations induced by KD (Data not shown).

Amelioration of oxidative stress markers in various brain regions

In general, CA reduced the basal levels of oxidative stress markers in both mitochondria (Table.2.1) and cytosol (Fig.2.6) of brain regions. Interestingly, the levels of MDA, ROS and HP in brain region cytosol and mitochondria were elevated among KD fed mice. While 34-47% increase in MDA levels were observed in mitochondria, there was 13-53% increase in cytosol. These increases in lipid peroxidation were brought down to levels comparable to that of control in all brain regions of CA supplemented KD fed group.

The ROS levels were also significantly diminished in both cytosol (17-22%) and mitochondria (12-17%) among CA fed group. A significant increase in ROS levels was observed both in mitochondria (Ct-49%; Cb-35%; Hc-19%; St-13%) and cytosol (Ct-63%; Cb-19%; Hc-19%; St-32%) in KD fed group, while varying degree of protection was observed in CA treated mice. Elevated HP levels observed in KD fed mice brain region mitochondria and cytosol were normalized in CA supplemented KD group.

Protein carbonyl levels

In general there was a decrease in protein carbonyl (PC) formation in both mitochondrial and cytosol fractions (Mitochondria: 24-39%; Cytosol: 12-28%) of brain region of mice fed CA in diet. There was significant increase in PC levels in KD fed group in both cytosol (13-21%) and mitochondria (22-32%). In CA supplemented KD group, total protection was observed against KD-induced oxidative damage (Fig.2.7).

Effect on Antioxidant enzymes

Feeding KD in diet resulted in significantly enhanced activities of GSH-Px, GST and SOD in brain region mitochondria. All alterations in antioxidant enzyme activities were brought back to normalcy in CA supplemented KD group (Table.2.2). Significant increases in antioxidant enzyme activities were evident among CA fed mice in the brain cytosol. In the KD group there was significant decrease in CAT activity (Ct-42%; Cb-16%; Hc-22%) and marginal increase in activity of GST (Ct-19%; Cb-14%; Hc-13%; St-21%). Significant increase was also observed in GPx (Ct-26%; Cb-28%; Hc-34%; St-37%) and SOD (Ct-37%; Cb-33%; Hc-12%) activities in KD fed animals. All the alterations in antioxidant enzyme activities were normalized in CA supplemented KD group (Table 2.3).

Modulatory effect on glutathione and thiol status

There was an increase in GSH (19-30%) and total thiol (20-34%) levels in cytosol of brain regions of mice fed CA *per se* in diet. In KD fed animals, there was a decrease in GSH (Ct-25%; Cb-14%; Hc-15%; St-12%) and TSH (Ct-23%; Cb-16%; Hc-20%; St-19%) levels in cytosol. All these decreases were normalized in mice supplemented CA along with KD in diet (Fig.2.8).

Similarly, in mitochondria of brain regions of mice fed *CA*, increase in levels of antioxidant molecules was observed (GSH: 16-20%; TSH: 21-25%) (Fig.2.8). Significant increase (npSH: 21-37%) in the levels of non-protein thiols were also observed in all brain regions of mice fed CA in diet (data not shown). In KD mice brain region mitochondria, there was significant decrease in GSH and TSH levels. GSSG levels were elevated (Ct-32%; Cb-19%; Hc-26%; St-13%) and a decrease in npSH (Ct-16%; Ct-21%; Hc-18%; St-20%) levels were also observed (Data not shown) in the KD fed group. In CA supplemented KD group, all these alterations were attenuated and the levels were comparable to that of controls.

Amelioration of MTT reduction and LDH activity

KD feeding resulted in significant decrease in MTT reduction (Ct-23%; Cb-30%; Hc-20%; St-13%) in all the brain regions and the decrease was normalized in CA supplemented KD group (Fig.2.9). There was no significant change in LDH activity in brain region cytosol of CA fed mice. Interestingly, in KD fed animals significant increase (Ct-46%; Cb-23%; Hc-30%; St-24%) in LDH

activity was observed in all brain regions while in CA supplemented KD group no significant changes in the activity was found (Fig.2.9).

Amelioration of Na^+ K^+ ATP-ase activity and NADH-Ubiquinone oxidoreductase

No appreciable alterations were observed in Na^+ K^+ ATP-ase and NADH-Ubiquinone oxidoreductase activity in mitochondria of brain regions of mice fed CA (Table. 2.4). While significant decrease in activity of Na^+ K^+ ATP-ase was observed in brain regions of KD fed animals (Ct-32%; Cb-33%; Hc-24%; St-18%), the decrease was normalized by CA supplementation. Marginal decrease in complex I activity was observed in KD fed mice brain while no observable alterations were seen in CA supplemented KD group (Table.2.4).

Acetylcholinesterase activity

Significant increase (14-27%) in AChE activity was observed in all brain regions of mice fed CA. However, KD feeding resulted in inhibition of AChE activity in all brain regions (Ct-18%; Cb-25%; Hc-23%; St-12%) and CA supplementation resulted in total restoration of AChE activity to normal levels (Fig.2.10).

SECTION B

(iii) Antioxidant potential of CA aqueous extract (CAAE)

In vitro Antioxidant potential of CAAE

The free radical scavenging activity of CAAE, evaluated by the DPPH method, is presented in (Figs. 2.11A, 2.11A_1). The extract significantly reduced the stable free radical DPPH to the yellow-colored 1, 1-Diphenyl-2-picrylhydrazyl with an IC_{50}= 12.18µg/ml. The synthetic antioxidant BHT exhibited an IC_{50} of 4.16µg/ml.The reducing power of the CAAE extracts steadily increased with increasing sample concentration (Figs.2.11.B, 2.11 B_1). In NO scavenging assay, IC_{50} of the CAAE extract was observed to be 42.36µg/ml (Fig.2.11.C).

The IC_{50} of CAAE for superoxide scavenging was 21.87µg/ml and for caffeic acid it was 4.15µg/ml (Fig.2.12A, 2.12 A_1). The IC_{50} of CAAE for hydroxyl radical scavenging was 1.22 mg/ml and for BHT it was 6.85µg/ml (Fig.2.12B, 2.12B_1). The IC_{50} value of CAAE was 200 µg/ml

where as of EDTA was 50 ug/ml (Fig.2.13 A, 2.13A_1). CAAE showed protection against deoxyribose oxidation also, with an IC_{50} value was 352.9µg/ml (Fig.2.13B). The total polyphenol content in CAAE was 11.2µg quercetin equivalents/ mg (Fig.2.13 C).

Modulation of 3- NPA induced Oxidative stress in synaptosomes (cortex & Whole brain)

Cortical synaptosomes: Among 3-NPA treated synaptosomes, there was a general increase in the level of oxidative markers *viz.,* MDA levels, ROS generation and hydroperoxides. Exposure of synaptosomes to CAAE *per se* significantly reduced the basal MDA levels (21-23%). On exposure to 3-NPA, the levels were elevated in cortical synaptosomes (26%) and pre treatment with CAAE resulted in 100% protection against this increase (Fig.2.14). Synaptosomes showed a marked increase (98%) in ROS generation following 3-NPA exposure. CAAE *per se* reduced the endogenous levels of ROS formation (by 26-45%) at both concentrations and offered complete protection against 3-NPA induced ROS generation. (Fig.2.14). Further, 3-NPA exposure resulted in marked elevation (136%) in hydroperoxide levels in synaptosomes. CA extract *per se* reduced the basal levels of hydroperoxides (by 35-45 %). 100% protection was observed among CAAE pretreated synaptosomes (Fig.2.14).

Whole brain synaptosomes: There was significant increase in the levels of MDA (30%), and ROS levels (96%) among 3-NPA exposed synaptosomes. A 14-21% reduction in basal levels was observed in CAAE *per se* treated synaptosomes. CAAE pretreatment offered in 87-100% protection (Data not shown).

Modulation of NPA induced Oxidative stress in striatal mitochondria

In general, there was significant induction of LPO (113%), ROS levels (120%) and hydroperoxide generation (126%) in striatal mitochondria exposed to 3-NPA (2mM). On the other hand which CAAE reduced the basal levels (by 15-46%). It offered significant protection against 3-NPA- induced LPO (80%) formation, ROS levels (34-80%) and HP levels (100%) (Fig.2.15).

Modulation of 3-NPA induced oxidative response in other brain regions

CAAE *per se* significantly reduced the basal levels of MDA in mitochondria (Ct-28%; Cb-20%; Hc-17%). Further, the extract provided marked protection (Ct-100%; Cb-80%, Hc-85%) against 3-NPA induced lipid peroxidation (Fig. 2.16). CAAE (0.5 µg/ml) provided complete protection to mitochondria of all brain regions against 3-NPA induced ROS generation. A similar

trend of protection was also evident in all brain regions against 3-NPA induced hydroperoxide formation (Ct-60%; Cb-85%; Hc-100%) (Fig. 2.16).

(iv) Prophylactic efficacy of CA extract against 3-NPA induced oxidative stress in brain regions of prepubertal mice

Growth, body weight and food intake

The body weight gain and food intake among mice given CA prophylaxis was comparable to those of controls. Mice injected 3-NPA showed a marginal reduction (10-15%) in body weight gain, accompanied with marginal reduction in liver (10%) and (17%) weights (Data not shown).

Effect of CA extract per se on endogenous oxidative markers in brain: preliminary study

In general, there was a dose dependent decrease in oxidative stress markers in all brain regions of mice fed CA extract, where at the highest dose of CA, a marginal increase was observed. While the decrease in MDA levels was marginal (10-15%) in all brain regions at 2.5mg/kg bw, significant decrease (Ct-31%; Cb-30%; Hc-38%; St-36%) was observed at 5mg/kg bw dose. At the higher dose hippocampus and striatum depicted significant decrease (43 and 18% respectively). Significant decrease in ROS generation was observed in all brain regions at all the doses with the highest decrease at 5mg/kg bw (Ct-31%; Cb-24%; Hc-27%; St-36%) (Data not shown).

Efficacy of CA prophylaxis (5mg/kg bw) on 3-NPA induced oxidative stress

Lipid peroxidation: In general there was an increase in oxidative markers in both cytosol and mitochondria of striatum of mice administered 3-NPA. There was significant increase in MDA formation in cytosol (83%) as well as mitochondria (88%) compared to controls. CAAE decreased the basal levels of MDA in cytosol (18%) and mitochondria (24%). Among mice given CA prophylaxis, while 100% protection was observed in cytosol, only 88% protection was observed in mitochondria against 3-NPA induced lipid peroxidation (Fig.2.17). The endogenous levels of lipid peroxidation measured as MDA levels were significantly lower among other brain regions of mice which received CAAE prophylaxis compared to the untreated controls. 3-NPA treatment resulted in differential increase in MDA in other brain regions both in cytosol (Ct-70%; Cb-57%; Hc-64%) (Table 2.5) and mitochondria (Ct-31%; Cb-38%; Hc-57%) (Fig.2.18). Interestingly, mice given CA prophylaxis exhibited no increase of oxidative markers suggests 100% protection against 3-NPA intoxication.

Generation of ROS: Striatal region of 3-NPA treated mice showed robust elevation in ROS levels (Cytosol-123%; Mitochondria-110%). CAAE alone significantly reduced the ROS levels in both fractions (by 25%). Among mice given CA prophylaxis, the levels of ROS were restored to normalcy in cytosol (100%), while mitochondria showed only 65% protection (Fig.2.17). Likewise the basal ROS levels were significantly lower (Cytosol: 24-43%; Mitochondria: 25-50%) among other brain regions of mice which received CA prophylaxis compared to the untreated controls. However, 3-NPA treatment caused marked increase in other brain regions cytosol (Ct-65%; Cb-89%; Hc-48%) (Table 2.5) and mitochondria (Ct-35%; Cb-30%; Hc-31%) (Fig.2.18) suggesting oxidative stress induction. In contrast, 3-NPA administration to mice given CA prophylaxis had no obvious elevation in the ROS levels in the other brain regions (100 % protection).

Formation of hydroperoxides: Striatal regions of 3-NPA treated mice showed a marked increase in hydroperoxide levels (cytosol-102%; mitochondria-131%) over the controls. CAAE alone marginally decreased the basal levels in both fractions (14-25%); among mice given CA prophylaxis, the HP levels were brought to complete normalcy in cytosol (100% protection), while in mitochondria it offered 85% protection (Fig.2.17). In other brain regions also significant increase in HP levels (Ct-85%; Cb-40%; Hc-57%) were observed in cytosol and nearly 100% protection was observed in animals pretreated with CAAE prior to 3-NPA exposure in all brain regions (Table 2.5). The HP levels among mitochondria of NPA administered mice were markedly elevated in other brain regions (Ct-64%; Cb-93%; Hc-50 %). In contrast, 3-NPA administration to mice given CA prophylaxis did not show any elevation in HP in other brain regions suggesting complete protection (Fig.2.18).

Protective effect of CA prophylaxis against protein oxidation

The endogenous levels of protein carbonyls were decreased marginally (13-15%) in cytosol as well as mitochondria of striatum region. 3-NPA exposure resulted in significant elevation of PC levels both in cytosol (37%) and mitochondria (41%). Total protection was evident among mice given CA prophylaxis against 3-NPA induced protein carbonyl oxidation (Fig.2.19). Further, the endogenous levels of PC were also significantly lower in both cytosol (15-30%) and mitochondria (15-35%) among brain regions of mice which received CA prophylaxis compared to the untreated controls. 3-NPA treatment resulted in a moderate elevation in protein carbonyl content in both cytosol (Ct-33%; Cb-25%; Hc-26%) and mitochondria (Ct-30%; Cb-20%; Hc-27%). Interestingly, 3-NPA

administration to mice received CA prophylaxis did not result in any noticeable increase in protein carbonyls in any of the brain regions (Fig. 2.19) indicating total protection.

Effect of CA prophylaxis on GSH, thiols and antioxidant enzyme activities

Among mice given CA prophylaxis, brain tissue showed marginal increase in GSH (15-22%), total thiols (14-27%) and non-protein thiols (11-20%). Although 3-NPA caused differential degree of depletion in GSH in all brain regions in normal mice with CA prophylaxis did not reveal any noticeable depletion of GSH levels suggesting complete protection by CA (Tables 2.6, 2.7). Further, 3-NPA administration resulted in a significant reduction in thiols in striatum (33%) and other brain regions (15-29%). On the other hand, 3-NPA administration to mice given CA prophylaxis showed depletion of thiol levels.

Among mice given CA prophylaxis, significant increases (20-35%) was evident in the activities of GSH-Px and SOD. 3-NPA administration caused significant reduction in the activities of GSH-Px (10-31%) and SOD (20-35%), while the activity of GST was significantly elevated (25-40%). On the contrary, CA prophylaxis offered protection against 3-NPA induced perturbations in the activities of antioxidant enzymes (Table 2.8, Fig: 2.20 and 2.21).

Effect on MTT reduction and activity of Citrate synthase (CS) and dehydrogenases

Mitochondria of brain regions of 3-NPA administered mice exhibited a significant reduction in the formation of formazan on exposure to MTT (St-33%; Ct-26%; Cb-28%, HC-61%). However, among mice given CA prophylaxis, the intensity was restored to complete normalcy in cerebellum and striatum, and significant protective effect was also evident in other regions (Ct-43%; Hc-54 % protection) (Fig.3A). Further, 3-NPA administration resulted in a marked reduction in the activity of CS in striatum (46%) and other brain regions (Ct-42%; Cb-39%, Hc-41%). In contrast, 3-NPA administration in mice given CA prophylaxis failed to induce any appreciable effect on the activity levels of CS (Fig.2.22). Interestingly, 3-NPA induced a significant decrease in the activity of SDH in striatum (76%) and other brain regions (71-76%) (Fig.2.22). On the other hand, 3-NPA administration to mice given CA prophylaxis afforded only a marginal protection (10-15%) against SDH reduction.

Mitochondrial electron transport chain (ETC) enzymes

3-NPA administration caused a marked and uniform reduction in the activity of succinate ubiquinone oxidoreductase enzyme in striatum (80%) and other brain regions (Ct-76%; Cb-76%; Hc-80%) (Fig.2.23). A general decrease was also observed in the activities of NADH-cytochrome C

oxidase (Ct-15%; Cb-18%; Hc-24%; St-26%) and succinate cytochrome C oxidase (Ct-65%; Cb-52%; Hc-74%; St-72%) (Fig. 2.24) among 3-NPA administered mice. On the contrary, varying degree of protection was evident following 3-NPA administration to mice given CA prophylaxis.

Effect on the activity of Na^+, K^+ ATP ase and mitochondrial swelling

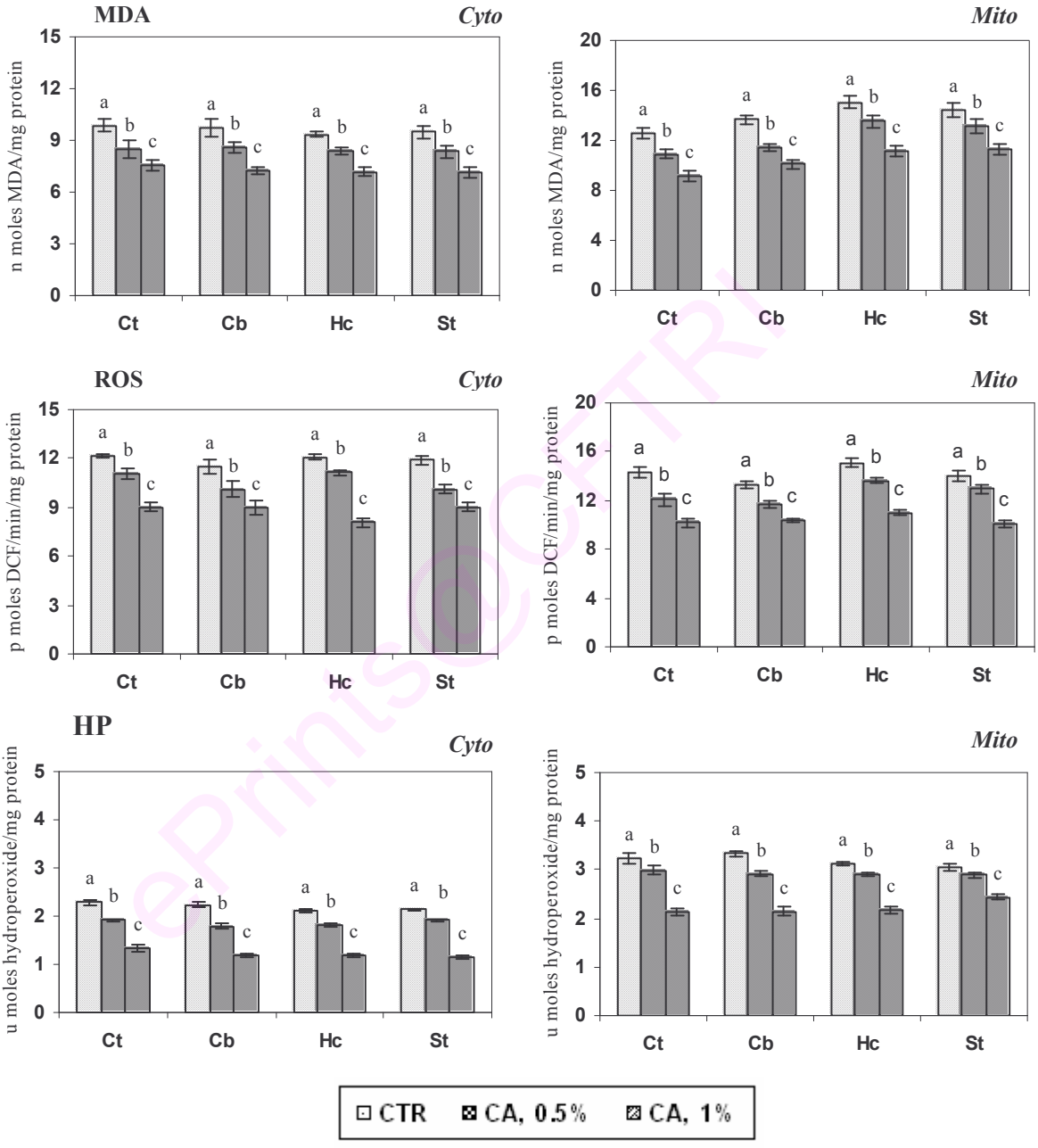
There was a dramatic reduction in the activity of Na^+ K^+ ATP ase in striatum (7.75 fold) and other brain regions (Ct-4.47fold; Cb-5.15 fold; Hc-8.42 fold) of mice administered with 3-NPA. Interestingly significant level of protection was observed among mice given CA prophylaxis in response to 3-NPA intoxication (Fig. 2.25). Mitochondrial swelling was quantified in terms of time taken for the reduction in absorbance at 540nm. A general reduction in time was observed among 3-NPA treated mice in all brain regions (Ct-25%; Cb-15%; Hc-16%; St-38%). Various levels of protection were shown by CA pretreatment to 3-NPA induced reduction in mitochondrial permeability transition (Fig. 2.25).

Effect on cholinergic system

There was significant increase in the activity of AChE in all brain regions (15-25%) of mice fed CA alone. Significant decrease (16-24%) in activity was observed in brain regions of mice administered 3-NPA while CA prophylaxis resulted in almost normalization of the enzyme activity (Table 2.11). A similar trend in increase in BChE was observed in all brain regions (10-40%). Marginal decrease was observed in BChE activity on 3-NPA exposure. Varying degrees of protection was offered by CA prophylaxis (Table 2.11).

Fig: 2.1

Status of oxidative stress markers in different brain regions of prepubertal mice fed CA incorporated diet for 30 days.

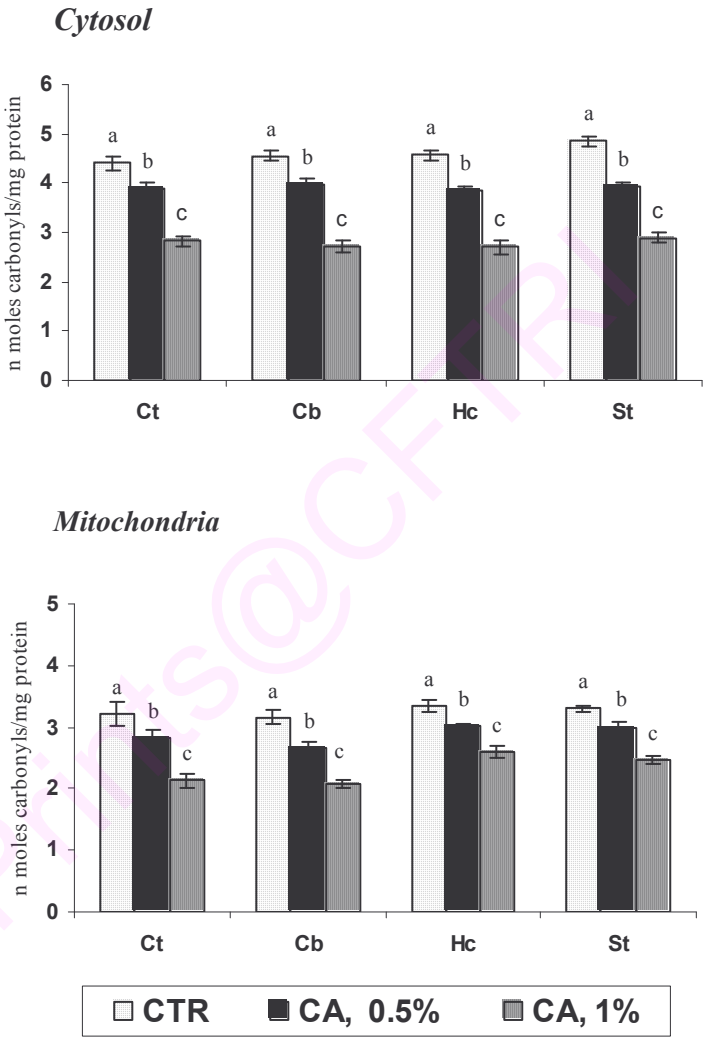


Values are mean ± S.D of six determinations each; data analyzed by ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

MDA: malondialdehyde; ROS: Reactive oxygen species; HP: hydroperoxides;
Cyto: cytosol; Mito: mitochondria. CA-*Centella asiatica* leaf powder

Fig: 2.2

Status of protein carbonyls in different brain regions of prepubertal mice fed CA supplemented diet for 30 days.

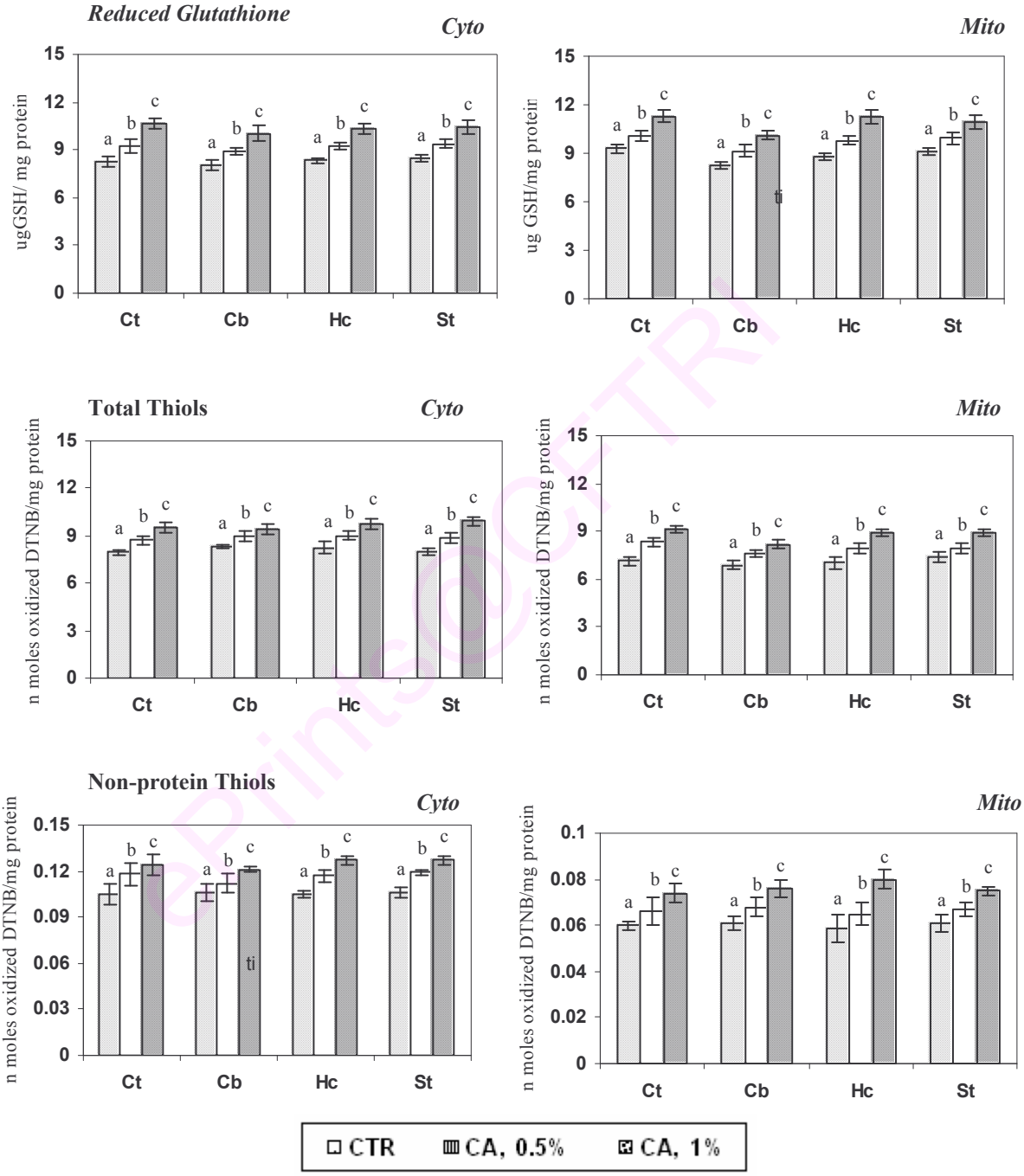


Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum; CA-*Centella asiatica* leaf powder

Fig: 2.3

Status of reduced glutathione, total thiols and non protein thiols in brain regions of prepubertal mice fed CA supplemented diet for 30 days.

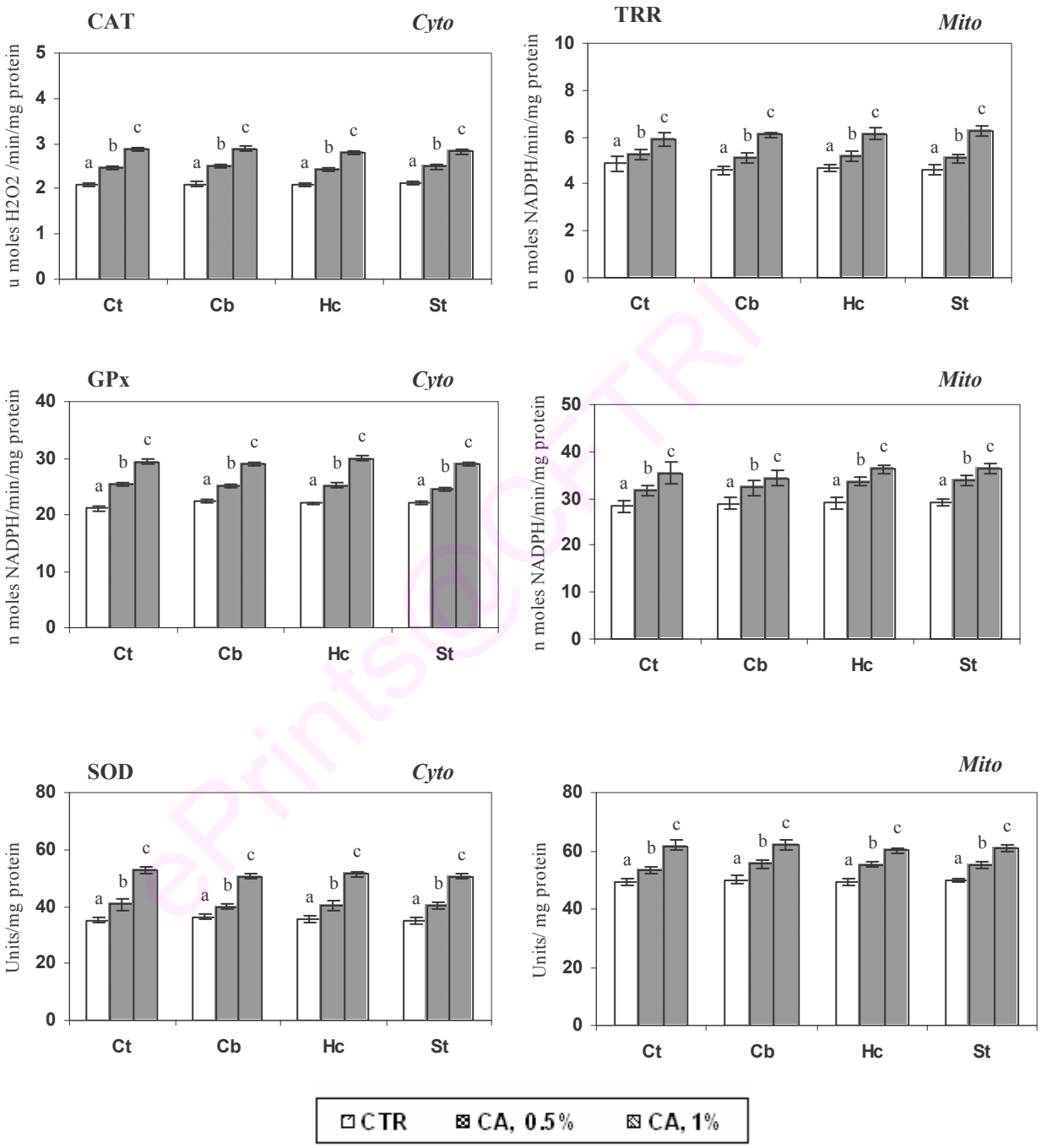


Values are ± S.D of six determinations each; data analyzed by ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum; CA-*Centella asiatica* leaf powder
Cyto-cytosol; Mito-mitochondria

Fig: 2.4

Activities of antioxidant enzymes and Thioredoxin reductase in cytosol and mitochondria of brain regions of prepubertal mice fed CA supplemented diet for 30 days.

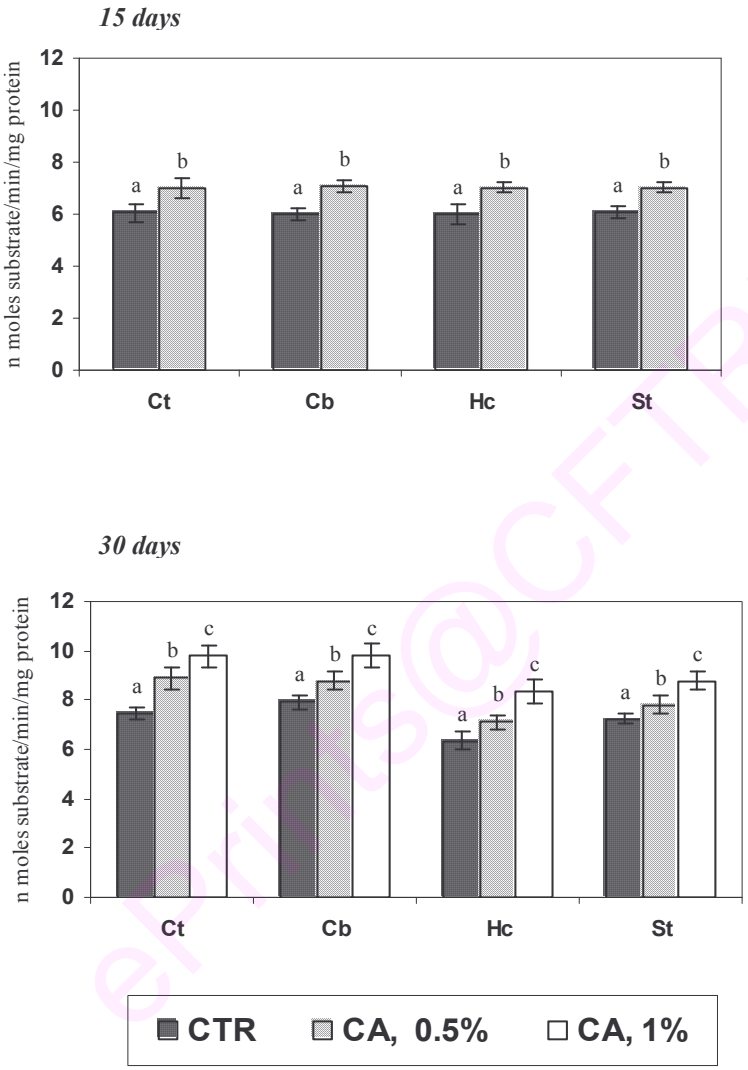


Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum; CA-*Centella asiatica* leaf powder
Cyto-cytosol; Mito-Mitochondria.

Fig: 2.5

Status of Acetylcholinesterase activity in brain regions of prepubertal mice fed CA supplemented diet for 30 days.



Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

Ct-Cortex; Cb-Cerebellum; Hc-Hippocampus; St-Striatum
CA-*Centella asiatica* leaf powder.

Table 2.1

Ameliorative effect of CA on KD-induced oxidative alterations in brain mitochondria of growing male mice

Group	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
MDA[1]				
CTR	15.08±0.76[a]	12.75±0.75[a]	13.24±0.58[a]	14.22±0.55[a]
CA	12.63±0.50[b]	9.56±0.59[b]	11.76±0.77[b]	12.26±0.67[b]
KD	22.18±1.12[c]	17.65±1.19[c]	20.58±0.98[c]	19.12±1.15[c]
CA /KD	15.93±0.85[a]	12.71±0.50[a]	13.68±0.67[a]	14.78±0.88[a]
ROS[2]				
CTR	14.16±0.53[a]	12.36±0.55[a]	12.97±0.64[a]	13.21±0.43[a]
CA	11.82±0.41[b]	10.85±0.63[b]	10.91±0.55[b]	11.32±0.51[b]
KD	21.15±0.93[c]	16.68±0.94[c]	15.46±0.67[c]	14.87±0.62[c]
CA /KD	15.55±0.63[d]	13.81±0.51[d]	12.68±0.59[a]	13.68±0.57[a]
HP[3]				
CTR	5.55±0.45[a]	5.34±0.13[a]	5.48±0.18[a]	5.34±0.23[a]
CA	4.12±0.35[b]	4.51±0.31[b]	4.80±0.20[b]	4.60±0.11[b]
KD	6.93±0.45[c]	6.71±0.19[c]	6.82±0.13[c]	6.88±0.45[c]
CA/KD	4.91±0.12[d]	5.05±0.10[a]	5.62±0.11[a]	4.84±0.56[a]

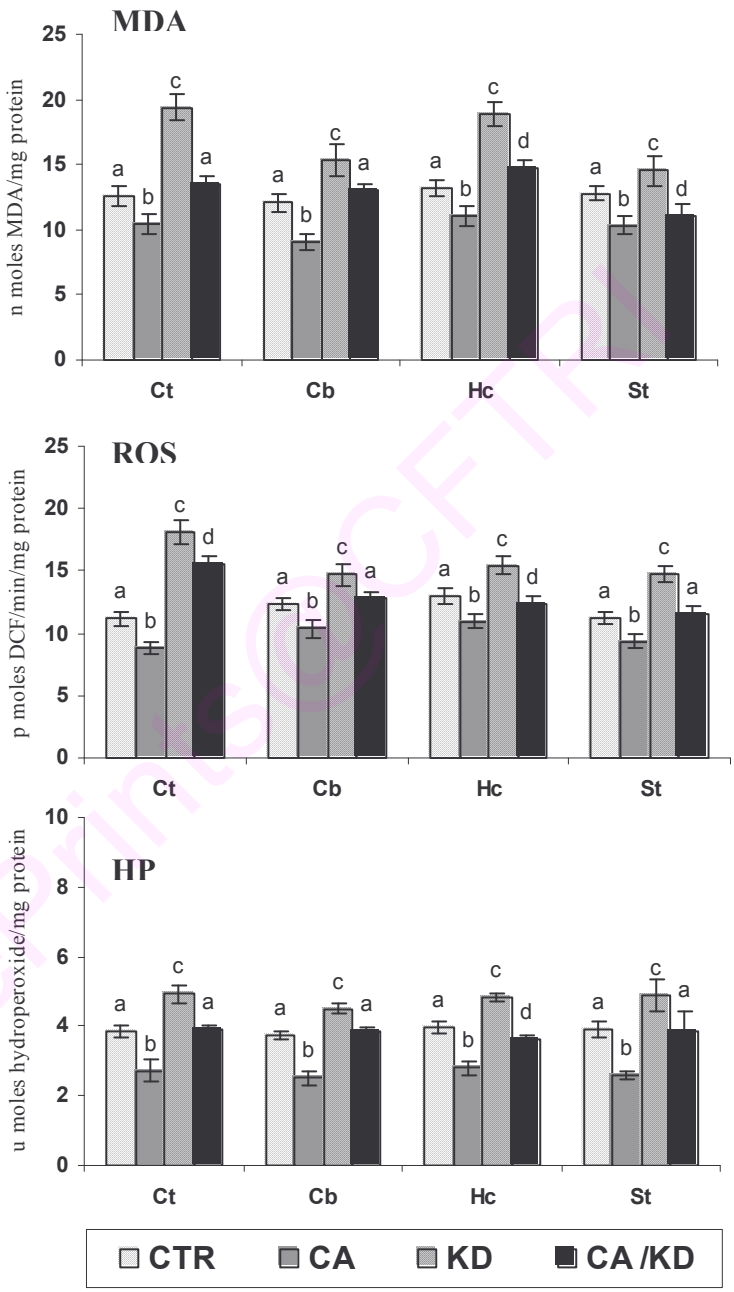
Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-Centella asiatica leaf powder (1%); KD- Khesari dhal (30%);
MDA-Malondialdehyde; ROS-Reactive oxygen species; HP-Hydroperoxides

1-η moles MDA/mg protein
2-ρ moles DCF/min/mg protein
3-μ moles hydroperoxides/mg protein

Fig: 2.6

Ameliorative effect of CA on KD-induced oxidative alterations in brain region cytosol of male mice fed Khesari dhal and CA incorporated diet for 30 days.

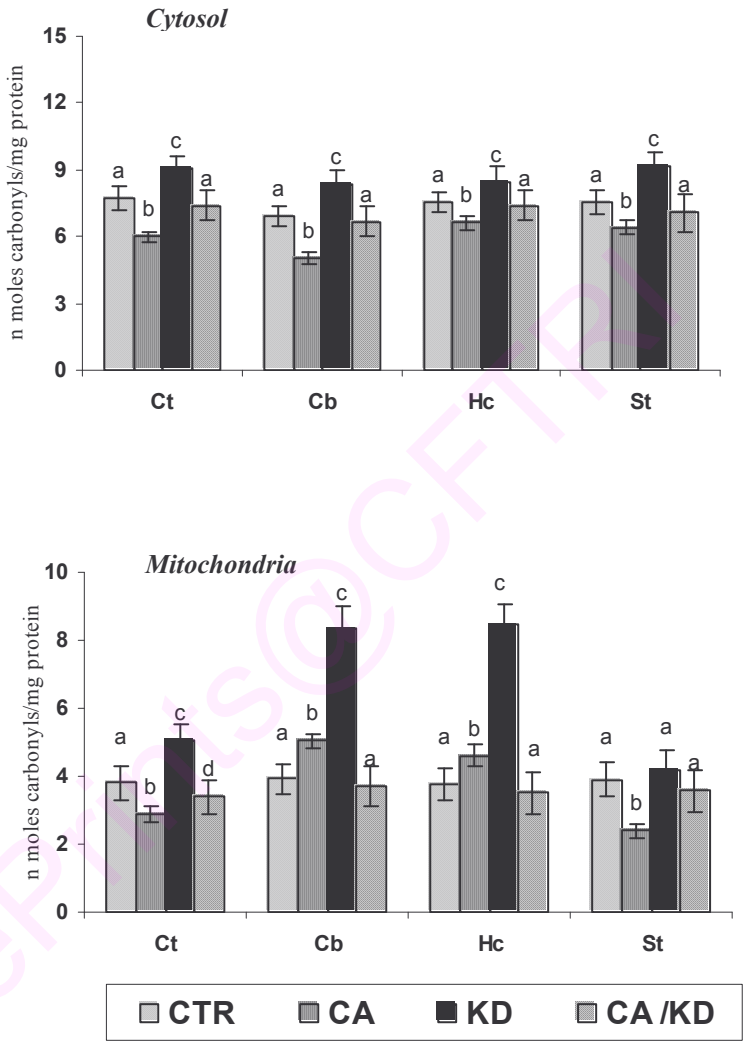


Values are mean± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA. *Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%); MDA-Malondialdehyde ROS-Reactive oxygen species; HP-Hydroperoxides.

Fig: 2.7

Ameliorative effect of CA supplementation on KD-induced protein carbonyls in brain region cytosol/ mitochondria of male mice.



Values are mean± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA.*Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%); MDA-Malondialdehyde
ROS-Reactive oxygen species; HP-Hydroperoxides

Table 2.2

Modulatory effect of CA on mitochondrial antioxidant enzymes in brain regions of mice fed KD incorporated diet for 30 days.

Group	Brain regions			
	Cortex	Cerebellum	Hippocampus	Striatum
GPx[1]				
CTR	24.72±0.72[a]	24.18±0.64[a]	23.89±0.59[a]	24.02±0.40[a]
CA	28.84±0.83[b]	27.56±0.67[b]	27.12±0.87[b]	28.24±0.73[b]
KD	27.77±0.94[b]	28.45±0.74[b]	27.12±0.81[b]	27.77±0.96[b]
CA /KD	23.91±0.78[a]	23.56±0.91[a]	24.99±0.78[a]	24.21±0.70[a]
GST[2]				
CTR	23.12±1.56[a]	25.21±1.02[a]	25.45 ±1.11[a]	23.12±1.59[a]
CA	24.02±0.98[a]	24.23±0.83[a]	25.67 ±0.78[a]	24.92±0.58[a]
KD	29.32±0.33[c]	28.01±0.45[c]	29.11 ±0.78[c]	27.32±0.35[c]
CA /KD	24.72±0.67[a]	24.67±0.54[a]	24.78±0.98[a]	24.16±0.60[a]
SOD[3]				
CTR	43.44±0.91[a]	44.23±0.67[a]	44.99±0.98[a]	45.44±0.91[a]
CA	49.12±0.89[b]	47.45±1.12[b]	50.18±0.99[b]	49.12±0.89[b]
KD	51.12±1.50[b]	49.21±1.12[b]	55.45±1.20[c]	50.12±1.30[b]
CA /KD	43.12±0.98[c]	43.23±0.83[c]	45.67±0.78[c]	45.90±0.98[c]

Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA- *Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%); GPx-Glutathione peroxidase; GST-Glutathione–S-Transferase; SOD-Superoxide dismutase

1-ηmoles NADPH/min/mg protein; 2-ηmoles conjugate/min/mg protein
3-Units/mg protein

Table 2.3

Effect of CA supplementation on KD- induced alterations in antioxidant enzymes in cytosol of brain regions of young mice.

Groups	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
CAT[1]				
CTR	2.19±0.10[a]	2.23±0.09[a]	2.30±0.05[a]	2.11±0.08[a]
CA	2.70±0.11[b]	3.02±0.12[b]	3.12±0.08[b]	3.05±0.11[b]
KD	1.28±0.08[c]	1.88±0.07[c]	1.79±0.06[c]	1.99±0.07[c]
CA /KD	2.30±0.11[a]	2.19±0.08[a]	2.35±0.10[a]	2.24±0.08[a]
GST[2]				
CTR	26.12±1.56[a]	26.21±1.02[a]	26.45±1.11[a]	25.12±1.59[a]
CA	26.02±0.98[a]	25.23±0.83[a]	25.67±0.78[a]	24.92±0.58[a]
KD	31.02±0.33[b]	30.01±0.45[b]	29.90 ±0.78[b]	30.32±0.35[b]
CA /KD	26.72±0.67[a]	27.67±0.54[a]	26.78±0.98[a]	25.16±0.60[a]
GPx[3]				
CTR	21.22±0.72[a]	22.18 ±0.60[a]	20.19±0.52[a]	20.20±0.49[a]
CA	25.14±0.83[b]	25.56 ±0.70[b]	25.12±0.81[b]	25.24±0.70[b]
KD	26.77±0.94[b]	28.45 ±0.74[c]	27.12±0.80[c]	27.77±1.02[c]
CA /KD	21.91±0.78[a]	21.56 ±0.90[a]	21.99±0.75[a]	20.21±0.76[a]
SOD[4]				
CTR	35.67±1.23[a]	36.77±0.89[a]	35.30±0.90[a]	35.67±0.97[a]
CA	42.56±1.40[b]	42.16±1.01[b]	40.21±1.02[b]	41.21±0.98[b]
KD	48.90±1.22[c]	48.81±1.03[c]	39.65±1.02[c]	38.23±0.90[c]
CA /KD	38.99±0.95[c]	39.77±1.07[c]	35.70±1.10[a]	35.78±1.12[a]

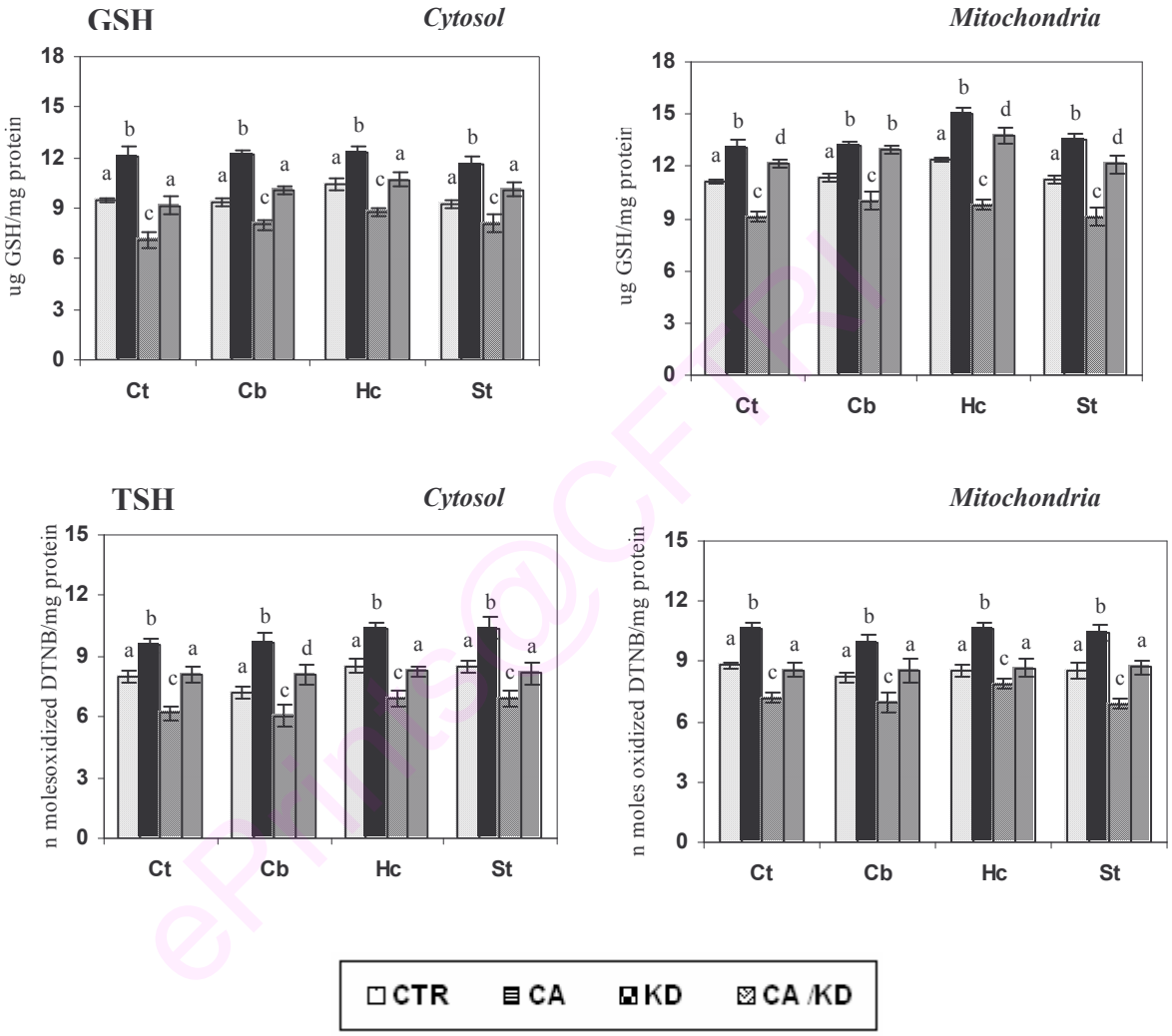
Values are mean± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-*Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%); CAT-Catalase
GPx-Glutathione peroxidase; GST-Glutathione –S-Transferase; SOD-Superoxide dismutase;

1-μ moles H_2O_2 degraded/min/mg protein; 2-η moles conjugate/min/mg protein
3-η moles NADPH/min/mg protein; 4-Units/mg protein

Fig: 2.8

Mitigation of KD induced changes in reduced Glutathione levels and total thiols in Cytosol and Mitochondria of brain regions of male mice given CA supplementation.

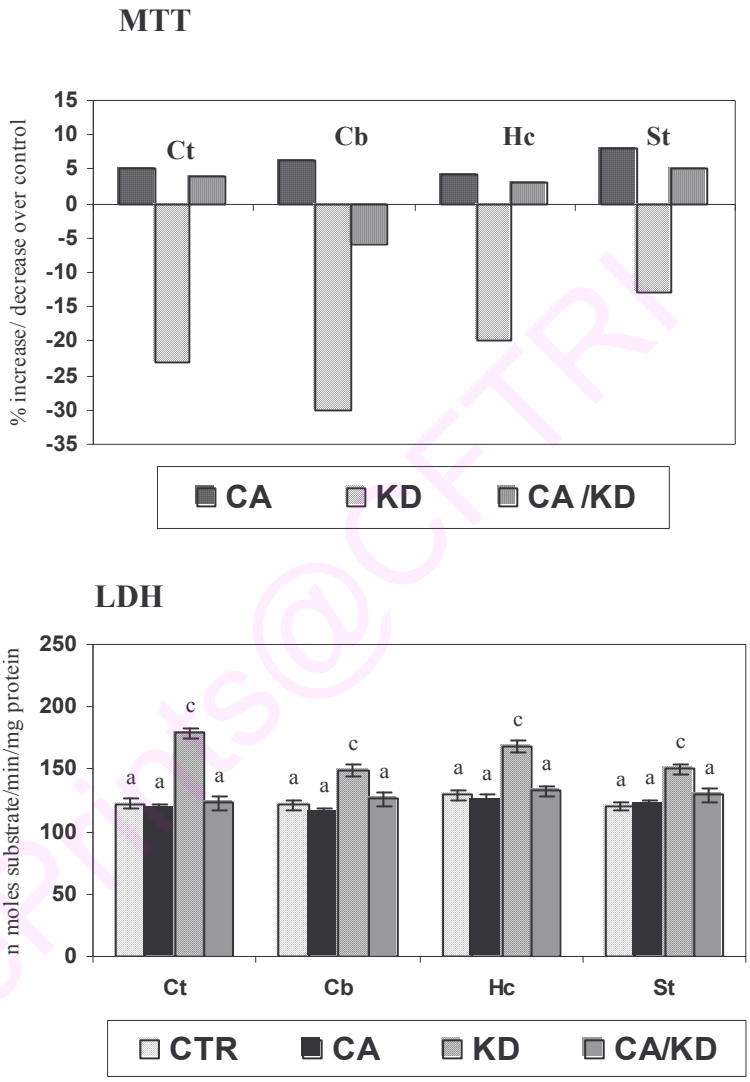


Values are mean± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-Centella asiatica leaf powder (1%); KD- Khesari dhal (30%); GSH-Reduced glutathione
TSH-Total thiols

Fig: 2.9

Effect of CA supplementation on KD- induced alterations in MTT assay and Lactate dehydrogenase activity mitochondria/cytosol of brain regions of mice.



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-*Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%).

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride
LDH-Lactate dehydrogenase;

Table 2.4

Effect of CA supplementation on KD- induced alterations in Na^+ K^+ ATP-ase and NADH-Ubiquinone oxidoreductase activity in mice.

Group	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
ATP-ase[1]				
CTR	24.50±0.50[a]	23.12±0.45[a]	24.13±0.55[a]	23.16±0.50[a]
CA	22.31±0.50[b]	24.12±0.75[a]	23.22±0.19[a]	23.15±0.54[a]
KD	16.45±0.23[b]	15.70±0.68[b]	18.35±0.50[b]	19.20±0.59[b]
CA /KD	23.76±0.36[a]	22.80±0.57[a]	24.19±0.36[a]	23.21±0.43[a]
Com-I[2]				
CTR	140.23±3.50[a]	141.13±4.20[a]	139.23± 3.59[a]	140.10±3.70[a]
CA	142.31±3.10[a]	143.11±3.50[a]	140.22±4.20[a]	141.66±3.75[a]
KD	128.90±4.27[b]	122.90±4.20[b]	128.00±4.25[b]	130.93±4.37[b]
CA /KD	142.45±4.20[a]	139.45±4.79[a]	138.25±4.50[a]	141.05±5.22[a]

Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

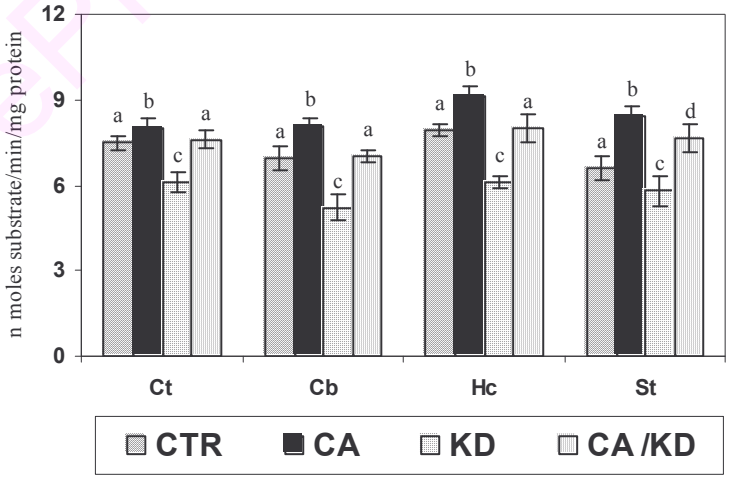
CA -Centella asiatica leaf powder (1%); KD- Khesari dhal (30%);

ATP-ase- Na^+ K^+ ATP-ase ; Com I-NADH-Ubiquinone oxido reductase

1-η moles pi released/min/mg protein; 2-η moles /min/mg protein

Figure: 2.10

Effect of CA supplementation on KD- induced alterations in Acetylcholinesterase activity in brain regions of mice.

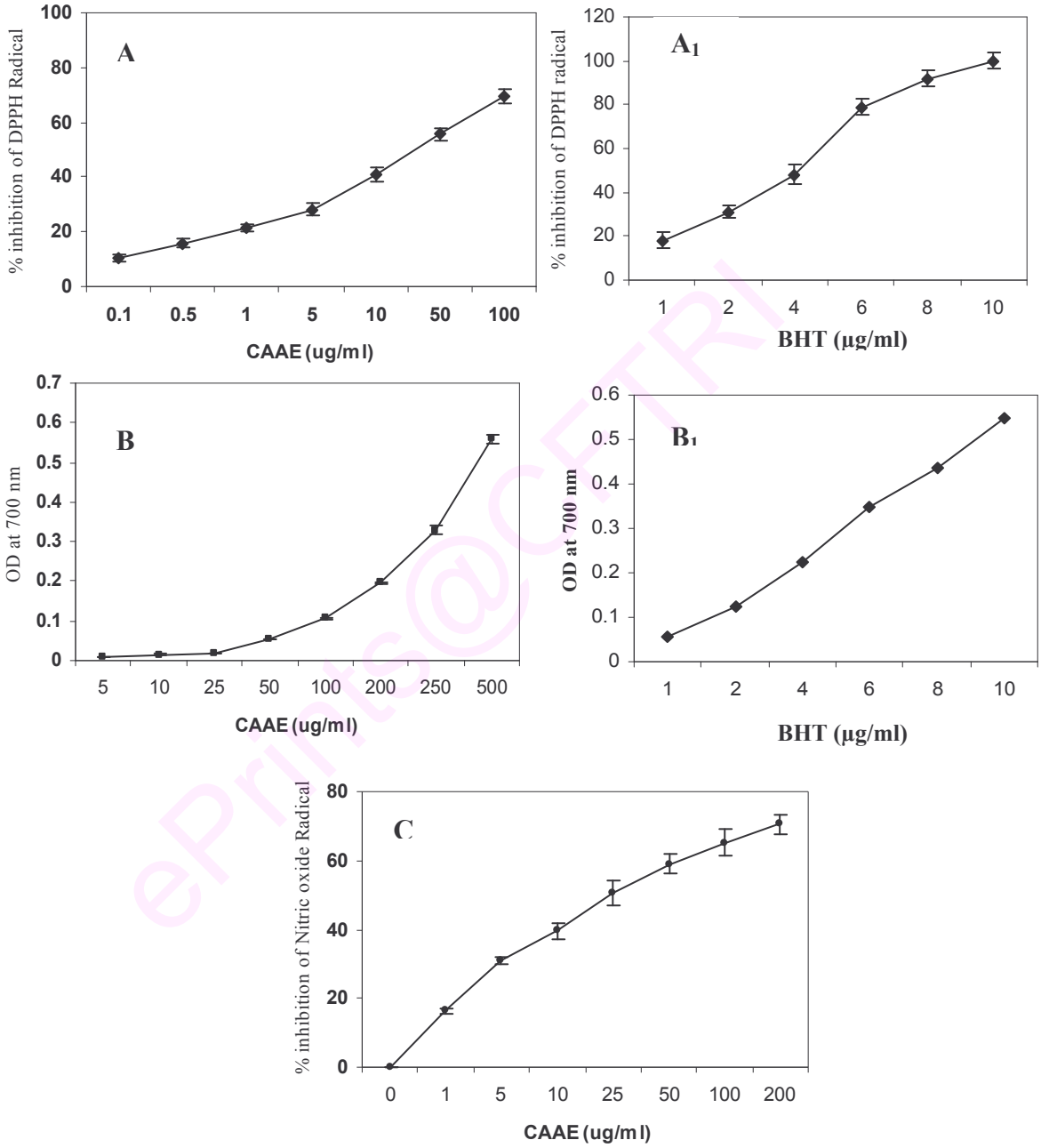


Values are mean± S.D of six determinations each; data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-*Centella asiatica* leaf powder (1%); KD- Khesari dhal (30%)

Fig: 2.11

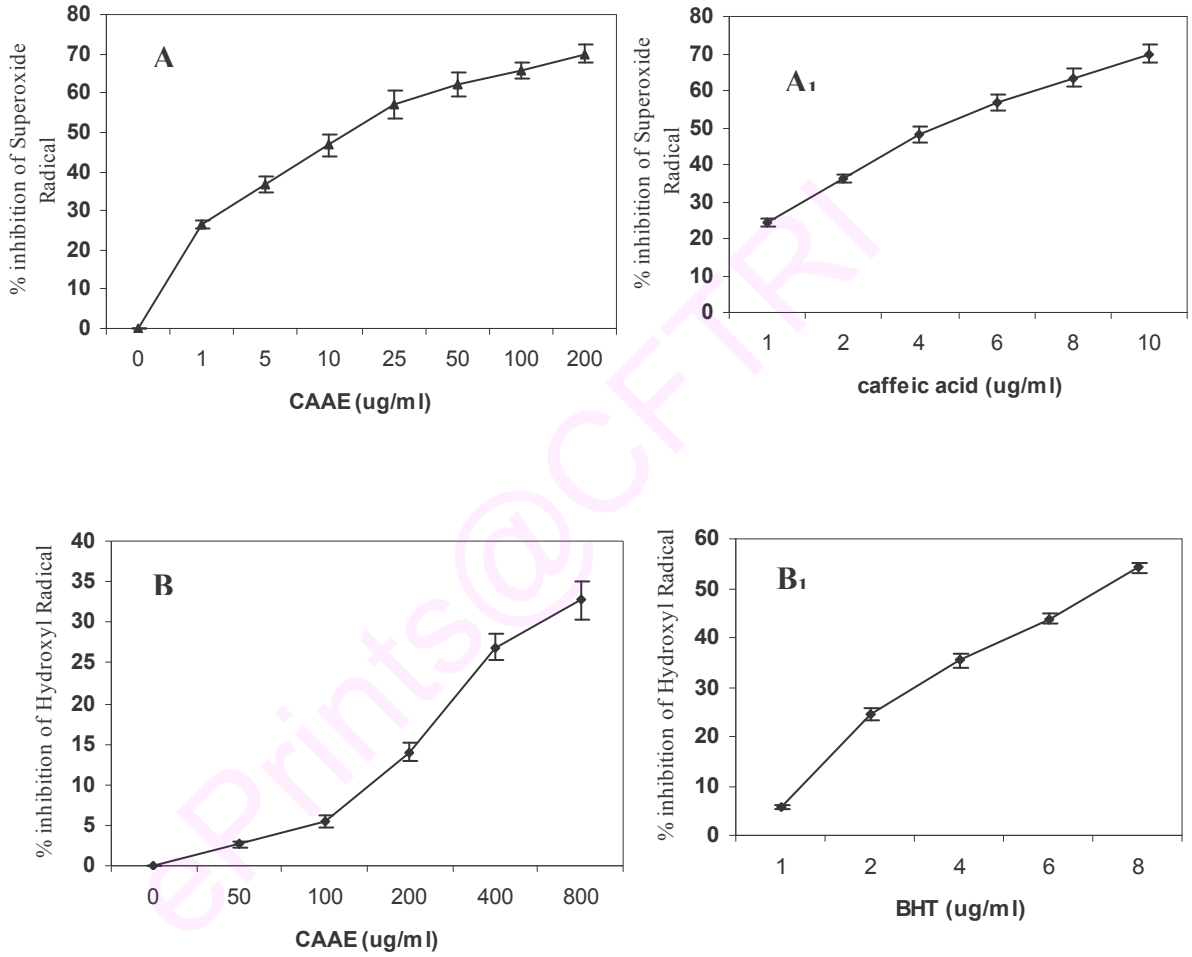
Free radical scavenging potential of CA aqueous extract in chemical systems



All values are represented as mean ±SD of six replicates.
A-DPPH assay; A_1 –BHT standard for comparison;
B-Reducing power assay; B_1 –BHT standard for comparison
C-Nitric oxide scavenging assay.

Fig: 2.12

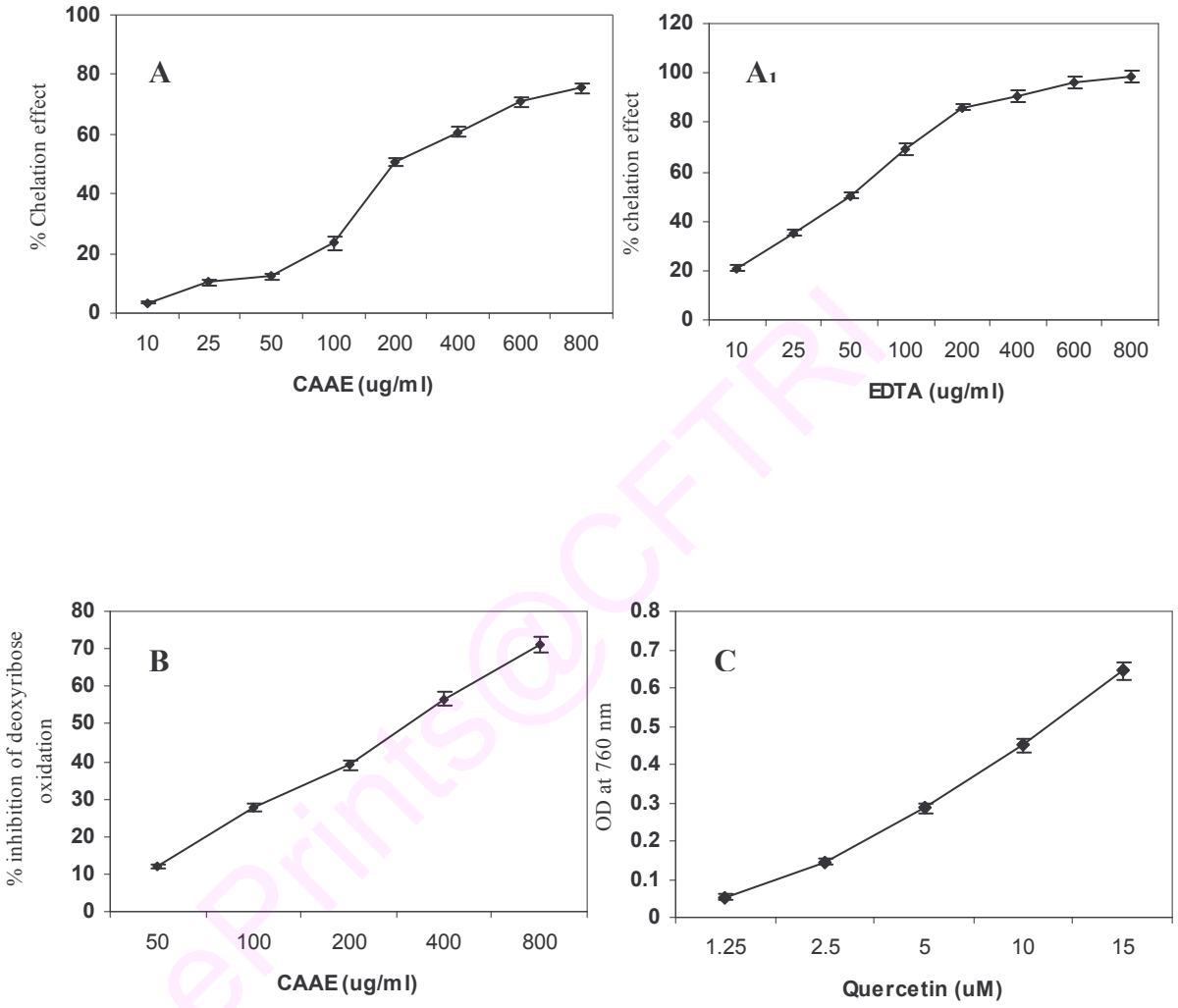
Assessment of antioxidant potential of *Centella asiatica* aqueous extract: Superoxide radical scavenging; Hydroxyl radical scavenging.



All the values are represented as mean ±SD of six replicates.
A-Superoxide scavenging assay; A_1 –Caffeic acid standard
B-Hydroxyl radical scavenging assay; B_1 –BHT Standard

Fig: 2.13

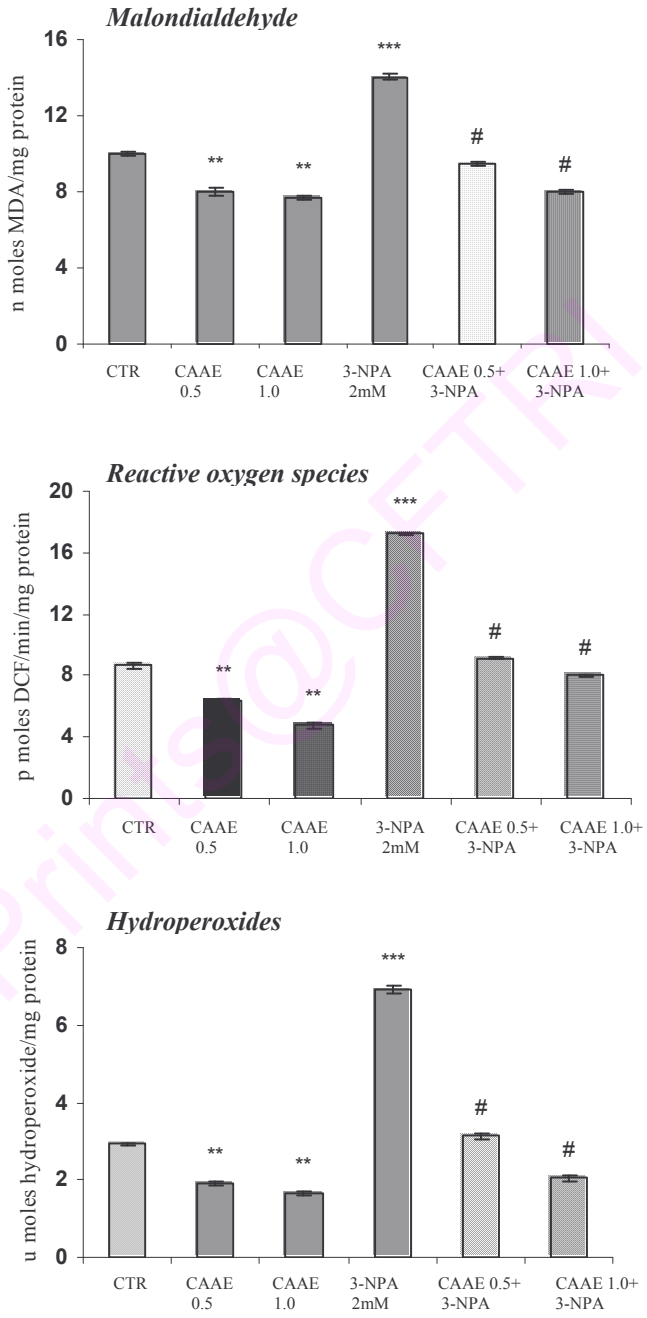
Assessment of antioxidant potential of *Centella asiatica* aqueous extract



All the values are represented as mean ±SD of six replicates.
A-Iron chelation; A_1 –EDTA standard
B-deoxyribose oxidation
C –Quercetin standard of total polyphenols assay.

Fig: 2.14

Modulatory potential of CA aqueous extract *in vitro* on 3-NPA induced oxidative alterations in synaptosomes of prepubertal mice brain cortex.

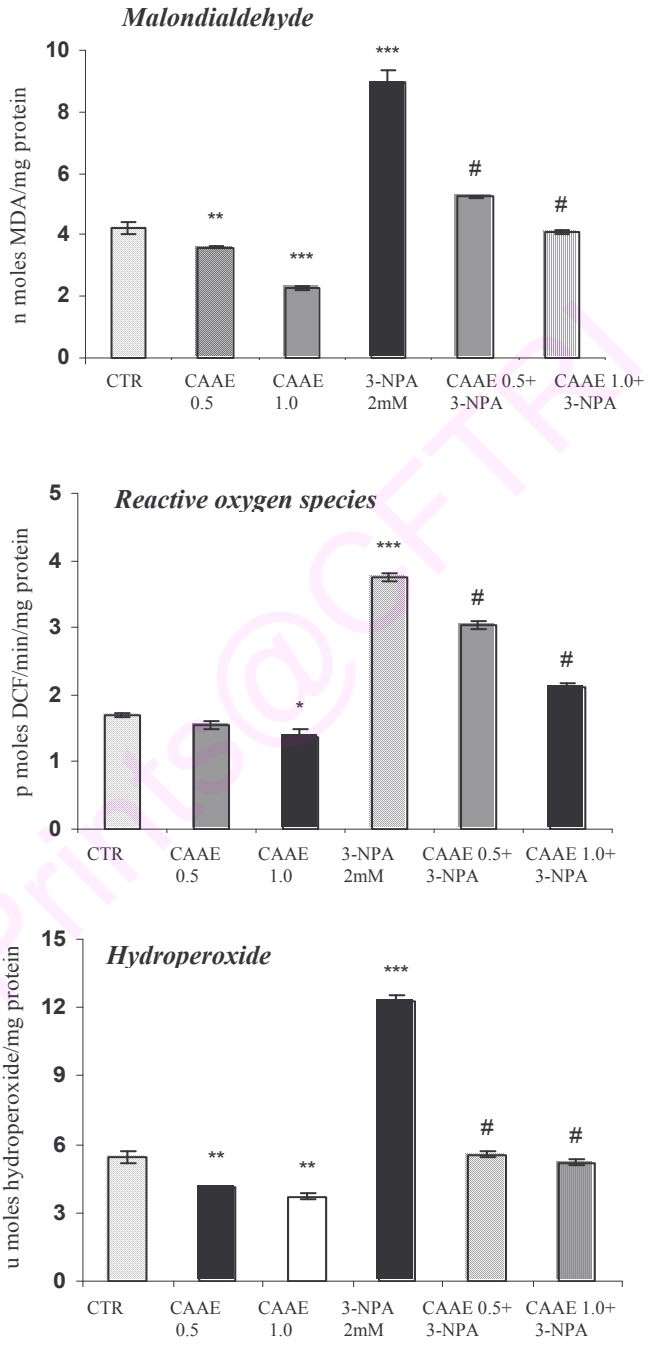


Values are mean± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05; **P<0.01; **P<0.001; #- Compared to 3-NPA (2mM),P< 0.01).

CAAE-*Centella asiatica* aqueous extract; 3-NPA-3-nitropropionic acid

Fig: 2.15

Modulatory potential of CA aqueous extract *in vitro* on 3-NPA induced oxidative alterations in striatal mitochondria of prepubertal mice brain.

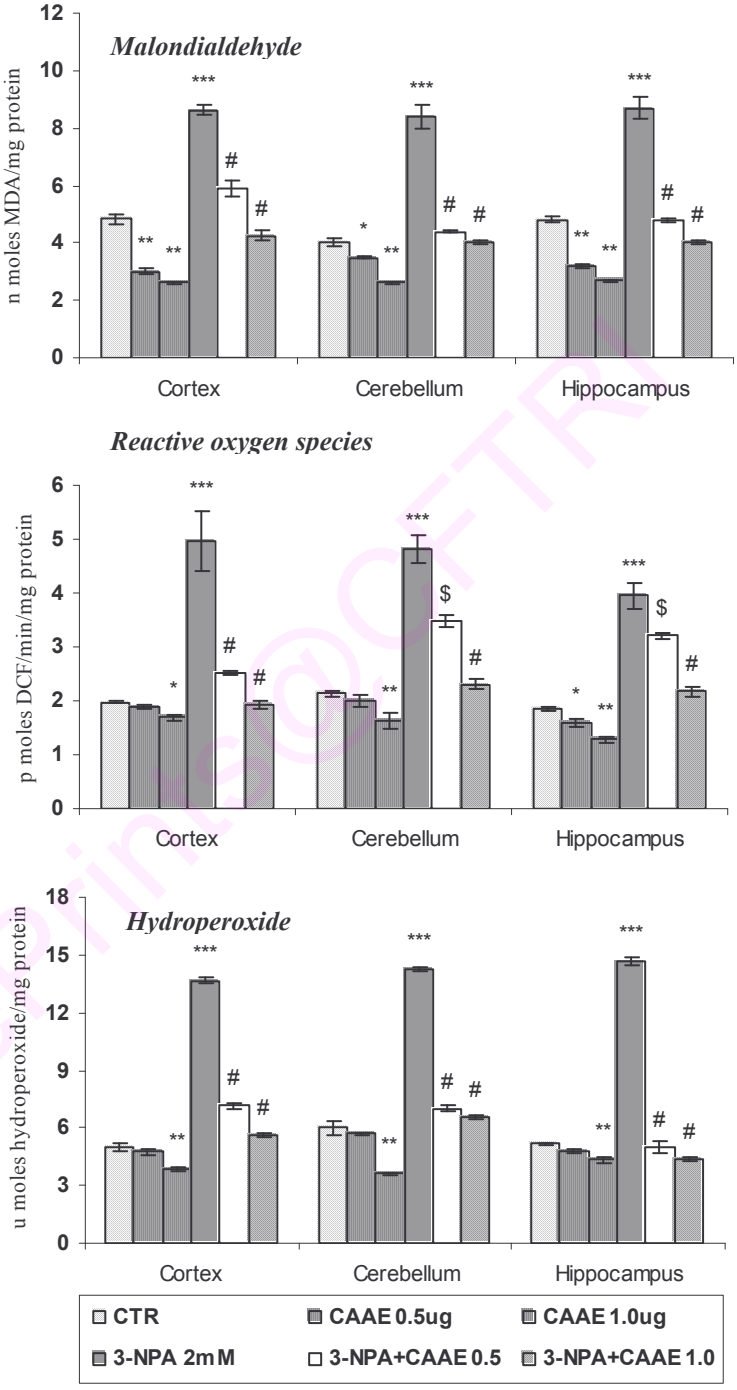


Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05; **P<0.01; **P<0.001); #- Compared to 3-NPA (2mM),P< 0.01)

CAAE-*Centella asiatica* aqueous extract; 3-NPA-3-nitropropionic acid

Fig: 2.16

Modulatory potential of CA aqueous extract *in vitro* on 3-NPA induced oxidative alterations in mitochondria of various brain regions of prepubertal mice.



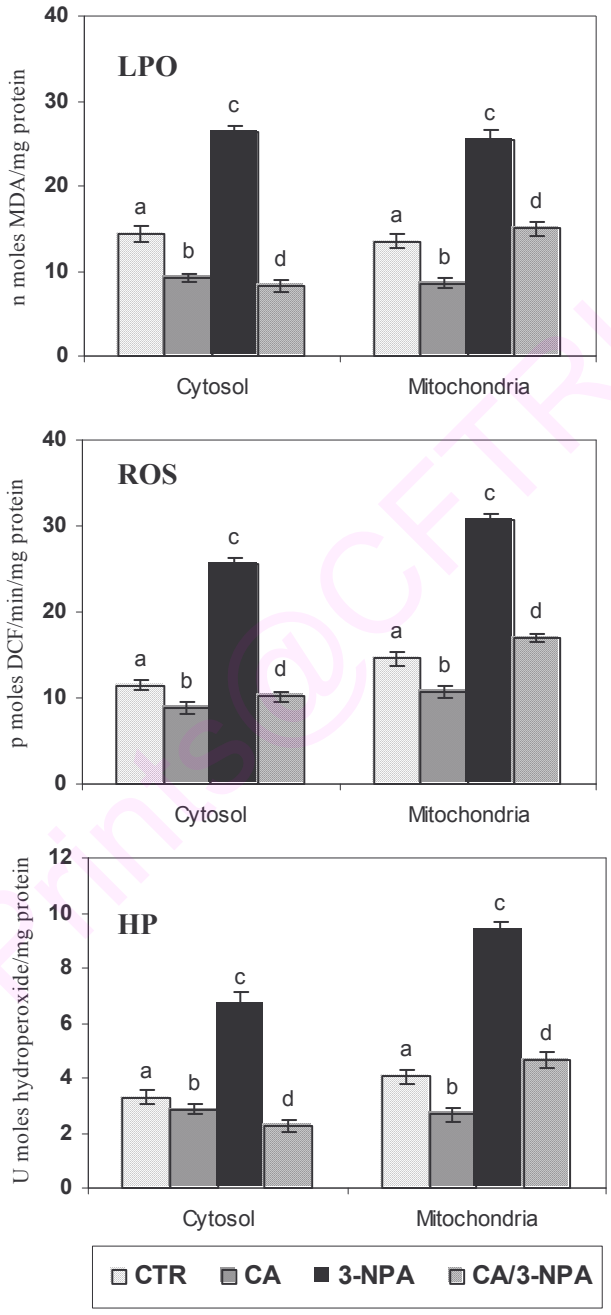
Values are mean ± S.D of six determinations; data analyzed by one way ANOVA (* P<0.05; **P<0.01; ***P<0.001).

#- Compared to 3-NPA (2mM),P< 0.01; $-Compared to 3-NPA (2mM),P< 0.05;

CA-*Centella asiatica* aqueous extract; 3-NPA-3-nitropropionic acid

Fig: 2.17

Effect of CA prophylaxis on 3-NPA induced oxidative stress in striatum of prepubertal mice.



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

MDA-malondialdehyde; ROS-Reactive oxygen species; HP-Hydroperoxides;

CA-*Centella asiatica* aqueous extract (5mg/kgbw, 10days)

3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Table 2.5

Oxidative markers in cytosol of brain regions of prepubertal mice given CA prophylaxis and challenged with 3-NPA.

Group	Brain Regions		
	Cortex	Cerebellum	Hippocampus
Malondialdehyde[1]			
CTR	9.06±0.50[a]	10.50±0.64[a]	15.61±1.12[a]
CA	6.26±0.64[b]	9.30±0.78[a]	9.62±0.89[b]
3-NPA	15.41±1.05[c]	16.50±1.04[b]	25.63±1.09[c]
CA/3-NPA	6.43±0.87[b]	6.31±0.78[d]	9.02±0.77[b]
Reactive oxygen species[2]			
CTR	12.95±0.40[a]	11.56±0.24[a]	12.45±0.22[a]
CA	9.05±0.24[b]	8.87±0.38[b]	9.11±0.29[b]
3-NPA	21.41±0.75[c]	21.80±0.56[c]	18.43±0.39[c]
CA/3-NPA	9.46±0.80[b]	9.07±0.84[d]	10.20±0.37[b]
Hydroperoxide[3]			
CTR	2.14±0.06[a]	2.51±0.04[a]	3.02±0.08[a]
CA	1.70±0.08[b]	1.81±0.08[b]	2.56±0.09[b]
3-NPA	3.98±0.25[c]	3.51±0.10[c]	4.74±0.09[c]
CA/3-NPA	2.05±0.10[a]	2.17±0.07[d]	1.23±0.07[d]

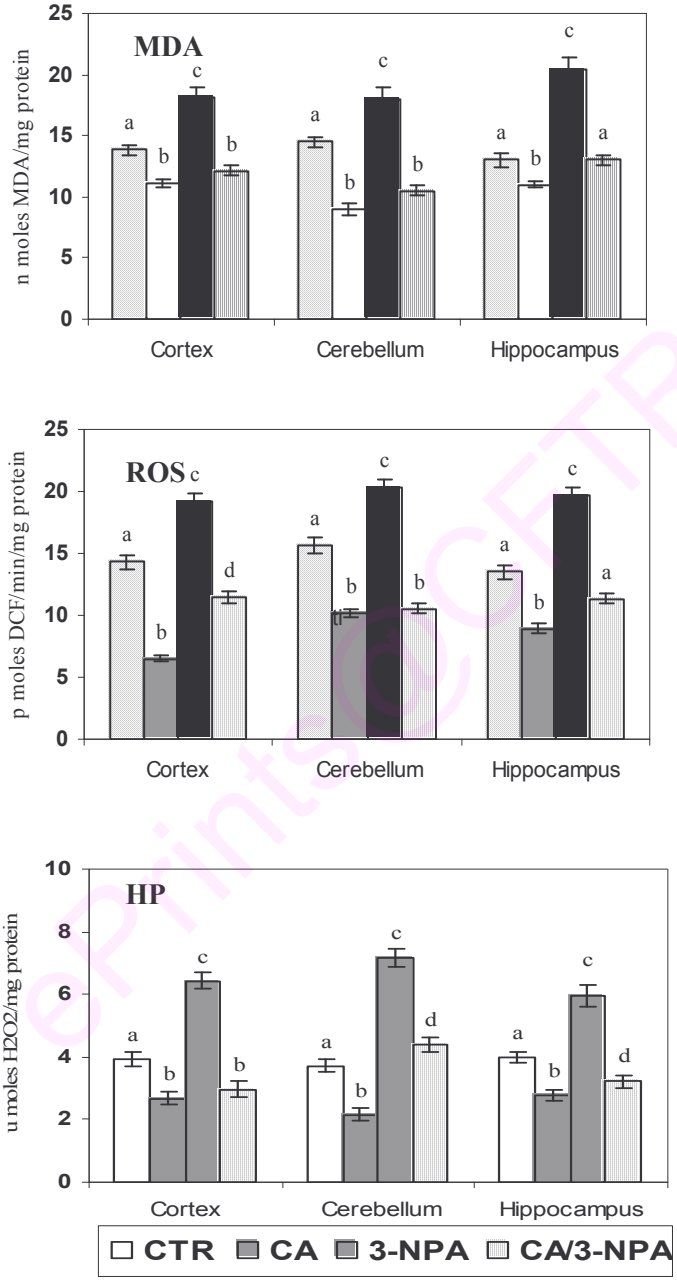
Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract-5mg/kg bw, 10days
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

1-η moles MDA/mg protein; 2-ρ moles DCF/min/mg protein
3-μ moles H_2O_2/mg protein

Fig: 2.18

Effect of CA prophylaxis on 3-NPA induced oxidative stress in mitochondria of brain regions of prepubertal mice.

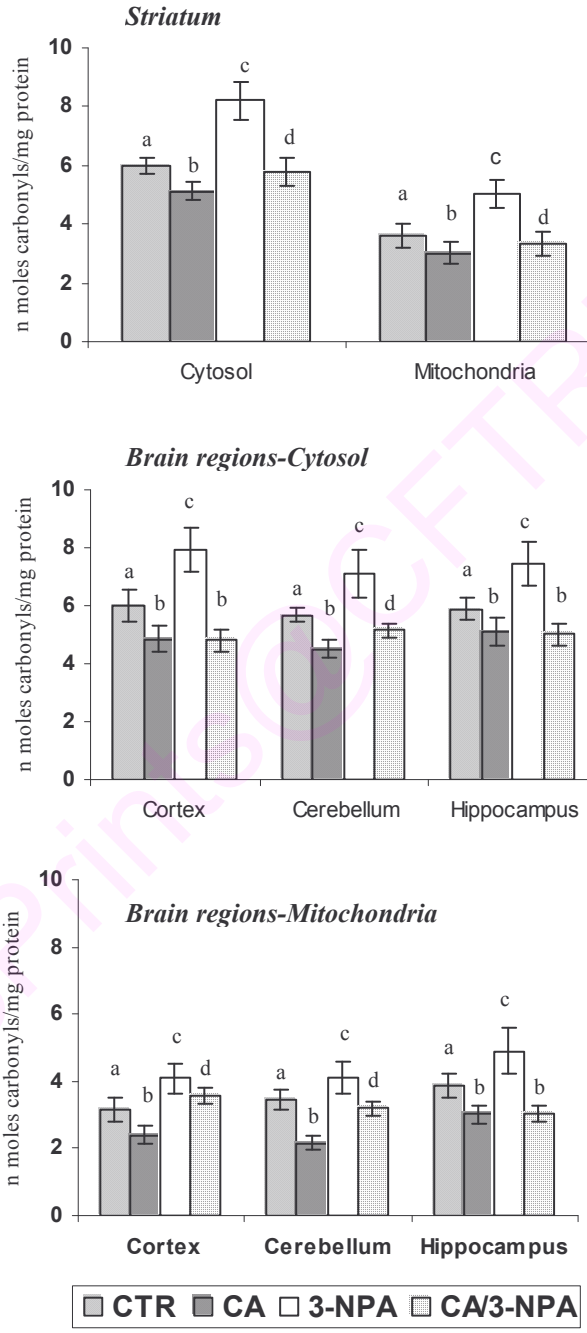


Values are mean± S.D of six determinations each; data analyzed by ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

MDA-Malondialdehyde; ROS-Reactive oxygen species
HP- hydroperoxide; CA-*Centella asiatica* (5mg/kgbw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.19

Effect of CA prophylaxis on 3-NPA induced Protein carbonyl formation in brain regions of prepubertal mice.



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA-*Centella asiatica* aqueous extract (5mg/kg bw, 10days)

3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Table 2.6

Effect of NPA on the status of reduced glutathione and total thiols in cytosol of brain regions of prepubertal mice fed CAAE and challenged with NPA.

Cytosol	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Reduced Glutathione(GSH)[1]				
CTR	8.01 ±0.10[a]	9.23 ±0.45[a]	10.01 ±0.78[a]	9.98 ±0.78[a]
CA	9.38 ±0.36[b]	10.45 ±0.66[b]	11.25 ±0.50[b]	11.31 ±0.56[b]
3-NPA	6.58 ±0.48[c]	8.12 ±0.34[c]	8.93 ±0.38[c]	7.99±0.90[c]
CA+3-NPA	8.39 ±0.36[a]	9.45 ±0.56[a]	9.88 ±0.69[a]	9.80±0.84[a]
Total thiols (TSH)[2]				
CTR	7.27 ±0.45[a]	6.85 ±0.67[a]	7.13 ±0.67[a]	6.98 ±0.56[a]
CA	8.55 ±0.31[b]	7.93 ±0.78[b]	8.28 ±0.78[b]	7.95 ±0.64[b]
3-NPA	6.29 ±0.33[c]	5.78 ±0.45[c]	6.01 ±0.89[c]	5.32 ±0.79[c]
CA+3-NPA	7.56 ±0.29[a]	6.90 ±0.69[a]	7.22 ±0.45[a]	7.01 ±0.56[a]

Table 2.7

Effect of NPA on the status of reduced glutathione and total thiols in mitochondria of brain regions of prepubertal mice fed CAAE and challenged with NPA.

Mitochondria	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Reduced Glutathione(GSH)[1]				
CTR	10.08 ±0.23[a]	10.11 ±0.25[a]	10.01 ±0.34[a]	10.14 ±0.23[a]
CA	13.51 ±0.11[b]	13.13 ±0.13[b]	13.35 ±0.35[b]	13.56 ±0.11[b]
3-NPA	8.15 ±0.13[c]	8.09 ±0.66[c]	8.70 ±0.17[c]	7.03 ±0.13[c]
CA+3-NPA	10.85 ±0.23[a]	10.12 ±0.51[a]	10.92 ±0.29[a]	9.92 ±0.23[a]
Total thiols (TSH)[2]				
CTR	8.26 ±0.31[a]	8.45 ±0.41[a]	8.31 ±0.12[a]	7.98 ±0.13[a]
CA	10.17 ±0.67[b]	10.56 ±0.37[b]	10.39 ±0.33[b]	10.19 ±0.23[b]
3-NPA	6.17 ±0.45[c]	6.99 ±0.19[c]	6.83 ±0.13[c]	5.06 ±0.45[c]
CA+3-NPA	9.07 ±0.12[d]	9.42 ±0.10[d]	9.28 ±0.11[d]	8.91 ±0.56[d]

Values are mean± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)
1-μg GSH/mg protein; 2-ηmoles oxidized DTNB/mg protein

Table 2.8

Effect of NPA on the status of antioxidant enzyme activity in cytosol of brain regions of prepubertal mice given CA prophylaxis.

Groups	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
CAT [1]				
CTR	1.83±0.10 [a]	1.94±0.05 [a]	1.81±0.08 [a]	1.89±0.78 [a]
CA	2.41±0.06 [b]	2.34±0.06 [b]	2.21±0.07 [b]	2.40±0.56 [b]
3-NPA	0.57±0.08 [c]	1.09±0.04 [c]	0.65±0.38 [c]	0.49±0.90 [c]
CA+3-NPA	1.26±0.06 [d]	1.78±0.06 [d]	1.32±0.09 [d]	1.57±0.84 [d]
GST [2]				
CTR	25.34±0.75 [a]	24.56±0.87 [a]	23.31±0.67 [a]	25.27±0.76 [a]
CA	24.17±0.91 [a]	25.12±0.78 [a]	22.80±0.78 [a]	26.35±0.84 [a]
3-NPA	35.45±0.93 [b]	36.57±1.35 [b]	35.42±1.19 [b]	42.23±2.29 [c]
CA+3-NPA	26.23±0.89 [a]	27.67±0.89 [c]	27.89±1.35 [c]	27.80±1.16 [d]
GPx [3]				
CTR	21.12±0.75 [a]	23.45±0.77 [a]	20.34±0.67 [a]	23.45±0.66 [a]
CA	24.98±0.81 [b]	29.78±0.88 [b]	26.30±0.78 [b]	28.79±0.74 [b]
3-NPA	15.78±0.83 [c]	17.67±0.85 [c]	16.70±0.69 [c]	16.50±0.69 [c]
CA+3-NPA	20.10±0.99 [a]	21.24±0.89 [d]	21.37±0.75 [a]	23.10±0.76 [a]
SOD [4]				
CTR	37.89±0.95 [a]	38.80±0.87 [a]	35.56±0.77 [a]	37.23±0.86 [a]
CA	42.34±1.01 [b]	44.43±0.88 [b]	40.35±0.88 [b]	43.23±1.24 [b]
3-NPA	32.14±0.63 [c]	32.14±1.35 [c]	30.12±0.79 [c]	29.80±0.89 [c]
CA+3-NPA	36.67±0.89 [a]	39.02±1.19 [a]	36.23±0.85 [a]	40.15±0.76 [d]

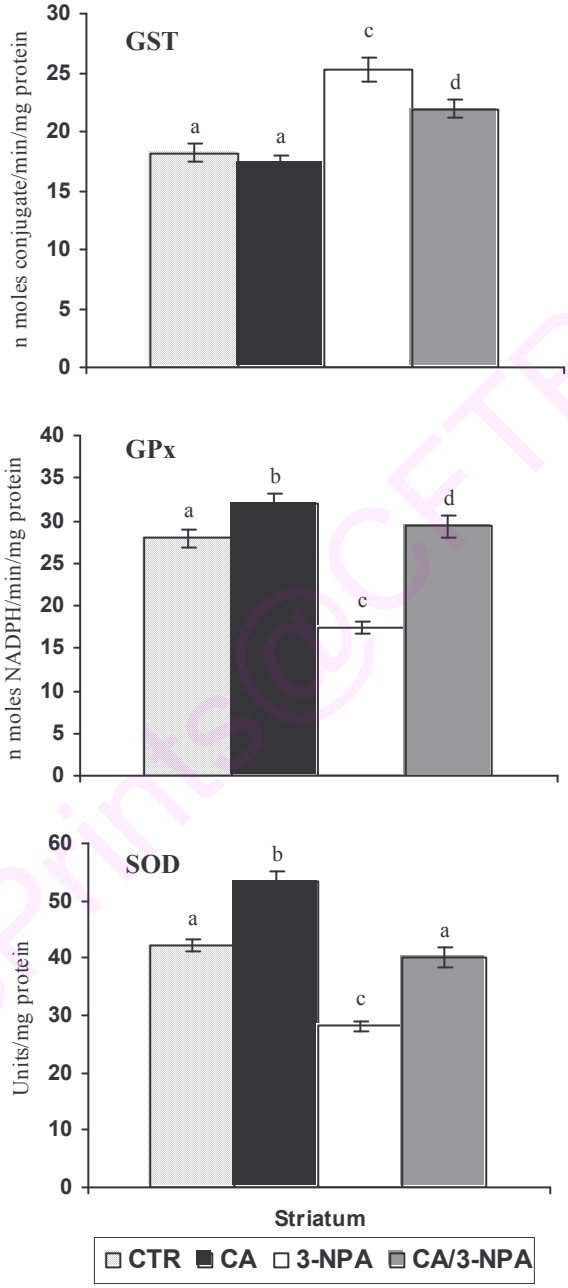
Values are mean± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kg bw, 2days)

1-μ moles H_2O_2/min/mg protein; 2-η moles conjugate/min/mg protein
3-η moles NADPH/min/mg protein; 4-Units/mg protein

Fig: 2.20

Effect of CA prophylaxis on 3-NPA induced alterations in antioxidant enzyme activity in striatum mitochondria of prepubertal mice.

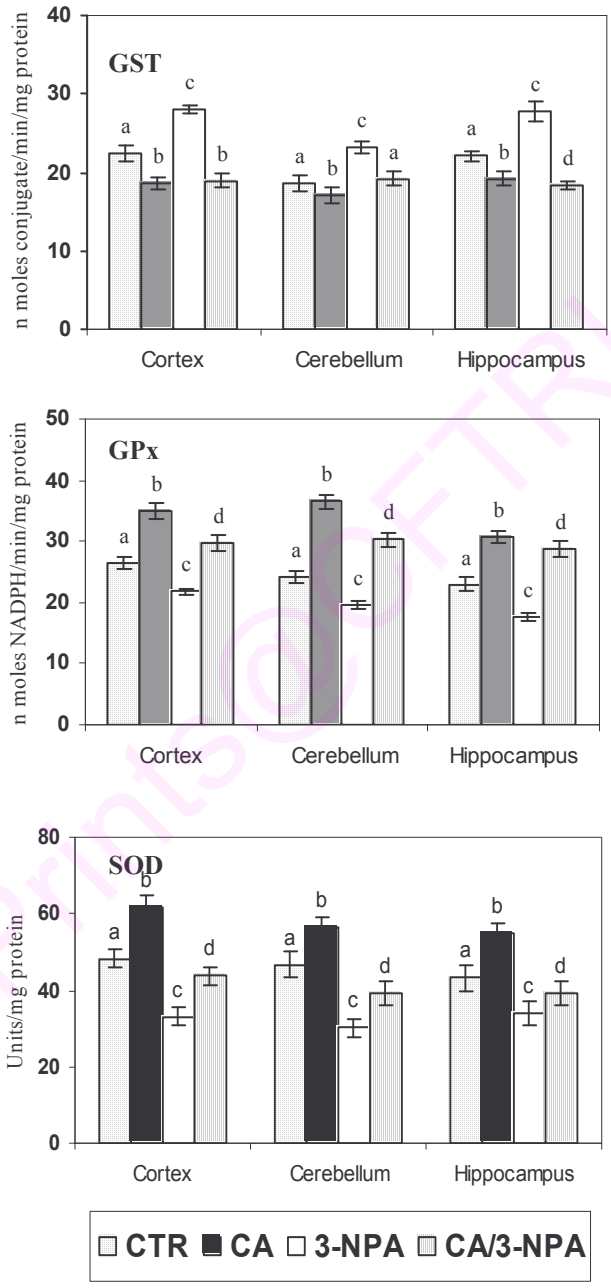


Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.21

Effect of CA prophylaxis on 3-NPA induced alterations in antioxidant enzyme activity in mitochondria of brain regions of prepubertal mice.

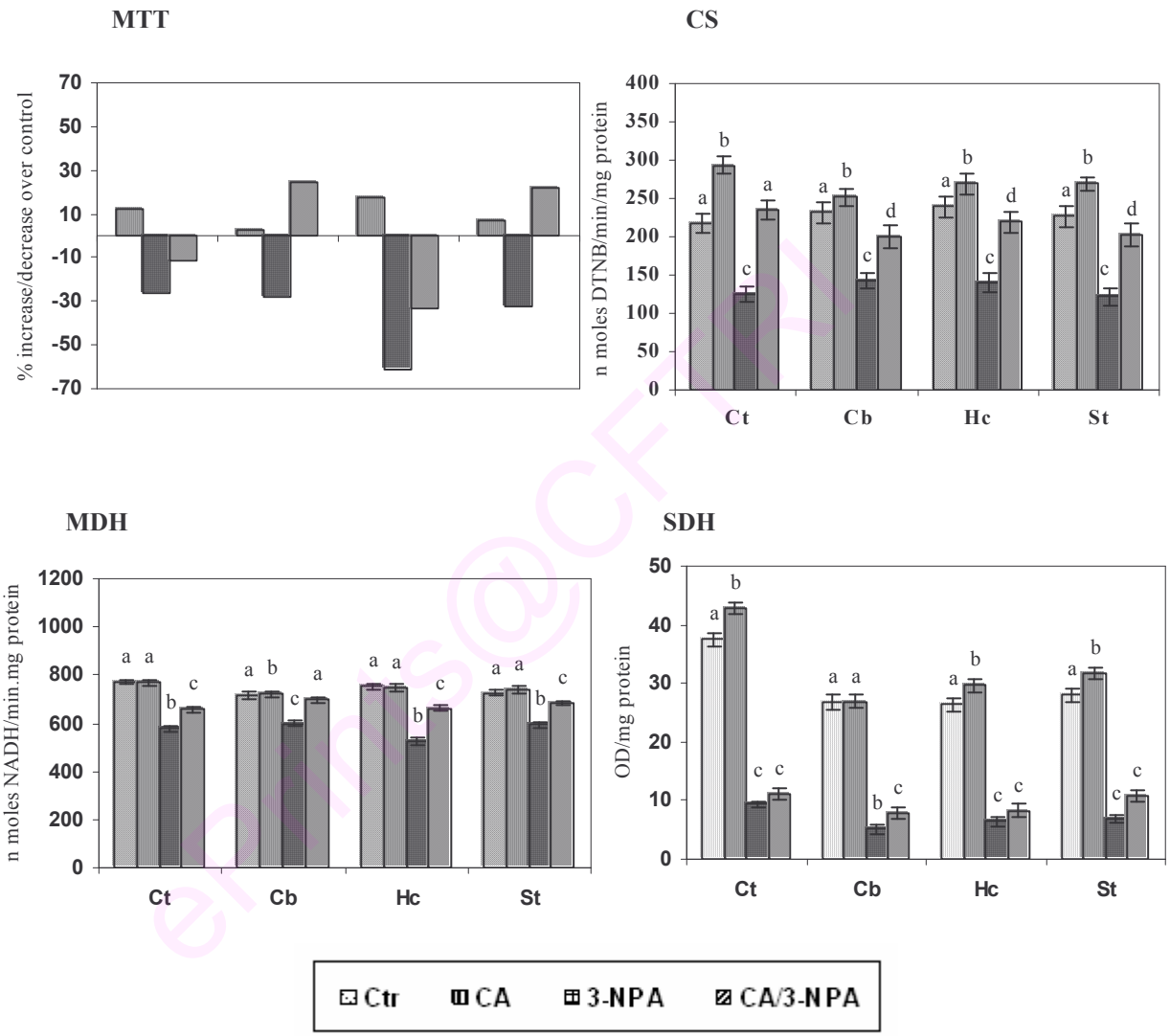


Values are mean± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.22

Effect of CA prophylaxis on 3-NPA induced alterations in viability of mitochondria and activity of mitochondrial enzymes in brain regions of prepubertal mice.

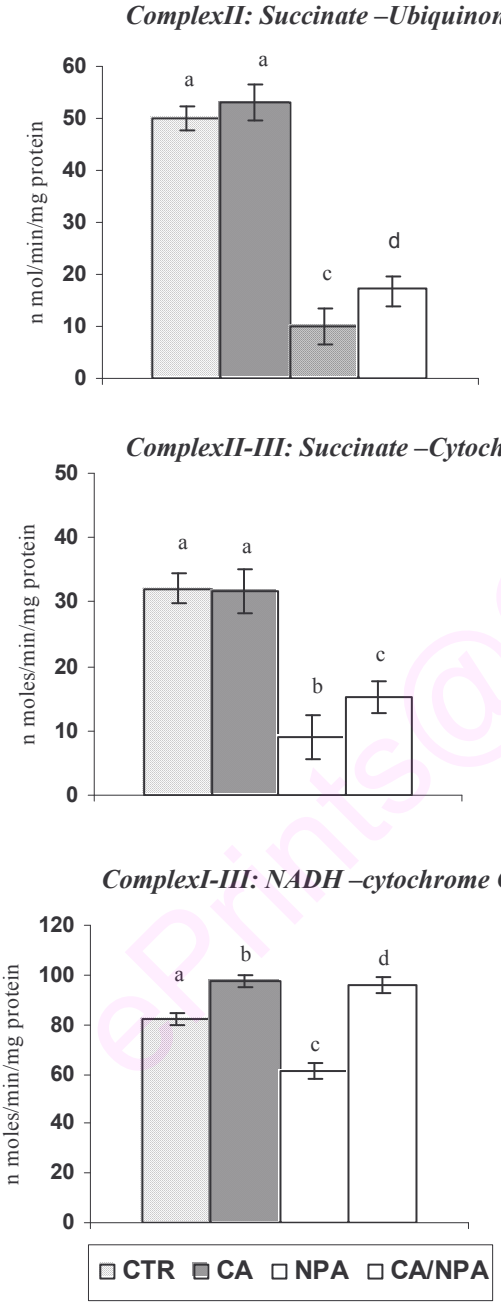


Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.23

Effect of CA prophylaxis on NPA-induced alterations ETC enzymes in striatum of prepubertal mice.

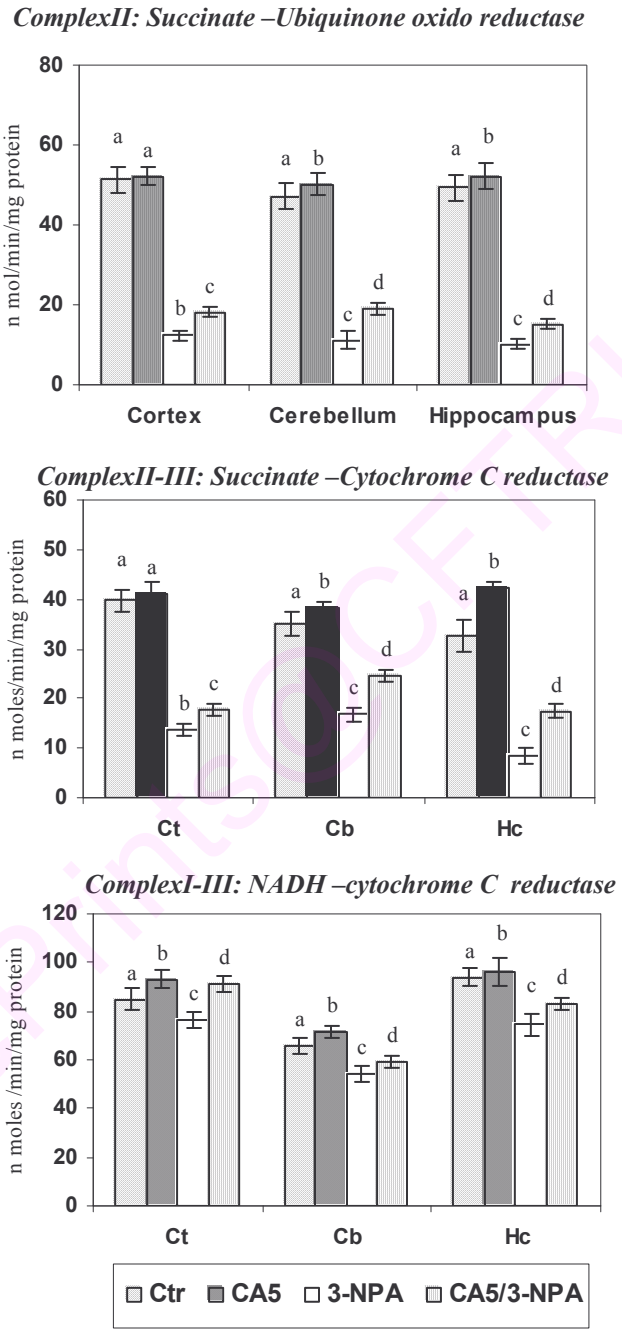


Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.24

Effect of CA prophylaxis on NPA-induced alterations in ETC enzymes in brain regions of prepubertal mice

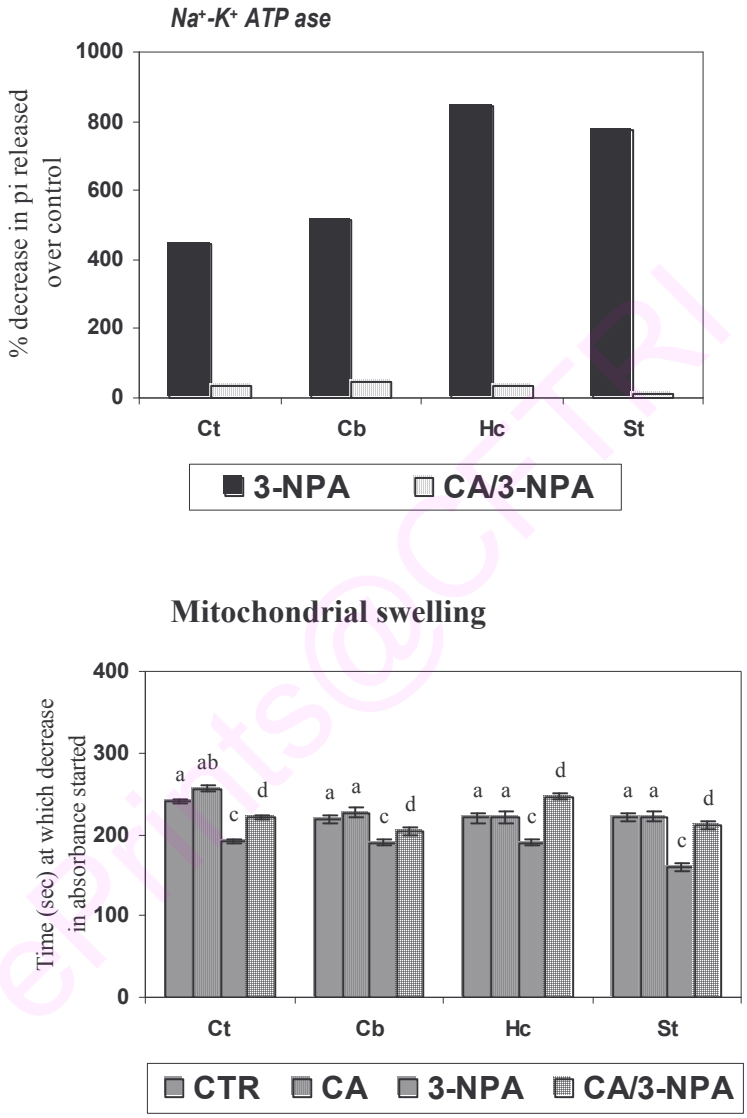


Values are mean± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

Fig: 2.25

Effect CA prophylaxis on NPA-induced alterations in Na+,K+ ATP-ase activity and mitochondrial swelling in brain regions of prepubertal mice.



Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days).

Table 2.9

Effect of NPA on the status of Acetylcholinesterase and Butyrylcholinesterase activity in cytosol of brain regions of prepubertal mice given CA prophylaxis.

Group	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
AChE [1]				
CTR	6.04±0.50 [a]	6.55±0.65 [a]	7.12±0.78 [a]	6.31±0.78 [a]
CA	7.41±0.46 [b]	7.92±0.86 [b]	8.21±0.56 [b]	7.89±0.56 [b]
3-NPA	5.10±0.68 [c]	5.12±0.74 [c]	5.78±0.38 [c]	4.77±0.90 [c]
CA+3-NPA	6.63±0.56 [a]	6.67±0.76 [a]	7.32±0.69 [a]	6.87±0.84 [a]
BuChE [2]				
CTR	2.26±0.25 [a]	2.09±0.17 [a]	2.04±0.27 [a]	2.25±0.16 [a]
CA	3.17±0.11 [b]	2.44±0.28 [b]	2.49±0.38 [b]	2.45±0.24 [b]
3-NPA	2.06±0.23 [c]	1.87±0.35 [c]	1.07±0.19 [c]	1.57±0.29 [c]
CA+3-NPA	2.49±0.19 [a]	2.76±0.19 [d]	1.87±0.35 [d]	2.10±0.16 [d]

Values are mean ± S.D of six determinations each; data analyzed by ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

CA: *Centella asiatica* aqueous extract (5mg/kg bw, 10days)
3-NPA: 3-nitropropionic acid (75mg/kgbw, 2days)

1-η moles substrate/min/mg protein
2-η moles substrate/min/mg protein

5.0 DISCUSSION

The extensive use of CA in Ayurvedic system of medicine necessitates a basic understanding of its possible mechanism of action (Dash et al., 1996). Recently investigators have speculated that the protective effects of CA may be related to its antioxidant properties (Hamid et al., 2002; Veerendrakumar and Gupta, 2002). However, evidences demonstrating the direct involvement of antioxidative action of CA *in vivo* are limited. More importantly, data on its potency to modulate endogenous markers of oxidative stress in different brain regions in prepubertal rodents is lacking. Hence, we determined the implications of feeding CA powder in the diet to prepubertal mice. The criterion for selection of prepubertal mice was based on the following: (i) the brain in a 4 wk old mice is still in the process of development of new inter neuronal connections and will continue during the postnatal development till the adult architecture is established by about 6 weeks (Rao et al., 2005) (ii) the prepubertal brain may be more responsive (iii) the enhancement of memory and increased antioxidant levels are necessary in growing stage. Hence, in the present study we examined (i) the efficacy of CA in diminishing the basal oxidative stress markers and antioxidant enzyme levels in different regions of pre pubertal mice (ii) The antioxidant ability of CA extract in chemical systems and to abrogate 3-NPA induced oxidative dysfunctions in brain mitochondria *in vitro* and (iii) The prophylactic efficacy of CA aqueous extract against 3-NPA induced early oxidative stress and mitochondrial dysfunctions.

Results of our dietary study clearly demonstrated that CA- fed mice exhibited a substantial reduction in the basal levels of ROS, MDA and hydroperoxides both in cytosol and mitochondria of all brain regions. Peroxidation of lipids represents a primary consequence of cellular oxidative stress (Halliwell, 2006) and the oxidation of membrane phospholipids in the plasma membrane, as well as within internal organelle membranes such as the mitochondria, leading to biophysical changes that disrupt membrane and organelle function which may promote cell death. In addition, lipid peroxidation may lead to the production of additional reactive species like 4-HNE (Awasthi et al., 2004). Our findings are consistent with earlier reports of reduced LPO in erythrocytes of CA fed adult rats (Hussin et al., 2007), reduction in MDA in whole brain of adult rats (Veerendrakumar and Gupta, 2002). However, our results for the first time demonstrate that CA has a uniform modulatory effect on oxidative markers in various brain regions of prepubertal mice.

ROS have been implicated to be the most likely candidate responsible for producing the neuronal changes in neurodegenerative disorders (Cantuti-Castelvetri et al., 2000) causing the progressive modification or degradation of cellular macromolecules including DNA, protein, lipids and carbohydrates when produced at elevated non physiological concentrations (Halliwell, 2006). Although H_2O_2 may freely diffuse out of mitochondria (Cadenas and Davies, 2000), it has a relatively long half life and a unique property of being soluble both in lipid and aqueous media (Droge, 2002). Moreover cytochrome C present in the mitochondrial inner membrane is an iron containing protein and mitochondrial oxidative damage can result in its release into the cytoplasmic compartment (Kroemer et al., 1998) promoting secondary ROS generation from nonspecific iron catalyzed reaction (Hazel et al ., 2006). CA may provide protection against this secondary reaction by chelating iron released from the inner mitochondrial membrane and thus prevent ROS generation. This is consistent with recent reports which have demonstrated CA to chelate metals such as arsenic *in vivo* (Gupta and Flora, 2005) and iron *in vitro* (Shinomol and Muralidhara, unpublished data).

In the present model, a significant increase in both GSH and thiols (total and non protein thiols) were discernible in all brain regions of mice fed CA both in cytosol and mitochondria, clearly suggesting that the active components of CA possess the propensity to enhance the thiol containing antioxidant molecules. The thiol compounds have critical importance in the 3 tier antioxidant defense of our body (Russel, 1998) and increasing the thiol containing antioxidants in brain provides protection against a wide range of oxidative and toxic insults in the prepubertal brain. Moreover these elevations may explain at least in part for the reduction in endogenous levels of ROS, MDA, hydroperoxides and protein carbonyls in brain regions of CA fed mice. Further, CA also caused a significant increase in the activities of antioxidant enzymes such as CAT, GSH-Px and SOD. This finding is consistent with the previous reports of elevated activities of SOD and Catalase in whole brain homogenate of adult rats fed CA (Veerendrakumar and Gupta, 2002).

Among the various oxidative modifications of amino acids in proteins, protein carbonyl formation may be an early marker for protein oxidation (Levine et al., 1990; Grune et al., 2004). Further, it also reflects a very low rate of oxidized protein degradation and or low repair activity since oxidized forms of some proteins and proteins modified by lipid peroxidation products are not only resistant to proteolysis, but also can inhibit the ability of proteases to degrade the oxidized forms of other proteins and form aggregates (Dalle-Donne, 2003). Following CA consumption,

protection against protein oxidative damage was also evident from the reduction in the endogenous protein carbonyl levels in all brain regions of growing mice. Since CA fed mice exhibited relatively lower basal ROS levels, we speculate that the lowered protein carbonyl level may also be due to the radical scavenging property of CA.

Another interesting observation was the significant enhancement in AChE activity in different brain regions of CA fed animals suggesting altered cholinergic function. Earlier workers have shown that oral administration of *Clitoria terneata* causes increased AChE activity and elevated acetylcholine content in rat brain (Taranalli and Cheeramkuzhy, 2000). Interestingly, up regulation of AChE activity is reported to be sufficient to reverse memory deficits (Parent and Baxer, 2004). Our finding of increased AChE levels might reflect the enhancement of acetylcholine release which would facilitate in synaptic transmission of CA3 pyramidal neurons which are still branching during growth spurt period (Rao et al., 2005). This thinking is consistent with a recent study in rats employing intracerebroventricular streptozotocin model of Alzheimer's disease in which CA was demonstrated to significantly mitigate oxidative stress and enhance cognitive behavior (Veerendrakumar and Gupta, 2003). The enhancement of cognitive functions by CA may be attributed to its ability to elevate brain AChE activity.

In conclusion, dietary CA markedly diminished the levels of endogenous oxidative markers, increased the antioxidant molecules and enzymes *in vivo* in both homogenates/ mitochondria. These results necessitate further understanding of the biochemical mechanisms underlying the neuroprotective effects of CA against select neurotoxicants *in vivo* in order to optimize its usage in humans. Our results provide direct evidence to show that antioxidant properties may in part be responsible for the modulatory effects of *Centella asiatica* leaf powder *in vivo* and can be better exploited to protect against neuronal dysfunctions among children.

In another study, the ability of CA leaf powder to ameliorate Khesari dhal (KD) induced oxidative damage in brain regions of male mice was examined. CA supplementation is known to possess a significant protective value as it showed potent antioxidant activity against arsenic sensitive biochemical variables in blood, moderate ability to chelate arsenic and is suggested for chelation therapy (Gupta and Flora, 2005). Further the effect on CA on various parameters like oxidative stress markers on cognition and memory are known (Gupta et al., 2003). However there are no reports on the efficacy of CA dietary supplementation to modulate KD induced

oxidative stress and mitochondrial dysfunctions in growing male mice. The nature and extent of oxidative impairments occurring in brain regions of mice following consumption of KD was described earlier (Chapter 1) KD induced significant elevations in oxidative stress markers in both cytosol and mitochondria of brain regions as evident from increased LPO and ROS, while CA supplemented mice showed normal levels of oxidative markers suggesting the protective efficacy of CA. Earlier studies have shown that β-ODAP- induced neurotoxicity, was prevented in a dose-dependent manner by focal co-injection of potential free radical scavengers; dimethyl sulphoxide (1750–7000 ηmol), dimethylthiourea (8000 ηmol), dimethylformamide (7000 ηmol) and mannitol (1000 ηmol). These findings suggest that hippocampal damage induced by β-ODAP- involves an interaction between AMPA receptors and free radicals (Willis et al., 1994). However, there are no reports on the use of phytochemicals in modulating KD–induced oxidative stress.

In the present study, there was significant increase in the levels of hydroperoxides indicative of the potency of KD to induce hydrogen peroxide formation which may partially account for the oxidative damages observed. Further the complete normalization of hydroperoxide levels in CA supplemented group is indicative of the ability of CA to scavenge hydrogen peroxide. GSH depletion is a robust and significant alteration in the antioxidant defense which is observed in many neurodegenerative diseases like PD and Neurolathyrism is no exception. We have reported earlier a marginal decrease in GSH following KD diet (Shinomol and Muralidhara, 2007). It has been suggested that GSH depletion particularly of mitochondria may precede the impairment of oxidative phosphorylation (Schulz et al., 2000). Apart from functions such as in GSH-peroxidase dependent metabolism of hydoperoxides and direct scavenging of reactive oxygen species, GSH may contribute to antioxidant defense by networking with other major antioxidants such as vitamins E and C (Packer et al., 1995). There was significant GSH depletion in both mitochondria and cytosol of all brain regions of KD fed mice indicating a state of compromised antioxidant status. As GSH is associated with many critical redox regulatory genes (Sen, 1997), GSH depletion (of about 20-30%) can impair the cell's defense against the toxic actions of ROS and may lead to cell injury and death (Reed, 1990). Further in the present study in CA supplemented KD group, there were no significant reduction in GSH levels indicative of the ability of CA to modulate redox status of cells. Additionally, there was significant depletion in total

thiol levels in both mitochondria and cytosol of brain regions of mice fed KD, while in CA supplemented group, no such alterations were discernible.

GSH depletion was associated with significant alterations in antioxidant enzyme activities in both mitochondria and cytosol of KD fed mice. Significant increase was observed in the activities of GSH-Px, GST and SOD in both cytosol and mitochondria followed by a decrease in catalase activity. The decrease in catalase and increase in GSH-Px and SOD may be due to the increase in ROS and hydroperoxides. GST is directly involved in detoxification mainly of xenobiotics (Menegon et al., 1998) and the significant increase in its activity in both mitochondria and cytosol of brain regions of KD fed mice can be explained as a response to β-ODAP toxin. Further, increase in GST may also be due to the increase in the levels of malondialdehyde formed in all brain regions following KD intake.

Carbonyl content of proteins are indicative of oxidative stress mediated protein-oxidation (Levine et al, 1990).The accumulation of oxidized proteins is a complex function of the rates and kinds of ROS formed, levels of numerous antioxidants systems ,and the rates of degradation of oxidized proteins by a multiplicity of proteases (Stadtman, 1990). Further protein oxidation *in vivo* affect variety of cellular functions involving proteins: receptors, signal transduction mechanisms, transport systems and enzyme (Samuel et al., 2005). The significant increase in protein carbonyls in both mitochondria and cytosol of brain regions of KD fed mice suggest a state of oxidative stress. CA supplementation resulted in normalization of those levels indicating its ability to protect against severe protein oxidation probably by scavenging the free radicals and thus preventing the chain reactions leading to protein oxidative damage.

Earlier reports have shown LDH leakage from brain slices on exposure to β-ODAP *in vitro* (Ravindranath, 2002). In the present study significant elevation in LDH activity was observed in brain regions of mice fed KD. LDH elevation may be due to increased ROS and subsequent oxidative stress leading to membrane damages. This result can again be explained more authentically as there was a decrease in Na^+ K^+ATP ase activity which is a membrane bound enzyme. Further, there was marginal decrease in the activity of Complex I enzyme, which is situated in the inner mitochondrial membrane. Inhibition of Complex I on exposure to β-ODAP toxin has been reported earlier (Diwakar et al., 2007). There was a decrease in mitochondrial respiration measured in terms of MTT reduction indicative of mitochondrial dysfunctions. CA

supplementation resulted in amelioration of these perturbations indicative of the protective property of CA against mitochondrial dysfunctions. Further there was a decrease in AChE activity on feeding KD which may be due to Increase ROS generated (Shinomol and Muralidhara, 2007). CA supplementation resulted in normalized AChE activity in all brain regions of mice fed KD indicative of the protective efficacy of CA against cholinergic dysfunctions.

CA aqueous extract is known to contain many bioactive compounds *viz.,* triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins *viz.,* madecassic and asiatic acid (Mangas et al., 2006) along with numerous caffeic acid derivatives and flavonols and in particular quercetin, kaempfownerol, catechin, rutin, and naringin (Zainol et al., 2003) some of which have been shown to be potent antioxidants (Hussin et al., 2007). We studied the efficacy of CA extract to abrogate 3-NPA induced oxidative stress in mitochondria, synaptosomes and slices isolated from various brain regions *in vitro.* Mitochondria exposed to *3*-NPA showed significant concentration dependent elevation in all the markers *viz.,* ROS, MDA and hydroperoxide levels suggesting induction of oxidative stress. This data is consistent with earlier findings of 3-NPA induced oxidative stress *in vivo* (Fu et al., 1995). In the presence of aqueous extract of CA, 3 -NPA -induced generation of ROS and hydroperoxides were brought to normalcy in mitochondrial fraction clearly suggesting its propensity to mitigate neuronal dysfunctions even at the sub cellular level. CA extract also provided protection against 3-NPA induced lipid peroxidation *in vitro* in mitochondria in all brain regions. Similar results were obtained for synaptosomes while more robust results were obtained for slices. These results are consistent with the results obtained in rat brain synaptosomes (Perez de-la Cruz et al., 2006).

Mammalian mitochondria are ubiquitous intracellular organelles that are responsible for the provision of ATP by aerobic metabolism and hence tissues with high aerobic activity, e.g., brain, skeletal and cardiac muscle have particularly high concentrations of mitochondria. Mitochondria also serve a variety of functions within the cell other than the production of ATP. They play a critical role in the regulation of apoptosis with pro- and antiapoptotic proteins being localised at the outer mitochondrial membrane and proapoptotic cytochrome c and apoptosis inducing factors located in the mitochondrial matrix. They also serve as cellular calcium buffers. The respiratory chain is also an important source of free radicals (Orth and Schapira, 2001). Many lines of evidence suggest that mitochondria have a central role in ageing-related

neurodegenerative diseases. However, despite the evidence of morphological, biochemical and molecular abnormalities in mitochondria in various tissues of patients with neurodegenerative disorders, the question ‘‘is mitochondrial dysfunction a necessary step in neurodegeneration?’’ is still unanswered (Petrozzi et al., 2007). Neuronal dysfunction and death have been ameliorated in animal and cell culture models of AD, PD, and HD (which are the major NDD) by manipulations that reduce levels of oxidative stress and by agents that maintain ATP levels and ion homeostasis (Mattson , 2003). There have been very few studies where phytochemicals have been used for therapeutic use in NDD. Hence we chose to examine whether CA prophylaxis would protect prepubertal mice against 3-NPA induced early oxidative damage and associated mitochondrial dysfunctions in striatum and other brain regions.

We chose 3-NPA as the model neurotoxicant and the basis of selection has been explained earlier (chapter 1). In the present study, we examined the prophylactic efficacy of CA aqueous extract on 3-NPA induced early oxidative stress in striatum and other brain regions in a prepubertal mice model. At the administered dose, 3-NPA caused significant oxidative impairments in brain regions as evidenced by marked elevation in ROS and MDA levels, perturbations in the activities of antioxidant enzymes, diminished GSH levels and elevated protein carbonyl content. These principal findings are in agreement with earlier reports of oxidative stress induction in brain regions of adult mice exposed to 3-NPA (Fu et al., 1995; Binienda et al., 1998). Striatal region showed relatively higher susceptibility to NPA-induced oxidative stress followed by cerebral cortex and hippocampus. The endogenous levels of oxidative markers in mitochondria and cytosol of brain regions were significantly diminished in mice given CA treatment *per se*. Interestingly, NPA intoxication among mice given CA prophylaxis did not induce any appreciable oxidative damage and mitochondrial dysfunctions in different brain regions clearly suggesting its neuroprotective propensity.

In the present model, 3-NPA treatment caused a significant decrease in brain GSH levels, a finding which conforms to earlier reports in adult animals (Binienda et al., 1998). The thiol group plays a key role in the cellular antioxidant defenses, the metabolism of xenobiotics and the regulation of cell cycle (Dringen, 2000). In particular, GSH efficiently reacts with electrophilic species by an addition reaction catalyzed by glutathione- S- transferase enzymes (Awasthi et al., 2004). CA prophylaxis offered significant protection against 3-NPA induced GSH depletion as

evidenced by restoration of GSH levels in striatum as well as in other brain regions. Hence it is likely that GSH related mechanism/ s may in part be responsible in protecting the various brain regions against 3-NPA induced oxidative stress. Further, the protective efficacy of CA extract may also be partly ascribed to its ability to enhance the antioxidant status in brain, since CA *per se* uniformly elevated the activities of GSH-Px and SOD.

In the prophylactic study, we observed elevated levels of protein carbonyls in all brain regions among 3-NPA treated mice suggesting protein oxidation. Among the various oxidative modifications of amino acids in proteins, protein carbonyl formation may be an early marker for protein oxidation (Levine et al., 1990; Grune et al., 2004). Further, it also reflects a very low rate of oxidized protein degradation and or low repair activity since oxidized forms of some proteins and proteins modified by lipid peroxidation products are not only resistant to proteolysis, but also can inhibit the ability of proteases to degrade the oxidized forms of other proteins and form aggregates (Dalle-Donne, 2003). CA prophylaxis afforded marked protection against 3-NPA induced protein oxidative damage as the protein carbonyl levels were significantly attenuated in all brain regions. Interestingly, CA extract *per se* fed mice also exhibited relatively lower endogenous levels, suggesting that this effect may be related to its radical scavenging property as observed in our *in vitro* studies. Alternatively it may also be due to an over expression of enzymes like carbonyl reductase apart from the direct free radical scavenging effect.

In the present model, 3-NPA administration to normal mice caused marked reduction in the activities of mitochondrial SDH, while the reduction in the activities of citrate synthase and MDH were less robust. In contrast, 3-NPA intoxication in mice given CA prophylaxis did not induce any significant effect on CS and MDH activities, while marginal restoration of SDH was evident (17% protection). Although the precise reason for this differential protective effects are not clear, it may be related to the fact that 3-NPA is an irreversible inhibitor of SDH (Brouillet et al., 1995). Further, it is unlikely that the protective effects of CA extract are due to direct interaction with 3-NPA, activation of SDH or reversal of blocking action of SDH inhibition, since CA *per se* had no significant effect on SDH activity.

Further, 3-NPA administration resulted in a significant degree of reduction in activity of enzymes, complex II and Complex II-III, in different brain regions, while the effect on Complex I-III was marginal. An increased ROS production in prepubertal brain mitochondria can inhibit ATP

synthesis, release cytochrome c and induce mitochondrial permeability transition (mPT) (Ichas and Mazat, 1998; Rizzuto et al., 2000). Further it is also probable that the elevated hydrogen peroxide levels and ROS levels following 3-NPA exposure can be the result of inhibition of ETC enzymes. Interestingly, CA prophylaxis offered significant degree of protection against these alterations suggesting its potential to preserve the integrity of the mitochondrial respiratory chain and thus energy metabolism. Although speculative, these results suggest that CA extract may serve as useful mitochondrial protectant and can be further exploited in the treatment of neuronal dysfunctions.

Marked decrease in the activity of Na^+, K^+ ATP ase observed in the mitochondria of 3-NPA treated mice suggested significant membrane damage. Recent studies have implicated a vital role for mitochondria in cell death mechanisms, since mitochondrial dysfunction results in the release of factors that initiate, amplify and execute various signals resulting in apoptotic cell death (Kroemer and Reed, 2000). Additionally, mitochondrial dysfunction and associated bio-energetic failure can lead to abnormal cellular ion homeostasis, as a result of which cells undergo swelling and cellular disruption, eventually leading to necrotic death (Nieminen, 2003). Significant degree of protection against decrease in Na^+ K^+ ATP ase activity was discernible among 3-NPA administered mice given CA prophylaxis clearly indicating the protective effect of CA extract against membrane damage. Increased mitochondrial swelling in various brain regions following 3-NPA intoxication is probably suggestive of the opening of the voltage gated channels in the mitochondrial membrane. Oxidative stress and increased inorganic phosphate levels are causative agents in the induction of mitochondrial swelling (Norenberg and Rao, 2007). Induction of the mPT can clearly result in cell death since the mPT leads to cessation of the ATP synthesis leading to loss of ion homeostasis, cell disintegration and death. mPT would collapse the mitochondrial membrane potential ($\Delta\psi m$) and uncouple the electron transport system from production of ATP. Additionally mPT results in mitochondrial swelling and can lead to the release of pro-apoptotic proteins. This significant mitochondrial dysfunction and the resulting energy deficit could trigger the onset of neuronal degeneration and death (Hansson et al., 2004). CA prophylaxis markedly prevented 3-NPA induced mitochondrial swelling suggesting its potential to protect against critical membrane damaging events.

In conclusion, our findings demonstrate the prophylactic neuroprotective efficacy of an aqueous extract of CA, although the precise mechanism/s are not clear. Based on our results, we hypothesize that it may be due to its ability to act as an antioxidant in scavenging ROS, ability to preserve the mitochondrial metabolic state and integrity of ETC. Further, it may also be related to the potential of CA extract in maintaining the key antioxidant enzyme status and the thiol redox status in mitochondria. Based on these findings it is suggested that *C.asiatica* may be specifically employed as a therapeutic adjuvant in protecting the prepubertal brain against neurotoxicant induced oxidative insult and can find therapeutic applications under other neurodegenerative disorders exhibiting elevated oxidative stress status.

6.0 SUMMARY

1. *Centella asiatica* leaf powder fed to prepubertal mice at dietary levels (0.5 and 1.0%) caused significant diminution in the levels of oxidative markers (MDA, ROS and HP) in both cytosol and mitochondria of different brain regions. Reduced levels of protein carbonyls in brain regions of CA fed mice suggested decreased oxidation of proteins.

2. CA fed mice showed enhanced GSH levels, total thiols and non-protein thiols in both cytosol and mitochondria of brain regions of mice and concomitant elevation in the activity of various antioxidant enzymes *viz.,* catalase, GSH-Px and SOD indicating the potential of CA to augment the levels of antioxidant defense in brain tissue .

3. CA consumption caused a significant increase in the activity of AChE in all the brain regions of mice suggesting its ability to modulate cholinergic functions *in vivo*

4. A standardized CA aqueous extract exhibited significant antioxidant activity in selected *in vitro* chemical systems.

5. CA aqueous extract exhibeted marked free radical scavenging activity in mitochondria, synaptosomes and slices under 3-NPA –induced oxidative stress *in vitro* suggesting the antioxidant potential of the CA components.

6. Dietary supplementation of CA leaf powder was able to significantly attenuate Khesari dhal induced oxidative stress in mitochondria and cytosol of various brain regions of mice

as ascertained by varying degrees of protection against oxidative dysfunctions. Further the alterations in antioxidant enzymes were restored to near normalcy by CA supplementation.

7. CA supplementation offered considerable protection against alterations in TCA cycle enzymes and ETC enzymes. The decreased Na^+ K^+ ATP-ase activity was also normalized among CA supplemented mice.

8. Prepubertal mice given CA extract prophylaxis were markedly protected against 3-NPA-induced increased oxidative stress in both cytosol and mitochondria. There was complete protection against neurotoxicant–induced elevations in oxidative markers (MDA, ROS, HP and PC) and alterations in enzymic antioxidant defenses in both cytosol and mitochondria.

9. CA prophylaxis (10 days) offered marked protection against 3-NPA induced depletion of GSH, total thiols and non-protein thiols in both mitochondria and cytosol of striatum and other brain regions. Further various mitochondrial impairments caused by 3-NPA were prevented to a greater extent among mice given CA prophylaxis. These comprised of alterations in the activity of citrate synthase, TCA cycle enzymes, ETC complex enzymes, and Na^+K^+ ATP ase.

10. 3-NPA-induced increase in mitochondrial swelling property as well as reduced viability of mitochondria (MTT assay) was significantly modulated by CA prophylaxis.

11. In general 3-NPA- induced perturbations in the activity of cholinergic enzymes (AChE and BChE) was also modulated by CA prophylaxis.

12. Collectively these experimental evidences demonstrate that CA leaf powder possesses considerable neuromodulatory activity at low dietary levels in prepubertal male mice. Further the aqueous extract exhibited marked prophylactic efficacy as evidenced by abrogation of 3-NPA induced early oxidative stress and mitochondrial dysfunctions. It is hypothesized that the neuroprotective action of CA extract may be related to its multiple free radical scavenging potency, ability to enhance antioxidant defenses GSH levels and redox status.

1.0 INTRODUCTION

The medicinal efficacy of *Bacopa monnieri*, (Brahmi), Family-Scrophulariaceae is extensively reported in Indian traditional literature such as Athar-Ved, Charak Samhita, Susrutu Samhita for treatment of epilepsy, insomnia and anxiety and also as a mild sedative and memory enhancer (Tripathi et al., 1996, Ernst 2006). Besides, *B. monnieri* displays antioxidant (Tripathi et al., 1996), antistress (Chowdhuri et al., 2002), and anxiolytic (Singh and Singh, 1980; Shanker and Singh, 2000) activities in animals. It improves the performance of rats in various learning situations such as shock-motivated brightness discrimination reaction, an active conditioned flight reaction, and continuous avoidance response (Singh and Dhawan, 1982). The protective effects of *B. monnieri* (BM) extract are explained to be due to several mechanisms such as chelation of metal ions (Tripathi et al., 1996), scavenging of free radicals (Russo et al., 2003) and enhancement of the activities of antioxidative defense enzymes (Bhattacharya et al., 2000). Further, the antistress activity in experimental animals is ascribed to the modulation of Hsp70, Cytochrome P450, and SOD (Chowdhuri et al., 2002), enhanced kinase activity, neuronal synthesis coupled with restoration of synaptic activity, and nerve impulse transmission (Singh and Dhawan, 1997Kishore and Singh, 2005). Recently, the antioxidative, anticytotoxic and DNA protecting capability of BM extract were demonstrated in human non-immobilized fibroblasts (Russo et al., 2003).

Bacopa extract contains two prominent constituents namely, bacoside-A and bacoside-B (Chatterji et al., 1965; Singh et al., 1988). Bacoside A is shown to alleviate the amnesic effects of scopolamine (Dhawan and Singh, 1996; Russo and Borrelli, 2005) and provided protection against phenytoin (an antiepileptic drug)-induced deficit in cognitive function in mice (Vohora et al., 2000). Several clinical studies suggest that Bacopa extract can significantly improve the speed of visual processing, learning rate and memory consolidation (Stough et al., 2001). Few studies have reported that Bacopa revitalizes the intellectual functions in children (Sharma et al., 1987). In clinical studies, the choice of dose and duration of treatment appears to be critical in obtaining optimal protective effects. For example, chronic oral administration of Bacopa (300 mg) for 5–12 weeks is shown to substantially improve the higher order of cognitive process in healthy humans (Stough et al., 2001). Likewise brahmi treatment was shown to decrease the rate of forgetting of newly acquired information in healthy humans (Roodenrys et al., 2002).

Evidences suggest that several endogenous and exogenous antioxidants (such as coenzyme Q10, N-acetylcysteine, melatonin, S-allylcysteine, L-carnitine and dehydroepiandrosterone) offer significant protective effects against the neuronal damage induced by 3-NPA both *in vitro* and *in vivo* (Nam et al., 2005; Herrera-Mundo et al., 2006; Pérez-de la Cruz et al., 2006). Although the precise mechanism/s by which antioxidants may protect against 3-NPA toxicity still remain unclear, it is speculated that they may be related either to the scavenging of free radicals or by preventive actions on the blockade of SDH. Although the role of phytochemicals as neuromodulators is gaining attention, not many studies have examined the possible modulation of 3-NPA induced neurotoxicity. In a recent study ginseng saponins were shown to protect against 3-NPA induced striatal degeneration in rats (Kim et al., 2005). In this context we have comprehensively studied the propensity of *Bacopa monnieri* to ameliorate 3-NPA induced mitochondrial dysfunctions and early oxidative damage in brain regions of prepubertal mice.

The neurotoxicant rotenone is a classical, well-characterized and high affinity specific inhibitor of mitochondrial NADH dehydrogenase (complex I), one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation (Thiffault et al., 2000). A defect of mitochondrial function due to complex I inhibition is postulated to be the cause of rotenone-induced neurodegeneration (Betarbet et al., 2000; Jenner, 2001). Rotenone is highly hydrophobic, crosses biological membranes easily and does not depend on the dopamine transporter for access to the cytoplasm of dopaminergic neurons (Betarbet et al., 2000; Greenamyre et al., 2001). Rotenone causes dopamine release, as evidenced by microdialysis and neurochemical data (Thiffault et al., 2000) and this may also contribute to the degeneration of dopaminergic neurons. Further, rotenone is the only neurotoxin known today that induces the formation of Lewy bodies, which are the most characteristic histopathological feature of Parkinson's disease (Betarbet et al., 2000). Inhibitors of complex I are known to increase ROS generation, which produces a state of oxidative stress leading to mitochondrial dysfunction (Wang et al., 2005; Mao et al., 2007). Subsequently the mitochondrial permeability transition pore is activated resulting in loss of mitochondrial membrane potential and triggers a cascade of caspases including caspase-3, with the cleavage of cellular proteins and the orderly demise of the neuron (Debatin et al., 2002). In addition, the Bcl-2 family of proteins are suggested to play a certain role in regulation of apoptosis. The interaction between the anti-apoptotic and pro-

apoptotic Bcl-2 family members can alter the permeability of mitochondrial membrane and control the release of cytochrome c (Budd, 2002).

There have been attempts to modulate rotenone induced mitochondrial dysfunctions using various therapeutic agents both *in vitro* and *in vivo.* In a recent study, protocatechuic acid was shown to inhibit the rotenone-induced apoptotic cell death in PC12 cells *via* amelioration of mitochondrial dysfunctions (Liu et al., 2007). Compounds such as fratexin (Sanchez-Reus et al., 2005) and N-acetyl cysteine (Bahat-Stroomza et al., 2005) have been shown to prevent rotenone -induced apoptotic death of DA cells. The behavioral and neurochemical effects of rotenone in rats was shown to be antagonized by exogenous administration of 1-methyl-1,2,3,4-tetrahydroisoquinoline (1MeTIQ) (Antkiewicz-Michaluk et al., 2003). In a recent study, a standardized extract of H*ypericum perforatum* (Sanchez-Rheus et al., 2007) and L-Deprenyl (Saravanan et al., 2006) have shown to attenuate rotenone-induced oxidative stress in rats.

Hence in a series of investigations, the neuroprotective properties of *B. monnieri* leaf powder and ethanolic extract have been studied. The results are presented separately under two sections. In **section A**, there are two subsections. In *subsection (i*) data on the propensity of dietary *BM* leaf powder to modulate endogenous levels of oxidative markers and related antioxidant levels are described, where as in *subsection (ii),* the *ex vivo* modulatory potential of BM against 3-NPA *in vitro* is emphasized. In **Section B** there are three sub sections where in under *subsection (i)* data on the *in vitro* antioxidant potential of an ethanolic extract of BM both in chemical and biological systems are presented. In *subsection (ii)* the prophylactic efficacy of BM extract against 3-NPA induced early oxidative stress and mitochondrial dysfunctions is enumerated. In subsection (iii) the prophylactic efficacy of BM extract against Rotenone induced oxidative stress, mitochondrial dysfunctions and motor behavior alterations are described.

2.0 OBJECTIVE

The primary focus of these investigations was to obtain evidences to illustrate that *Bacopa monnieri* leaf powder *per se* can reduce the basal levels of oxidative markers in mitochondria and cytosol of brain regions of prepubertal mice. Further, to establish the prophylactic efficacy of Bacopa ethanolic extract against 3-nitropropionic acid and Rotenone induced oxidative dysfunctions in mitochondria as well as behavioral alterations.

3.0 EXPERIMENTAL DESIGN

SECTION A

Propensity of *Bacopa monnieri (BM)* leaf powder to modulate endogenous oxidative markers in brain of prepubertal mice

Preparation of Bacopa monnieri supplemented diet

The *Bacopa monnieri leaf* powder (preparation described in materials and methods section) was mixed homogenously with commercial diet at dosages of 0.5, 1.0 and 2%.

Experimental protocol

Prepubertal mice were randomly assigned to control and treatment groups. While the control mice received normal powder diet, mice of the treatment groups received BM incorporated diet (0.5, 1.0 and 2.0 %) for 30 days. Daily food intake was recorded by weighing the residual diet. Body weight gain was also recorded every week. In order to assess the progressive effect of BM, on endogenous oxidative markers, an interim sampling was conducted after 15 days. Terminally, mice of both control and treatment groups were sacrificed by cervical dislocation and vital organs such as brain, liver and kidney were excised, blotted and weighed. Brain regions were dissected out over ice and processed to obtain cytosolic and mitochondrial fractions. The following biochemical investigations were conducted to determine the effect of BM on basal oxidative markers.

Effect of BM on oxidative markers in brain regions

The status of oxidative markers was assessed by determining the extent of lipid peroxidation quantified as MDA levels, ROS generation and hydroperoxides levels, while protein oxidation was measured in terms of protein carbonyls content in cytosol and mitochondria of various brain regions.

Perturbations in antioxidant status

To understand the possible up regulation in antioxidant status induced by BM treatment, the levels of GSH, total thiols and non-protein thiols were determined at the terminal sampling in freshly prepared samples of cytosol and mitochondria of all brain regions. The antioxidant enzyme status was ascertained by selected antioxidant enzymes in cytosol and mitochondria.

Effect on mitochondrial enzymes

The status of redox sensitive enzymes *viz.,* thioredoxin reductase and TCA cycle enzyme- malate dehydrogenase in different brain regions was measured in fresh mitochondrial preparations of different brain regions.

Effect on cholinergic systems

The activity of AChE in different brain regions following BM consumption was determined in freshly prepared cytosol samples.

Ex vivo response of brain regions to neurotoxicant exposure in vitro

In this study, fresh homogenates (Whole brain; cortex) and synaptosomes of both control and BM leaf powder fed mice (1 month) were exposed to varying concentration of 3-NPA (0.5, 1.0 and 1.5 mM). Oxidation induction pattern was determined in the form of malondialdehyde (MDA levels), ROS generation and hydroperoxide levels.

SECTION B

(i) Antioxidant potential of BM ethanolic extract: *in vitro* evidences in chemical and biological systems

Preparation of BM *ethanolic* extract

BM leaf powder was extracted using 90% ethanol, flash evaporated, lyophilized to a green powder (as described under materials and methods section). The extract was dissolved in saline and used for *in vitro* studies

Chemical systems

The antioxidant potential of *Bacopa monnieri* ethanolic extract was assessed employing various chemical systems such as DPPH radical scavenging assay, nitric oxide scavenging assay, hydroxyl radical scavenging assay and superoxide scavenging assay. Further, reducing power assay, total polyphenols assay and protection against deoxyribose oxidation assays were also conducted.

Biological systems

In vitro response in mitochondria: Mitochondria isolated from various brain regions (cortex, cerebellum, hippocampus and striatum) were pre incubated with BM extract (0.5 and

1µg/ml) for 30 minutes. Further untreated and BM pretreated mitochondria were exposed to 3-NPA (2mM) for 1 hr. The extent of oxidative induction was quantified in terms of malondialdehyde formation, ROS generation and hydroperoxide levels. Similar experimental regime was followed for slices obtained from various brain regions.

(ii) Prophylactic efficacy of BM extract against 3-NPA-induced oxidative stress

Experimental protocol

Preliminary study: In a preliminary study, the efficacy of BM prophylaxis to modulate endogenous oxidative markers in brain regions was investigated. For this, prepubertal mice were orally administered with BM extract at dosages viz., 1.25, 2.5 and 5mg /kg bw for a period of 10 days and its ability to modulate endogenous oxidative markers in brain were assessed.

Determinative study: Dosages of 3-NPA and BM ethanolic extract were selected based on the preliminary study. Prepubertal male mice were orally administered (5mg/kgbw) with an ethanolic extract of BM for a period of 10 days (prophylaxis group). Both normal and BM prophylaxis mice were administered 3-NPA (i.p. 75mg/kg bw) on days 9 and 10 and sacrificed 24 hrs after the last dose. Mice given physiological saline served as the normal controls. Biochemical analysis was carried out in mitochondria and cytosol isolated from striatum and other brain regions *viz.,* cortex, cerebellum and hippocampus.

Effect of BM extract on 3-NPA-induced oxidative damage

To determine the protective efficacy of BM on 3-NPA induced oxidative stress damage, various markers such as generation of ROS, lipid peroxidation and hydroperoxide levels were quantified in mitochondria and cytosol. Further, protein oxidation was quantified as protein carbonyls.

Protective effect of BM extract against perturbations in antioxidant status

To determine the protective effect of BM, the activities of antioxidant enzymes were measured in cytosol/mitochondria of different brain regions. Further, the status of GSH, total thiols and non–protein thiols were also quantified in cytosol/mitochondria.

Effect on the status of Vitamin C and Iron in brain regions

The effect BM prophylaxis on 3-NPA-indued alterations in vitamin C and iron levels in brain regions were quantitated in cytosol in different brain regions.

Cholinergic activity

Analysis was made to determine the effect of BM extract prophylaxis on 3-NPA induced alterations on the activities of cholinergic enzymes *viz.,* AChE and BChE in various brain regions.

Effects on mitochondrial dysfunctions

Activities of Enzymes: Thioredoxin reductase and LDH activity was measured to determine the protective efficacy of BM prophylaxis on membrane damage induced by 3-NPA. Activities of Citrate synthase, malate dehydrogenase, isocitrate dehydrogenase and fumarase were monitored to determine the effects of BM on 3-NPA-induced alterations in TCA cycle enzymes.

MTTreduction and Succinate dehydrogenase: The prophylactic effect of BM extract in modulating the inhibition in the activity of SDH was monitored since 3-NPA is a known Complex II inhibitor.

Membrane damage: To determine the ameliorative effect of BM extract prophylaxis on toxic effects of 3-NPA on membrane bound enzymes, Na^+ K^+ ATP-ase activity was measured.

Membrane potential: In order to determine the effect of 3-NPA on brain mitochondrial voltage gaited channels, mitochondrial swelling was measured in terms of mitochondrial swelling.

Electron transport chain enzymes: In order to ascertain the prophylactic efficacy of CA prophylaxis on 3-NPA induced alterations in ETC enzymes, the activity levels of complexes *viz.,* Complex II (succinate ubiquinone oxidoreductase), Complex II-III (succinate-Cytochrome C reductase), Complex I-III (NADH-cytochrome C reductase), Complex-IV (Cytochrome C oxidase) in various brain regions were determined.

(iii) Neuroprotective efficacy of BM extract against Rotenone - induced oxidative stress

Experimental protocol

Prepubertal mice were randomly assigned to control and treatment groups (n=6). There were four groups: Group I- DMSO alone; group II- BM extract (5mg/kg bw) alone; group III- rotenone (i.p. 0.5 and 1.0 mg/kg bw) and group IV-mice given BM three hours before rotenone administration. Body weight gain and food intake was monitored during the experimental period

of 7 days. 24hrs after the last dose of toxin, animals were sacrificed by cervical dislocation and brain, liver and kidney were excised, blotted and weighed. Brain regions were dissected out and various biochemical investigations were carried out in both mitochondria and cytosol. The protective effect of BM prophylaxis on rotenone induced mortality was also determined.

Effect of BM extract on Rotenone-induced oxidative damage

To determine the protective efficacy of BM extract on Rotenone-induced oxidative damage various biochemical parameters *viz.,* generation of ROS, lipid peroxidation and hydroperoxides levels were determined. Protein oxidation was quantified as protein carbonyls in freshly prepared mitochondria and cytosol of all brain regions.

Protective effect of BM extract against perturbations in antioxidant status

Activity of antioxidant enzymes *viz..,* catalase, GSH-Px, GST and SOD were measured in cytosol and mitochondria of different brain regions. Further GSH levels, total thiols and non–protein thiols were also quantified to assess the prophylactic effects of BM on rotenone induced alterations on major thiol antioxidant molecules.

Lactate dehydrogenase

LDH activity was measured to determine the extent of cytoplasmic membrane damage induced by Rot and its amelioration by BM pretreatment

Mitochondrial dysfunctions

Malate dehydrogenase: MDH activity was monitored to determine the prophylactic effects of BM on Rot-induced alterations in TCA cycle enzymes

Thioredoxin reductase: In order to ascertain the prophylactic efficacy of BM extract in ameliorating the Rot-induced alterations in redox sensitive enzymes, Thioredoxin reductase activity was measured in mitochondria.

Mitochondrial swelling: In order to determine the effect of 3-NPA on prepubertal brain mitochondrial voltage gaited channels mitochondrial swelling was measured.

MTT reduction: MTT assay was conducting in mitochondria to determine the viability of mitochondrial.

Electron transport chain enzymes

To assess the prophylactic affects of BM the activities of various ETC complexes, *viz.,* Complex I (NADH-ubiquinone oxidoreductase) and Complex I-II *(*NADH-cytochrome C reductase) were determined.

Cholinergic activity

Studies were carried out to determine the effect of BM extract prophylaxis on Rot-induced inhibition of the cholinergic enzyme *viz.,* AChE and BChE in cytosol of brain regions

Dopamine levels

The dopamine levels in striatal tissue were quantified by HPLC-UVD in striatal tissue to determine the ameliorative effect of BM extract on Rot- induced perturbations in dopamine levels.

Histopathology of brain samples

In order to determine the pathology associated with rotenone intoxication, histopathological examinations were carried out in the whole brain tissue following standardized histopathological techniques (as described in materials and methods).

Effects on behavioral dysfunctions

To determine the protective effect of BM prophylaxis on rotenone –induced the motor behavior alterations, selected motor behavior tests were conducted. In this context, pole test (to ascertain the grip strength), rotarod (to determine the motor coordination) and stride length (which gives a direct correlation with gait abnormalities and dopaminergic neurodegeneration) were conducted. Mice of control and treatment were subjected to these studies as described in materials and methods section.

4.0 RESULTS
SECTION A

Propensity of *Bacopa monnieri (BM)* leaf powder to modulate endogenous oxidative markers in brain of prepubertal mice

Body weight, organ weights and food intake

No significant alterations in body weights were noticeable among mice fed BM for 30 days. At the highest concentration (2%) marginal decrease in liver weight (7%). No significant alterations were observed in daily food intake among BM fed mice (Data not shown).

Effect of BM on oxidative markers in brain

Interim sampling data: Analysis of brain of prepubertal mice fed BM (1%) showed significant decrease in various oxidative markers during interim sampling (15 days). Among BM fed mice significant decrease in MDA levels (15-21%), protein carbonyls (17-24%) was accompanied by elevated GSH levels (14-27%) and levels of antioxidant enzymes (Catalase,26%; GSH-Px, 22% and SOD, 24%) in all brain regions (Data not shown).

Terminal sampling data: Biochemical data on only two dietary doses of BM (0.5 and 1.0%) have been presented. There was a decrease in basal levels of oxidative markers both in cytosol and mitochondria of all brain regions of mice fed BM (Fig.3.1). Significant decrease in MDA levels in both cytosol (27-30%) and mitochondria (22-25%) was accompanied with varying degree of decrease in ROS formation in cytosol (14-29%) and mitochondria (10-29%). However, the decrease in hydroperoxide levels was more robust in both cytosol (10-44%) and mitochondria (14-40%).

Effect on Protein carbonyls formation

Significant decrease in protein carbonyls levels was evident in both cytosol (14-34%) and mitochondria (18-44%) of brain regions of mice fed BM (Fig.3.2).

Status of glutathione (GSH) and thiol levels

The levels of reduced glutathione and thiols were significantly enhanced in both cytosol and mitochondria of various brain regions of mice fed BM (Fig.3.3). In cytosol, the elevations in

GSH levels were: 10-23% and increase in total thiols were: 11-29%, while the increase in non-protein thiols was less robust. Likewise, significant increases were also observed in reduced glutathione (10-32%), total thiols (16-35%) and non-protein thiols (10-31%) in mitochondria of brain regions among mice fed BM.

Effect on antioxidant enzymes

In general, the activities of antioxidant enzymes in brain regions of mice fed BM were enhanced (Table 3.1). A dose dependent increase in activity of Catalase (17-47%), Glutathione peroxidase (12-37%) and super oxide dismutase (10-38%) were observed in cytosol. A similar trend of increase was also observed in mitochondria. While the SOD activity was significantly increased (10-23%), the increase was less robust in case of GPx activity. However, no significant alterations were observed in GST activity (Data not shown).

Effect on mitochondrial enzymes

There was a general increase in the activities of mitochondrial enzymes such as malate dehydrogenase (MDH) (maximum 20% increase in striatum) and Thioredoxin reductase (maximum increase of 36% in striatum) in brain regions of mice fed BM (Data not shown).

Effect on Cholinergic enzymes

In general, a significant dose dependent decrease in AChE activity was evident in all brain regions of mice fed BM (Fig.3.4).

Ex vivo response of brain regions to neurotoxicant exposure in vitro

Induction response in cortex and whole brain homogenates

Among untreated mice, 3-NPA exposure induced a concentration dependent increase in ROS levels in both cortex (16-27%) and whole brain homogenates (16-84%). The basal ROS levels among BM fed mice were diminished significantly (24%) in both cortex and whole brain. On exposure to 3-NPA, no induction of ROS at the lower concentrations while only marginal induction was evident at the highest concentration (1.5mM). Interestingly, the induction levels among BM fed mice were relatively lower (cortex: 35%; whole brain: 70%) (Fig:3.5).

Like wise among untreated mice, 3-NPA exposure induced a concentration dependent increase in LPO levels in both cortex (10-36%) and whole brain homogenates (10-27%). The

basal LPO levels among BM fed mice were diminished (14%). On exposure to 3-NPA, no induction of LPO at the two lower concentrations while only a marginal (11%) induction was evident even at the highest concentration. Interestingly the induction levels among these mice were significantly lower (cortex: 64%; whole brain: 20% lower compared to untreated control) (Fig. 3.5)

Induction response of cortex and cerebellar synaptosomes

Among untreated mice, NPA exposure induced a concentration dependent increase in ROS levels in both cortex (12-54%) and cerebellar (11-36%) synaptosomes. On exposure to NPA the synaptosomes of BM fed mice showed no induction of ROS at the two lower concentrations while only a marginal induction was observed even at the highest concentration (1.5mM). Interestingly, the induction response among these mice were significantly lower (cortex: 60%; cerebellum: 41% lower compared to control) (Fig.3.5, lower panel).

Like wise among untreated mice, NPA exposure induced a concentration dependent increase in MDA levels in both cortex (29-95%) and cerebellar (27-79%) synaptosomes. On exposure to NPA the BM fed mice showed no induction of MDA at the two lower concentrations, while only a marginal induction was evident at the highest concentration (1.5mM). Interestingly the induction levels were among these mice was less robust compared to the control mice (Fig.3.5, lower panel).

SECTION B

(i) Antioxidant activity of BM ethanolic extract: *in vitro* evidences in chemical and biological systems

Radical scavenging property and reducing power

In general, BM extract exhibited a concentration dependent DPPH radical scavenging activity. The IC_{50} concentration of BME was 9.41µg/ml and that of the standard BHT (a synthetic antioxidant) was 4.16µg/ml (Fig.3.6A, A_1). There was a significant concentration dependent increase in reduction of ferrous ion to ferric by BM extract compared to standard (BHT) (Fig.3.6B, B_1). Further a concentration dependent scavenging of nitric oxide radical was also observed with BM extract and the IC_{50} concentration was 9.21µg/ml (Fig.3.6C).

Superoxide and hydroxyl radical scavenging property

The two most potent radicals in living systems are superoxide and hydroxyl radicals. A concentration dependent increase in hydroxyl radical scavenging activity was observed with BM extract. The IC_{50} concentration was 436.77µg/ml and synthetic antioxidant BHT was used as a standard (IC_{50} concentration: 6.85µg/ml) (Fig.3.7A, A_1). Further, there was a concentration dependent increase in Superoxide radical scavenging activity by BM extract and the IC_{50} concentration was 10µg/ml and that of the standard Caffeic acid was 4.15µg/ml (Fig.3.7B, B_1).

Iron chelation, Deoxyribose oxidation and Total polyphenol content

A concentration dependent increase in iron chelation effect was observed with BM extract. The IC_{50} concentration was 160µg/ml and that of the standard EDTA was 50µg/ml, a known commercial iron chelator was 50µg/ml (Fig.3.8A, A_1). There was a concentration dependent increase in inhibition against Deoxyribose oxidation and LC_{50} concentration was 217.34µg/ml (Fig.3.8 B). The total polyphenolic content of BM extract was expressed was quercetin equivalents (11.58µg quercetin equivalents) (Standard quercetin graph: Fig.3.8C)

Modulation of 3-NPA-induced oxidative response in brain *in vitro*

Abrogation of oxidative stress in striatal mitochondria in vitro

Exposure of striatal mitochondria to BM extract alone diminished the basal levels to a significant degree (39-49%) at both concentrations while, 3-NPA (2mM) exposure markedly elevated the MDA levels (113%). BM pretreatment completely inhibited the induction of lipid peroxidation at both concentrations. Like wise, 3-NPA also caused marked enhancement in the levels of ROS (120%) and HP (126%) in mitochondria of striatum. Interestingly, BM extract pretreatment completely prevented oxidative stress induction of 3-NPA (Fig.3.9).

Abrogation of oxidative stress in brain region mitochondria in vitro

BM extract alone significantly diminished the basal levels of MDA (38-67%), ROS (15-47%) and HP levels (10-37%) in mitochondria of different brain regions. While, 3-NPA exposure markedly elevated the MDA levels. BM pretreatment completely inhibited the induction of lipid peroxidation at both concentrations. Like wise 3-NPA also caused marked enhancement in the levels of ROS and HP in mitochondria of other brain regions. Interestingly BM extract pretreatment completely prevented the induction of oxidative response by 3-NPA (Fig.3.10).

Modulation of oxidative stress in brain regions slices in vitro

On exposure to BM extract, brain region slices showed diminished levels of MDA (10-37%), ROS (15-70%) and HP (10-52%) (Data not shown). 3-NPA exposure resulted in dramatic enhancement in MDA (262-562%), ROS (90-260%) and HP (Ct- 220-390%) levels. BM extract pretreatment afforded marginal protection (20%) at lower concentration (0.5µg), while complete protection (100%) was observed at higher concentration of BME (1µg) (Data not shown).

(ii) Prophylactic efficacy of BM extract against 3-NPA-induced oxidative perturbations

Body weights, organ weights and food intake

3-NPA administration caused a marginal decrease (15%) in body weights, while significant decrease was observed in the weights of liver (21%), heart (20%), spleen (24%) and thymus (48%). Marginal decrease in food intake was also observed in 3-NPA group (Data not shown).

Effect of BM extract alone on the status of oxidative markers in brain

In the preliminary study, oral administration of BM extract resulted in significant dose related diminution of basal oxidative markers in all brain regions. While the effect was marginal (10%) at the lowest dose, the intermediate and higher dose showed significant decrease (25-35%) in MDA levels. Like wise, the ROS and levels in hydroperoxides all brain regions were also diminished (25-38%) among mice given BM extract (Data not shown).

Attenuation of NPA-induced oxidative implications by BM prophylaxis

Effects on striatum: BM treatment alone resulted in a significant decrease in the basal levels of oxidative markers in cytosol (MDA-23%; ROS-24%; HP-38%) and mitochondria (MDA-28%; ROS-21%; HP-30%). Among mice administered 3-NPA, the striatal tissue exhibited marked increase in MDA (cytosol-70%; mitochondria-92%), ROS (cytosol-65%; mitochondria-131%) and HP (cytosol-57%; mitochondria-77%) levels suggesting induction of oxidative stress. Interestingly, BM prophylaxis was observed to be neuroprotective in striatum, since 3-NPA caused no induction of oxidative impairments (Fig.3.11).

Effects on other brain regions: BM extract treatment alone caused a significant decrease in the basal levels of oxidative markers in both cytosol (25%) and mitochondria (18-34%) in other

brain regions. However, 3-NPA caused a marked increase in MDA levels in both cytosol (56-71%) and mitochondria (50%). Further, BM extract treatment alone also resulted in significant decrease in the basal ROS levels in cytosol (25%) and mitochondria (22%). 3-NPA treatment caused marked enhancement of ROS formation in cytosol (44-58%) and mitochondria (64-82%). BME treatment also caused a significant reduction in HP levels in cytosol (35%) and mitochondria (26-39%) of other brain regions. 3-NPA administration resulted in elevated HP levels in both cytosol (34-45%) and mitochondria (46-60%) in all brain regions. Interestingly, BM prophylaxis was observed to be neuroprotective in other brain regions, since 3-NPA caused no significant oxidative damage (Fig.3.12).

Effect on Protein carbonyls formation

BM treatment alone caused significant decrease in protein carbonyls content in striatum (cytosol: 26%; Mitochondria: 30%) and other brain regions (cytosol: 22-34%; mitochondria 26-39%). On the other hand, 3-NPA administration significantly enhanced the protein carbonyls content in striatum (cytosol: 38%; mitochondria: 77%) and other brain regions (cytosol: 23-48%; mitochondria 46-60%). However, BM prophylaxis mice were completely resistant to protein oxidative damage, since 3-NPA did not induce any appreciable increase in protein carbonyl content in various brain regions (Fig.3.13).

Effect of 3- NPA administration on reduced glutathione and thiols status

Effects on striatum: BM treatment alone resulted in a significant increase in the basal levels of GSH (cytosol-25%; mitochondria-34%), total thiols (cytosol-21%; mitochondria-28%), and non protein thiols (cytosol-20%; mitochondria-27%). Among mice administered 3-NPA, the striatal tissue exhibited decrease in GSH (cytosol-23%; mitochondria-31%), total thiols (cytosol-21%; mitochondria-37%) and non protein thiols (cytosol-19%; mitochondria-48%). Interestingly, among mice given BM prophylaxis, 3-NPA did not induce any noticeable alterations in the status of GSH and thiols suggesting complete protection. Further, marginal decrease was observed in GSSG levels in BM alone mice, while there was significant increase (cytosol-45%; mitochondria-14%) on NPA administration which was normalized on BM prophylaxis (Table 3.2).

Effects on other brain regions: BM treatment alone also resulted in significant increase in GSH levels (cytosol: 19-30%; mitochondria: 30-34%), TSH (cytosol: 19-25%; mitochondria:23-25%), and npSH (cytosol:10-23%; mitochondria:20-24%). On the other hand, 3-NPA treatment

resulted in varying levels of depletion in GSH, total thiols and non-protein thiols. A general increase (cytosol: Ct-50%; Cb-33%; Hc-20%; mitochondria: 12%) was observed in GSSG levels on exposure to 3-NPA, where as marginal decrease (10-15%) was observed in BM extract fed group. Mice given BM extract prophylaxis were not susceptible to 3-NPA induced depletion in antioxidant molecules suggesting total protection (Table 3.3, 3.4).

Effect on antioxidant enzymes

Effect on striatum: BM treatment alone significantly enhanced the activities of antioxidant enzymes in striatal tissue (cytosol-CAT: 34%; GPx: 17%; SOD: 35% and mitochondria-GPx: 25%; GR: 19%; SOD; 39%). 3-NPA administration resulted in significant decrease in cytosolic catalase (37%), GPx (25%) and SOD (19%), while GST was increased (15-27%) and marginal to moderate decrease in the activities of mitochondrial antioxidant enzymes (GPx: 14%; SOD; 32%; GR 17%), while an increase was observed with GST (40%). In contrast mice given BM prophylaxis exhibited normal levels of enzyme activities indicating the neuroprotective effect of BM (Fig.3.14).

Effect on other brain regions: In general, BM extract treatment enhanced the activities of antioxidant enzymes in all other brain regions (Table.3.5; Fig.3.15). The extent of increase of enzyme activities in cytosol were: (CAT: 13-35%, GSH-Px: Ct-10-30%; SOD: Ct-23-38%) while the elevations in mitochondria were: GPx (~25%), GR (21-32%) and SOD (33-44%). Interestingly NPA administration caused significant decrease in both cytosol (GSH-Px: 25%; SOD: 21-27%) and mitochondria (GSH-Px: 22-24%; GR: 13-21%; SOD: 25-31%). However, the activity of GST was increased in cytosol (14-27%) and mitochondria (29-35%) of 3-NPA administered mice brain regions. Interestingly, varying degree of normalization in enzyme activities occurred among mice given BM prophylaxis suggesting the neuroprotective effect of BM.

Status of Iron and Vitamin C levels

BM extract alone caused a general decease in free iron levels in striatum (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 striatum (40%) and other brain regions (Ct-21%; Cb-47%; Hc-33%). Interestingly, BM prophylaxis resulted in marked reduction in the free iron levels in striatum and other regions. The free iron levels were infact significantly lower than the endogenous levels in all brain regions except for hippocampus which showed normal levels. BM

extract alone did not have any effect on the vitamin C levels in any of the brain regions. However, 3-NPA treatment caused a marginal diminution in the vitamin C levels in striatum and cortex (St-10%; Ct-26%), while the levels were higher in cerebellum (36%) and hippocampus (21%). BM prophylaxis had no modulatory effect on 3-NPA induced alterations (Table 3.6).

Activity of thioredoxin reductase (TRR) and lactate dehydrogenase (LDH)

BM extract treatment alone marginally elevated the activity of TRR in striatum and other brain regions. However, 3-NPA treatment reduced the activity of TRR in striatum (33%) and other brain regions (10-21%). Mice given BM prophylaxis showed normal TRR activity in cortex and hippocampus while, the activities in striatum and cerebellum were partially restored. (Fig.3.17). 3-NPA administration resulted in significant elevation in LDH activity in striatum (27%) and other brain regions (14-30%). Mice received BM prophylaxis normalized the 3-NPA induced elevations in LDH activity, although BM alone had no significant effect (Fig.3.16).

Effect on Citric acid cycle enzymes

BM treatment alone had no appreciable effect on the activities of mitochondrial TCA cycle enzymes (Table 3.7). 3-NPA caused a significant reduction in the activity of various enzymes in striatum and other brain regions suggesting an increased susceptibility of striatum. Mice given BM prophylaxis showed varying degree of restoration of the enzyme activities excepting for SDH which showed a partial restoration in striatum (25%).

Interestingly, differential reduction in the activity of TCA enzymes was noticeable in other brain regions of 3-NPA administered mice (MDH: 18-25%; SDH: 59-84%); ICDH: 17-27%; Fumarase: 17-22% and Citrate synthase: 29-33%), while maximum decrease was observed in striatum. BM extract prophylaxis resulted in 100% protection in 3-NPA-induced alterations in TCA cycle enzymes in other brain regions (19-27%) of mice except for SDH (Table 3.7).

Effect on electron transport chain (ETC) enzymes

BM extract alone caused no appreciable alterations in the activities of mitochondrial ETC enzymes in striatum (Fig.3.17) and other brain regions (Table 3.8). In contrast 3-NPA administration caused a decrease in the activity of ETC enzymes in striatum (Complex I-III: 17%, Complex II-III: 81%, Complex I: 13%, Complex II: 83%, Complex IV: 10%) and other brain regions (Complex I-II: 12-21%, Complex II-III: 75-78%, Complex I: 10-15%, Complex II: 80%,

Complex IV: 10%). BM prophylaxis resulted in varying degree of protection against 3-NPA-induced perturbations in activity in striatum and other brain regions.

Effect on mitochondrial swelling, MTT reduction and Na^+ K^+ ATPase activity

BM extract alone caused only a marginal increase in mitochondrial swelling and Na^+ K^+ ATPase activity in all brain regions, while no significant change was observed for MTT. Differential reduction of MTT was evident in striatum (60%) and other brain regions (29-60%) of mice administered 3-NPA. Mice given BM prophylaxis showed varying degree of protection in striatum (88%) and other brain regions (67-77%) (Fig.3.18, 3.19).

A significant reduction (Ct-26%; Cb-13%; Hc-14%; St-27%) in swelling (measured as the time at which decrease in absorbance starts) (Fig.3.18, 3.19) was observed among 3-NPA administered mice. With BM prophylaxis, varying degree of protection was observed for swelling in mitochondria of all brain regions. 3-NPA caused marked decrease in the activities of Na^+ K^+ ATPase in striatum (82%) and other brain regions (69-80%), while BM prophylaxis had no modulatory effect on 3-NPA–induced alterations.

Activity of Cholinergic enzymes

BM treatment alone caused significant decrease in the activities of AChE in striatum (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 striatum (20%) and other brain regions (14-32%). Like wise NPA administration also marginally decreased the activities of BChE in all the brain regions (10-45%). While BM prophylaxis had no modulatory effect on AChE activity, it normalized the 3-NPA induced alterations in BChE activity (Data not shown).

(iii) Neuroprotective efficacy of BM extract against rotenone induced neurotoxicity: attenuation of oxidative stress and mitochondrial dysfunctions

Effect of BM extract on general characters

The body weight and general health condition of rotenone treated mice were determined daily. Rotenone administration caused a marginal (15%) decrease in body weights and significant

decrease (15%) in food intake. Further, there was a decrease in final organ weights (liver-11%; spleen- 26%) among rotenone administered mice (Data not shown).

Mortality profile in Rotenone administered mice

Short term administration (7 consecutive days) of rotenone caused no mortality at dosages of 0.5 and 1.0mg/kgbw. However, dosages of 2mg/kg bw resulted in 100% mortality. Interestingly, mice co-administered with BM extract showed a lower (66.6%) incidence of mortality at the highest rotenone dose (2mg/g/bw) (Data not shown).

Amelioration of rotenone- induced oxidative implications by BM treatment

BM extract treatment alone resulted in significant decrease in the basal levels of oxidative markers in cytosol (Fig 3.20) and mitochondria (Fig 3.21) of all brain regions. Among mice administered rotenone, marked increase in MDA levels in both cytosol (St: 47%; other brain regions: 30-43%) and mitochondria (St: 33%; other brain regions: 18-35%) were observed. Further, the cytosolic ROS (St: 20%; other brain region: 75-95%) and HP levels (St: 77%; other brain regions: 54-88%) were markedly elevated all brain regions of rotenone administered mice. Likewise, rotenone also caused marked increase in ROS (St: 87%; other brain regions: 67-80%) and HP (28-32%) levels in mitochondria of all brain regions. Interestingly, BM treatment was observed to be neuroprotective in striatum as well as in other brain regions, since complete protection was evident.

Protein carbonyl formation

BM extract alone resulted in significant decrease in endogenous protein carbonyl levels in both cytosol (St: 21%; other brain regions: 15-28%) and mitochondria (St: 40%; other brain regions:15-41%) of brain regions (Table 3.9). Rotenone exposure resulted in a dramatic increase in PC in cytosol (St: 144%; other brain regions: 60-127%) and mitochondria (St: 119%; other brain regions: 48-83%). Interestingly, there were no prominent alterations in protein carbonyls levels in both cytosol and mitochondria of brain regions of mice treated with BM (Table 3.9) suggesting total protection.

Protective effect on reduced glutathione levels

BM treatment alone resulted in marginal increase in GSH levels in all brain regions (cytosol: 10-20% and mitochondria: 11-20%) (Table 3.10). Significant decrease was observed in

all brain regions both in cytosol (11-15%) and mitochondria (St: 45%; other brain regions: 25-37%) of mice administered rotenone. BM treatment afforded 100% protection against this decrease in GSH induced by rotenone in all brain regions, except in mitochondria of cortex where only 65% protection was observed (Table 3.10).

Modulatory effect of BM extract on antioxidant enzymes

BM treatment resulted in significant increase in the activities of antioxidant enzymes in cytosol of brain region cytosol (Table 3.11) and mitochondria of brain regions (Fig: 3.22). Rotenone administration resulted in significant decrease in antioxidant enzymes in both cytosol and mitochondria. Among rotenone mice, the activity of GST was enhanced markedly in cytosol (St-64%; other brain regions: 41-52% and mitochondria (St-45% other brain regions: 24-39%). Like wise among rotenone mice, the activity of SOD was also elevated markedly in cytosol (St-7%; other brain regions: 55-72%). Interestingly treatment with BM extract resulted in 100% protection against alterations in GSH-Px, GST, GR and catalase in cytosol of all brain regions, while varying degree of protection (75-100%) was observed with SOD activity (Table 3.11). Further, BM extract treatment completely protected all brain regions against alterations in mitochondria (Fig: 3.22).

Modulation against alterations in activities of LDH, MDH, TRR

BM treatment alone had no effect on the activities of LDH and MDH, while marginal increase was observed in the activity of TRR in all brain regions (Table 3.12). Rotenone caused a significant increase (St: 61%; other brain regions: 33-50%), in the activity of LDH in cytosol of brain regions, while significant decrease was observed with MDH activity (St: 25%; other brain regions: 13-35%). BM extract provided 100% protection against LDH increase and MDH decrease in all brain regions of mice (Table 3.12). Further significant decrease (St-50%; other brain regions: 38-51%), in the activity of TRR was observed among rotenone administered mice. Varying degree of protection was afforded by BM extract treatment in rotenone mice (Table 3.12).

Ameliorative effect of BM extract on cholinergic systems

BM treatment alone resulted in a marginal decrease in the activity of AChE (15-23%), and BChE (10-16%) enzymes. Rotenone caused an increase (Ct-34%; Cb-21%; Hc-45%), in AChE activity in all brain regions except striatum where there was 18% decrease over control. BM extract treatment provided 100% protection in all brain regions except in striatum where only 55% protection was observed (Fig.3.23). Significant decrease in BChE activity was observed in

all brain regions of mice administered rotenone and BM extract treatment gave 100% protection (Fig.3.23).

Modulatory effect of BM extract on ETC enzymes

BM treatment alone did not cause any significant alterations in the activity of ETC enzymes. Rotenone treatment resulted in marked decrease in complex I (Ct-72%; Cb-68%; Hc-44%; St-67%) and Complex I-III (Ct-30%; Cb-22%; Hc-27%; St-47%) in all brain regions. Varying degree of protection was offered by BM pretreatment in all brain regions of mice (Table 3.13).

Protective effect on MTT and mitochondrial swelling

No significant alterations in MTT and mitochondrial swelling in brain regions of mice fed BM extract alone. However rotenone caused significant decrease (St: 26%; other brain regions: 10-25%) in MTT reduction in all brain regions of mice (Fig.3.24). Complete protection was afforded by BM extract pretreatment to all brain regions administered rotenone. Marked decrease (St: 100%; other brain regions: 55-97%) in mitochondrial swelling was observed in all brain regions of mice administered rotenone. Differential protective effect was evident (St: 33%; other brain regions: 30-100%) among BM extract treated mice.

Protection against dopamine depletion

There was a marginal (10%) increase in dopamine levels in mice treated with BM extract alone. However rotenone caused significant decrease (33%) in dopamine levels in the striatal tissue, while BM treatment resulted in significant protection (70%) against dopamine depletion (Fig: 3.25).

Histopathological examinations

The histopathological findings in the striatum of prepubertal mice are illustrated in photomicrographs (Plate:1a, b, c, d). Tissue sections from mice treated with BM extract alone showed normal histological structure. Treatment of mice with rotenone for 7 days resulted in striatal focal degeneration and necrosis with marginal loss of cell details and architecture and appearance of basophilic nuclear remnants. In mice treated with BM extract there were only marginal gliosis associated with endothelial lining of blood vessels (Plate:1)

Effect of BM extract on rotenone induced behavioral dysfunctions

Mice of both control and treatment groups were individually tested for neurological reflexes (eye blink, ear twitch and whisker orientation, postural and righting reflex) and examinations of motor functions (muscle strength, catalepsy and gait analysis). The neuronal reflexes except the postural and righting reflex were not affected during the treatment period. Marginal decrease (10-12%) in postural and righting reflexes were observed in rotenone administered animals while in BM extract prophylaxis group exhibited no such significant alterations were observed (Data not shown).

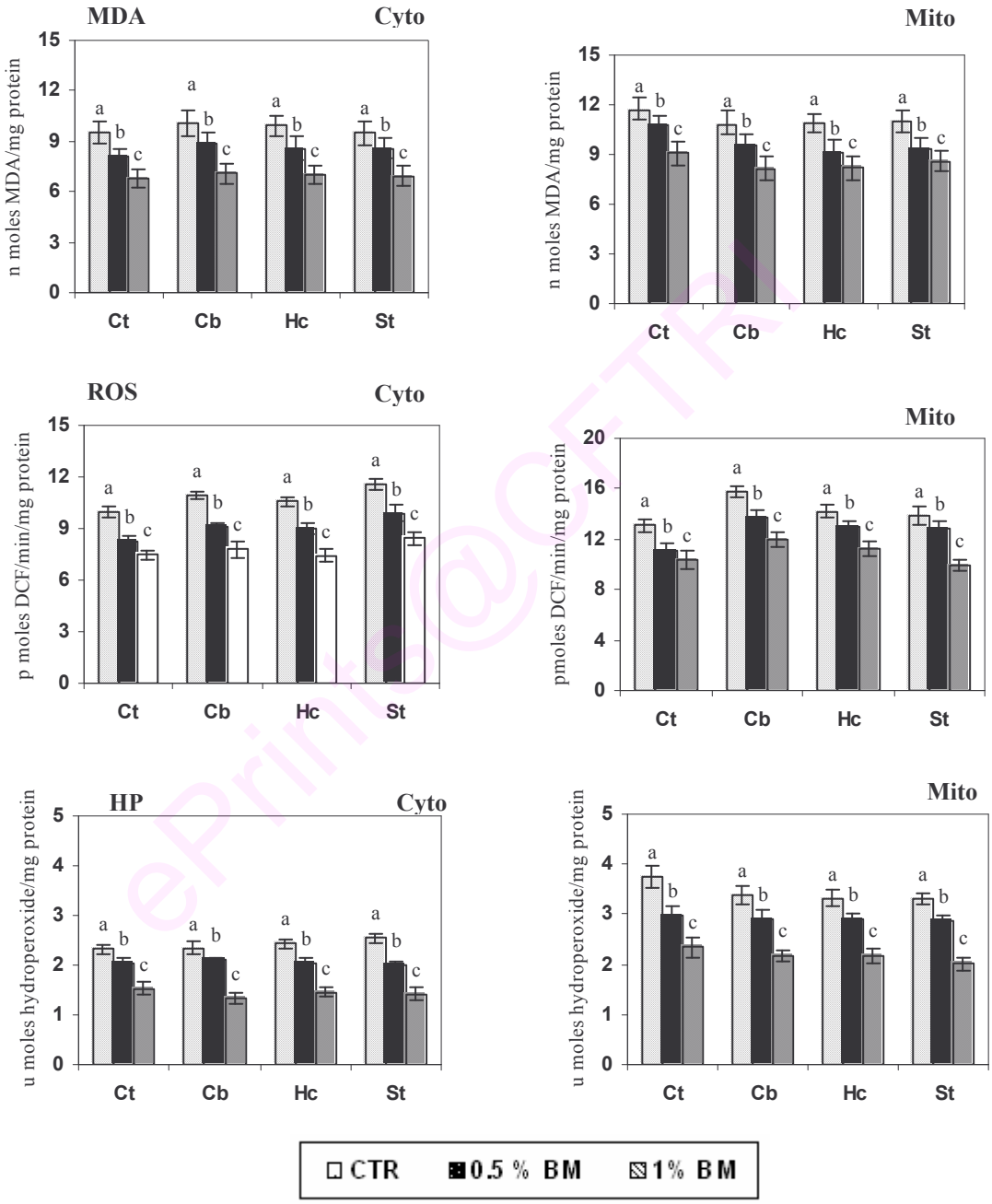
*a) Pole test: A g*eneral decrease in efficiency of in motor behavior was evident with rotenone administration observed. There was a gradual increase (6-143%) in time taken to reach the ground on four paws in rotenone (1mg/kgbw) treated group (Table 14a). A Similar trend was observed for T-turn in the pole test (Table14b). There was an increase in time taken (12-57%) for T-turn in rotenone administered mice after 6th and 7th doses. Varying degree of protection was evident among BM extract treated mice suggesting significant neuroprotective effects in motor dysfunctions.

b) Rota rod: There was a general decrease in the time for which the animals were able to stay in the rotarod among rotenone administered mice (Table 15a). Significant latency to fall was observed in rotenone administered animals. The BM extract treatment resulted in substantial degree of protection against rotenone –induced co-ordination abnormalities

c) Stride length: In the foot print test, rotenone treated mice developed gait abnormalities such as irregular footing of the fore-and hind limbs and shorter distance between stepping of forelimbs. Mean stride length measures during walking/trotting were very stable across individuals of 4week-old mice and were consistent within repeated sessions performed , with no training effect throughout the sessions (*n*=8). Mean forelimb stride length was significantly longer than that of the hind limbs. A mild akinesia was induced by rotenone. Stride lengths could only be measured 3 hrs after the injection, when mice began to ambulate again. There was a significant increase in the mean forelimb and hind limb stride length compared with baseline and these dysfunctions were fully reversed by BM extract treatment (Tables 16 a,b,c).

Fig: 3.1

Status of oxidative markers in cytosol and mitochondria of brain regions of prepubertal male mice fed *Bacopa monnieri* (BM) leaf powder for 30 days.

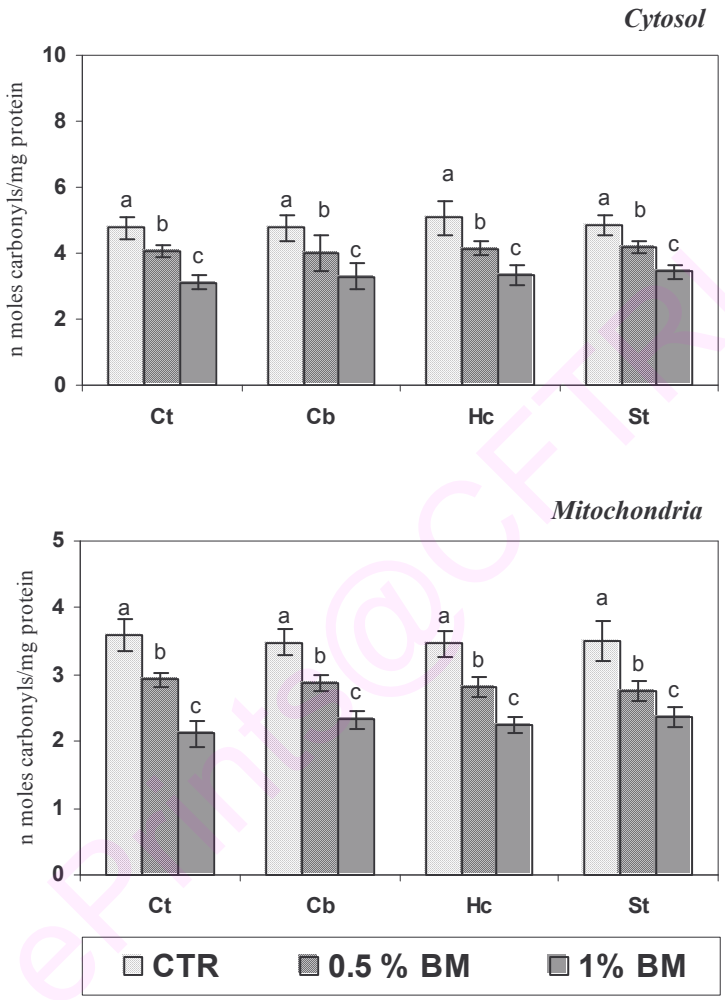


Values are mean ± S.D of six determinations each.
Data analyzed by one way ANOVA (P<0.05); appropriate to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT.

ROS-Reactive oxygen species; MDA-Malondialdehyde; HP- hydroperoxide
Cyto-Cytosol ; Mito-Mitochondria

Fig: 3.2

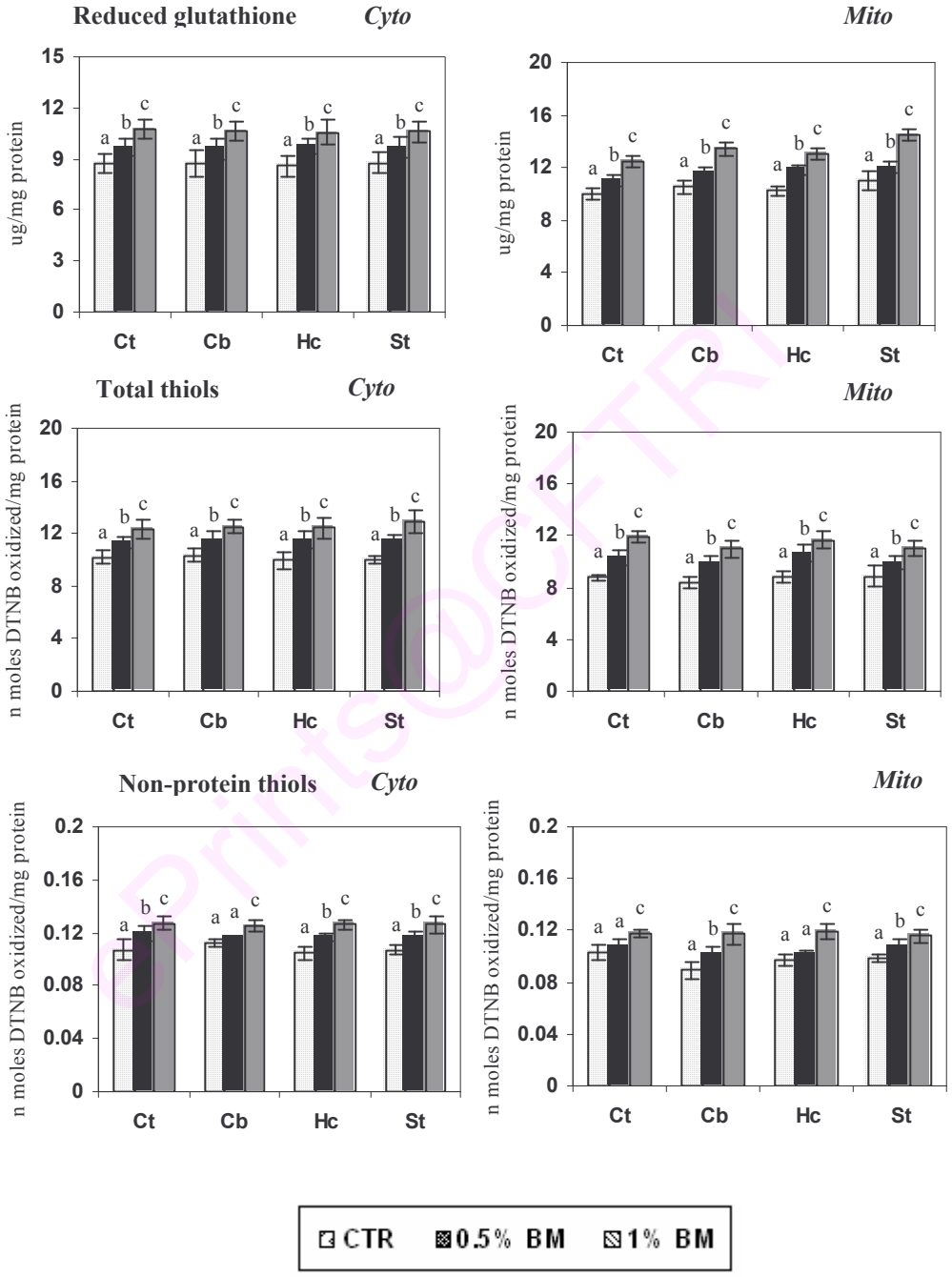
Status of protein carbonyls in cytosol and mitochondria of brain regions of prepubertal male mice fed *Bacopa monnieri* (BM) leaf powder for 30 days.



Values are mean ± S.D of six determinations each.
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT.

Fig: 3.3

Status of antioxidant molecules in cytosol and mitochondria of brain regions of prepubertal male mice fed *Bacopa monnieri* (BM) leaf powder for 30 days.



Values are mean ±SD (n=6). Cyto-Cytosol; Mito-Mitochondria

Data is analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates.

Means followed by different letters differ significantly according to DMRT

Table 3.1

Status of antioxidant enzymes in cytosol and mitochondria of brain regions of prepubertal male mice fed *Bacopa monnieri* (BM) leaf powder for 30 days.

BM (%)	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
Cytosol				
CAT[1]				
0	2.0±0.08[a]	2.0±0.07[a]	2.0±0.07[a]	2.0±0.04[a]
0.5	2.3±0.11[b]	2.5±0.09[b]	2.43±0.08[b]	2.3±0.05[b]
1	2.9±0.10[c]	2.9±0.06[c]	2.95±0.07[c]	2.7±0.07[c]
GPx[2]				
0	20.6±0.89[a]	20.8±0.90[a]	19.11±0.38[a]	20.1±0.60[a]
0.5	23.4±0.78[b]	23.3±0.56[b]	23.25±0.50[b]	23.0±0.45[b]
1	26.2±1.21[c]	26.4±0.24[c]	26.09±0.45[c]	26.8±0.56[c]
SOD[3]				
0	34.9±1.12[a]	36.0±1.50[a]	36.17±1.10[a]	36.0±1.23[a]
0.5	38.8±1.11[b]	38.4±1.66[b]	39.77±1.34[b]	39.1±1.23[b]
1	47.2±2.34[c]	48.4±2.10[c]	49.09±1.01[c]	49.7±1.01[c]
Mitochondria				
GPx[2]				
0	27.0±0.98[a]	29.1±0.65[a]	29.7±0.75[a]	28.3±0.80[a]
0.5	30.5±1.12[b]	31.1±1.21[b]	32.0±1.22[b]	31.7±1.05[b]
1	34.0±1.0[c]	33.0±1.15[c]	32.7±1.21[c]	32.6±1.07[c]
SOD[3]				
0	49.6±1.23[a]	50.5±1.34[a]	50.0±1.67[a]	49.1±1.80[a]
0.5	55.1±2.31[b]	54.8±1.13[b]	54.9±1.23[b]	55.2±1.22[b]
1	58.9±2.14[c]	59.1±2.03[c]	61.4±2.15[c]	60.5±2.09[c]

Values are mean ±SD (n=6).

Data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

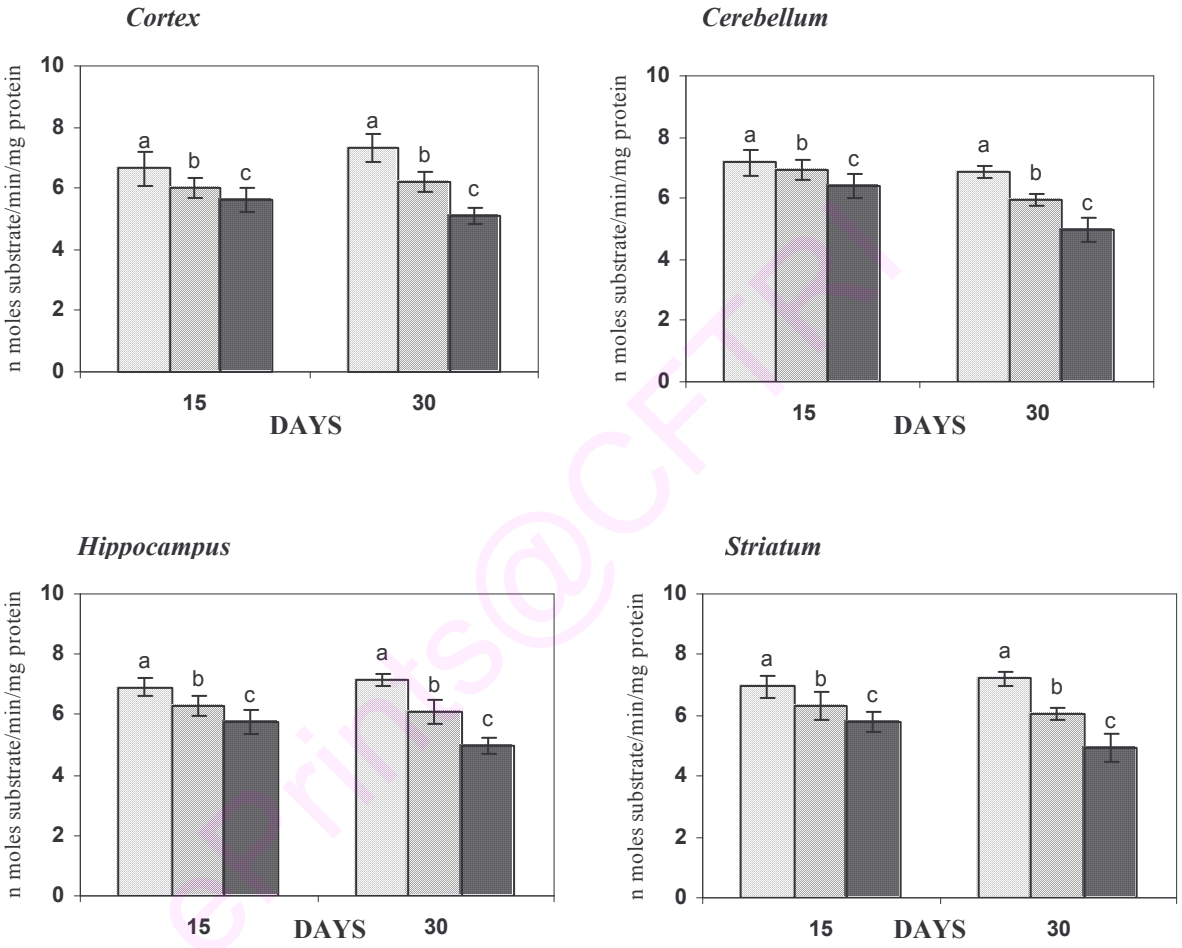
1-μ moles H_2O_2 degraded/min/mg protein

2-η moles NADPH/min/mg protein; 3-Units/mg protein

CAT-Catalase; GPx-Glutathione peroxidase; SOD-Superoxide dismutase;

Fig: 3.4

Comparison of the status of acetylcholinesterase activity in brain regions of prepubertal male mice fed *Bacopa monnieri* (BM) leaf powder for 30 days.



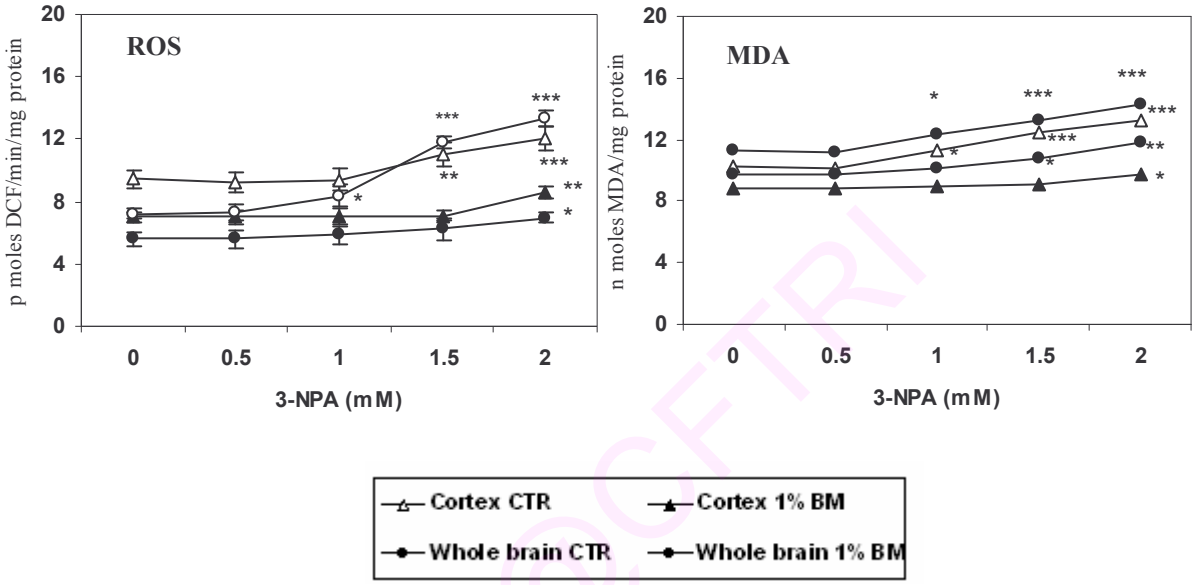
Values are mean ±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

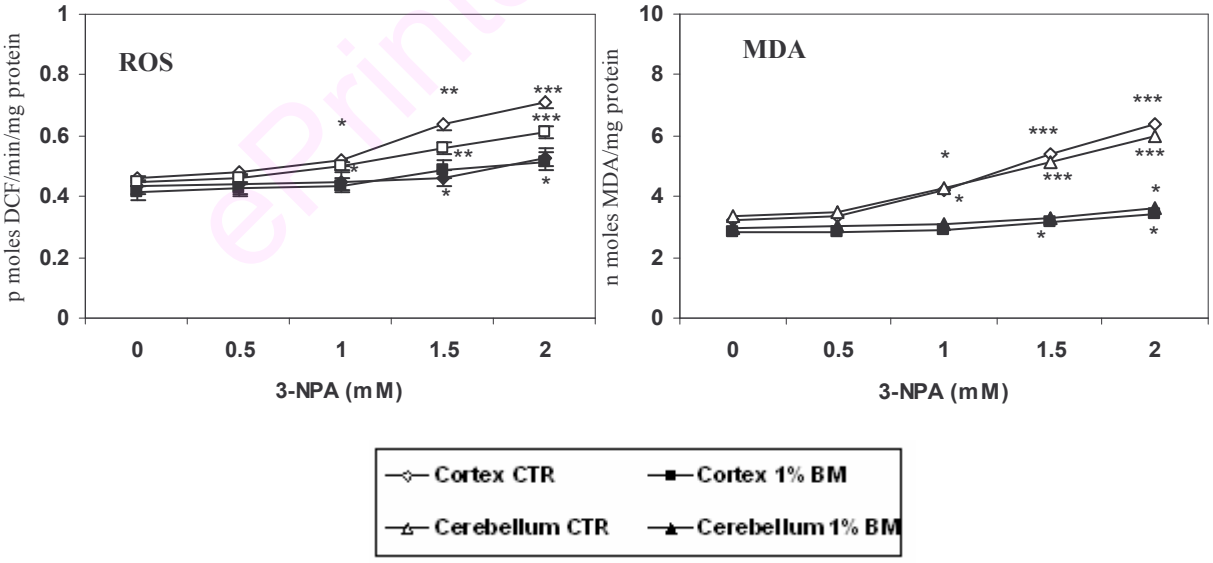
Fig: 3.5

3-NPA–induced induction (*in vitro)* of ROS and LPO in brain cortex and whole brain synaptosomes of prepubertal mice fed *Bacopa monnieri* leaf powder (BM) for 30 days.

Homogenates



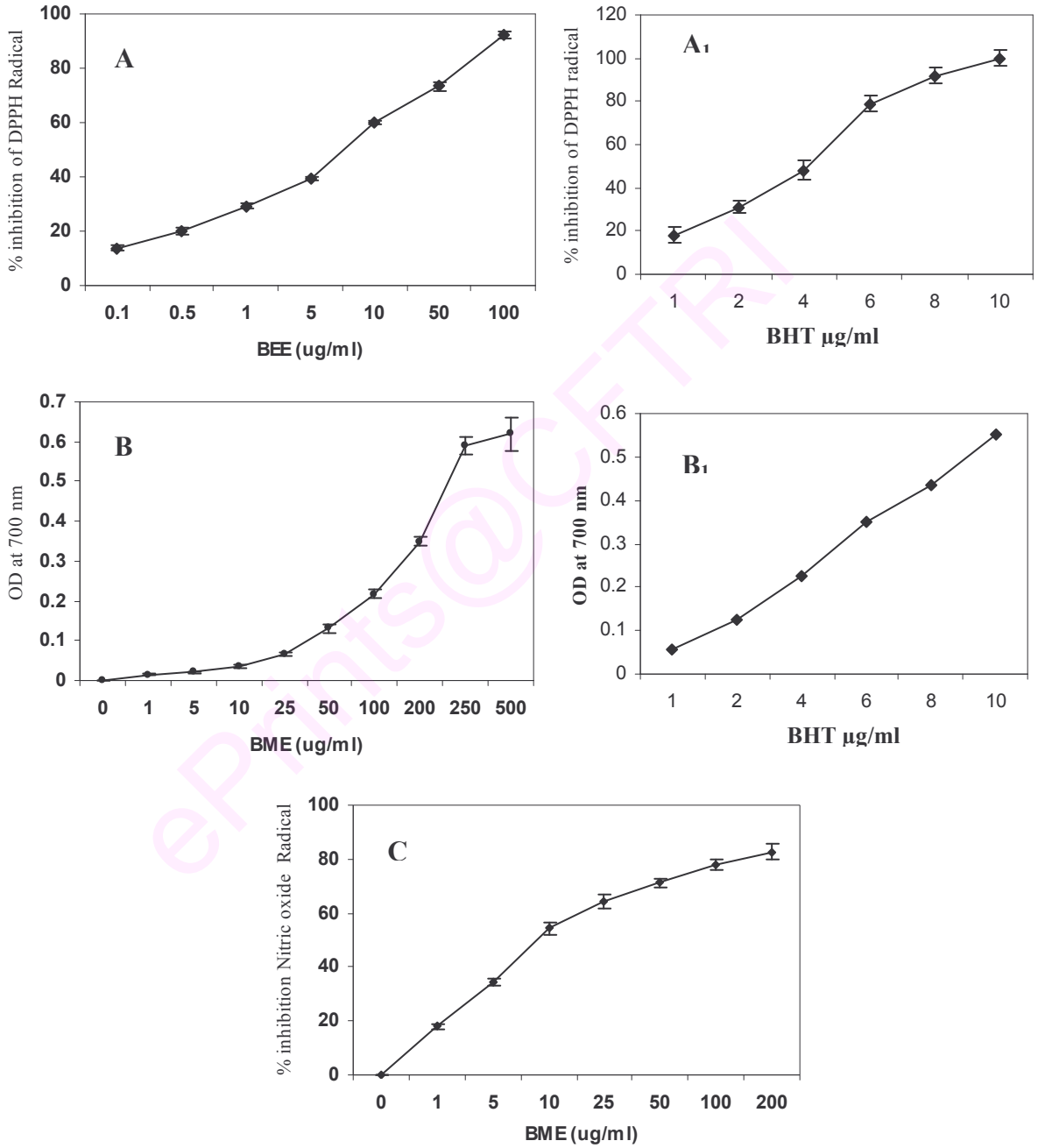
Synaptosomes



Values are mean ± S.D of six determinations each.
Data analyzed by one way ANOVA (* P<0.05; **P<0.01; **P<0.001).
ROS-Reactive oxygen species; MDA-Malondialdehyde; HP- hydroperoxide; NPA-3-nitropropionic acid;

Fig: 3.6

Free radical scavenging efficacy of *Bacopa monnieri* ethanolic extract (BME) as determined by DPPH assay, reducing power and nitric oxide scavenging potency.



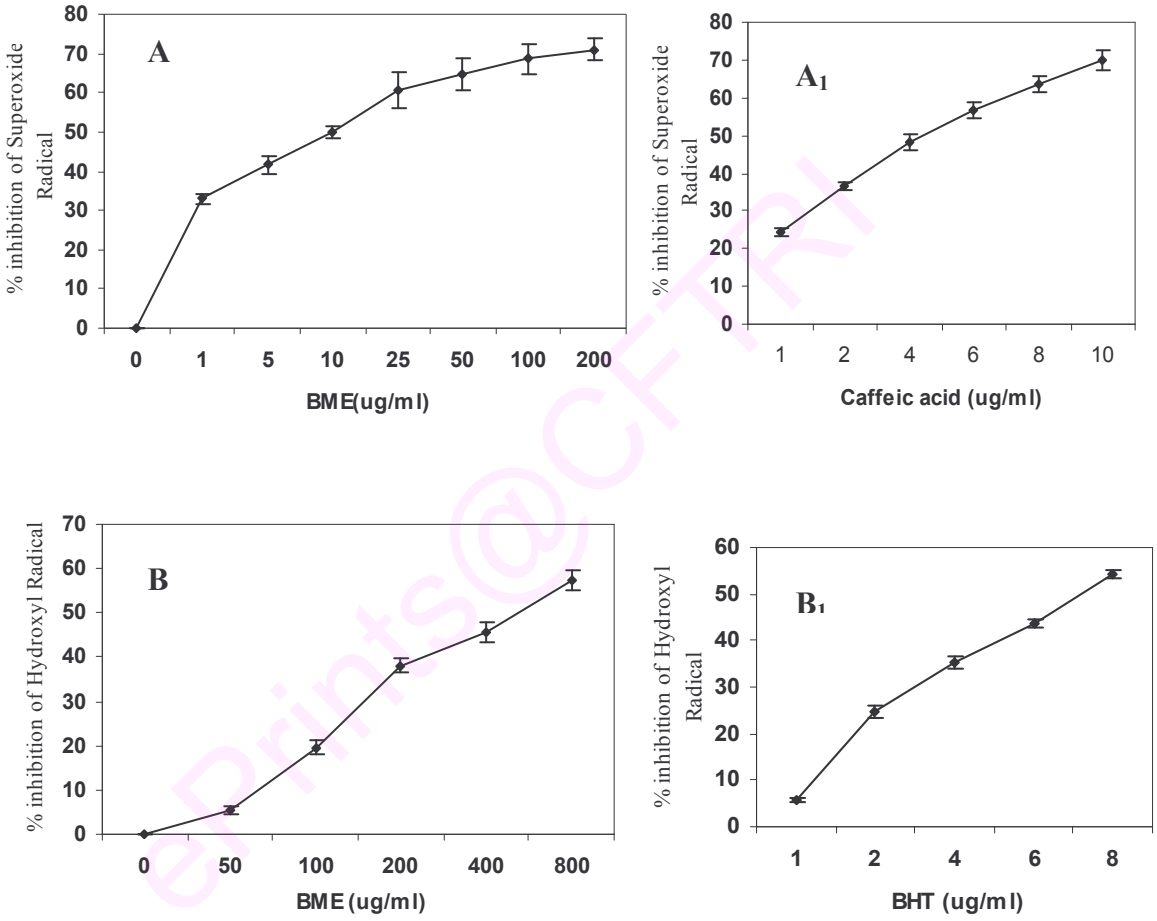
Values are represented as mean ±SD of six replicates.

A-DPPH assay; A_1 –BHT standard for DPPH; B-Reducing power assay;

B_1 –BHT standard for reducing power assay ; C-Nitric oxide scavenging assay

Fig: 3.7

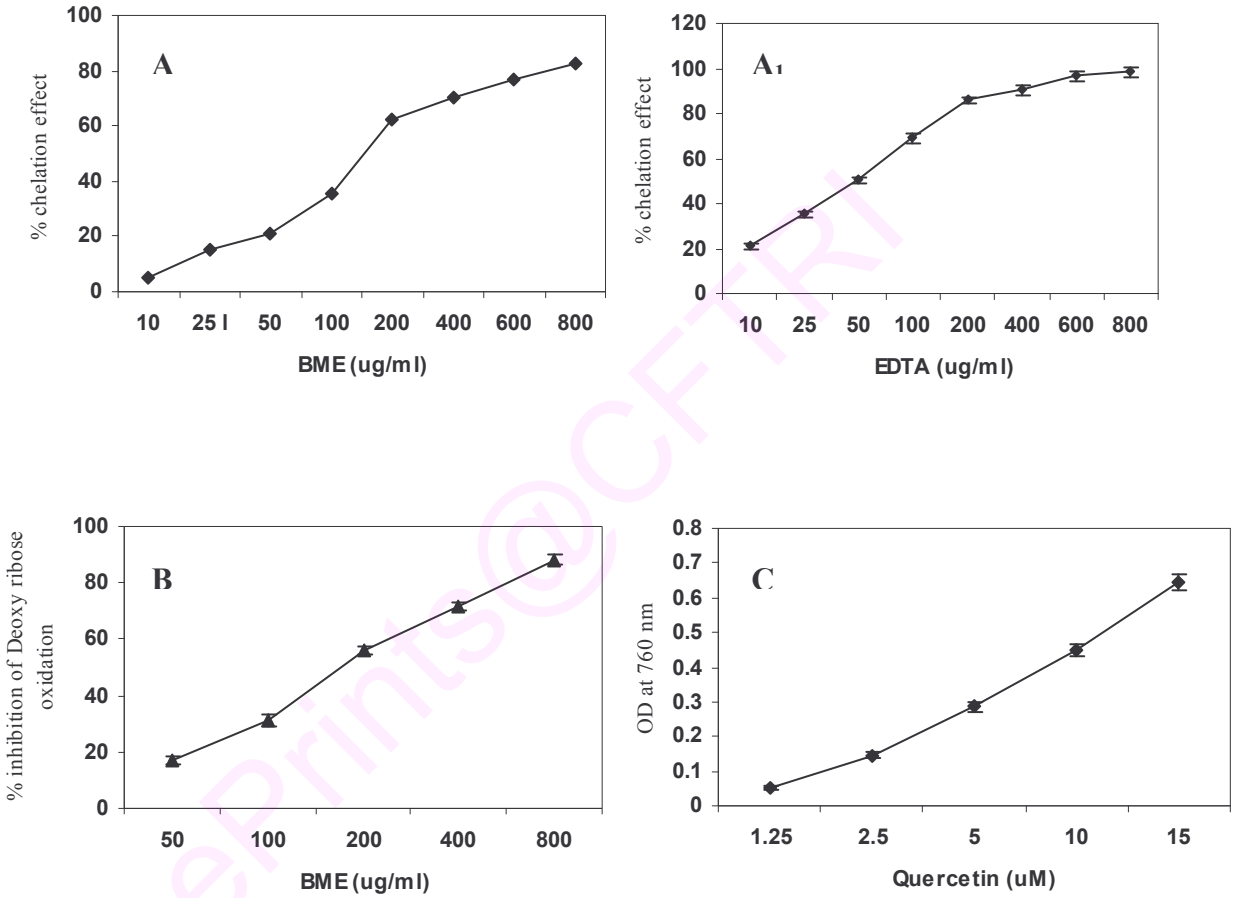
Superoxide and Hydroxyl radical scavenging potency of *Bacopa monnieri* ethanolic extract (BME) *in vitro.*



Values are represented as mean ±SD of six replicates.
A-superoxide scavenging assay; A_1 –Caffeic acid standard
B-Hydroxyl radical scavenging assay; B_1 –BHT standard

Fig: 3.8

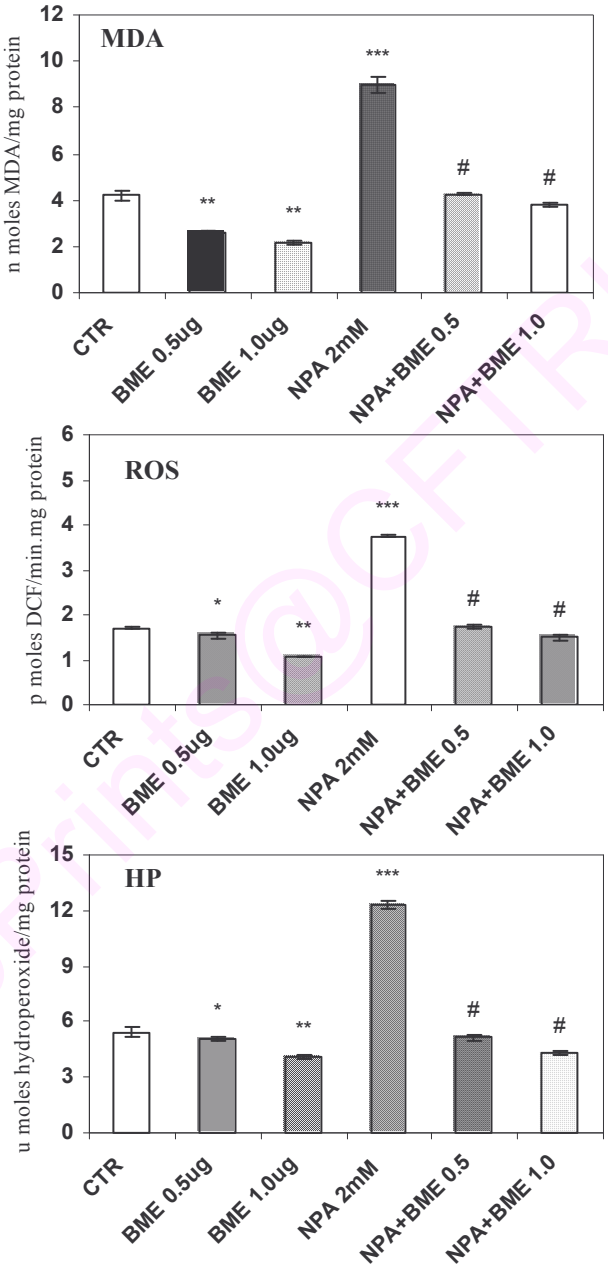
Determination of Iron chelation, Deoxyribose oxidation property and total polyphenol content of *Bacopa monnieri* ethanolic extract (BME).



Values are represented as mean ±SD of six replicates
A-Iron chelation; A_1 –EDTA standard; B-Deoxyribose oxidation
C–Quercetin standard of total polyphenol assay

Fig: 3.9

Attenuation of 3-NPA-induced oxidative stress *in vitro* by *Bacopa monnieri* ethanolic extract (BME) in striatal mitochondria of prepubertal male mice.

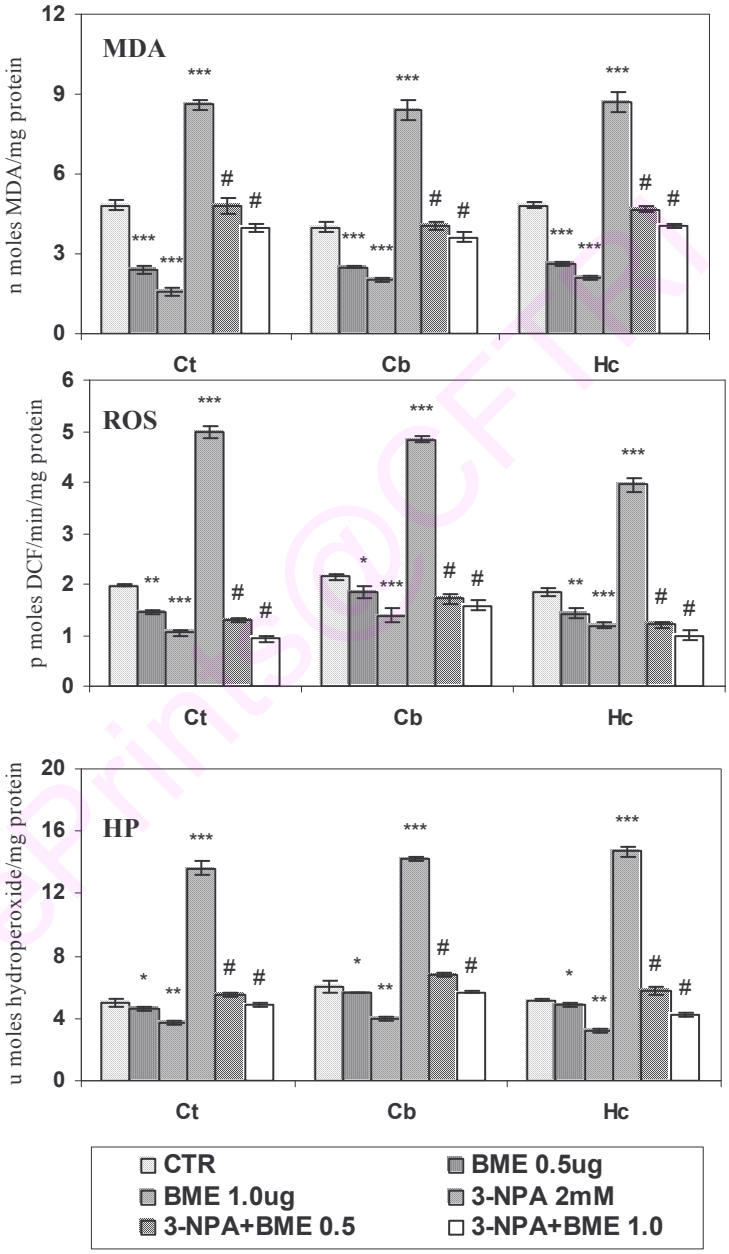


Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05; **P<0.01; ***P<0.001); #- Compared to NPA 2mM, P<0.001)

ROS: Reactive oxygen species; MDA: Malondialdehyde;
HP: hydroperoxide; NPA: 3-nitropropionic acid
(The concentration of BM extract is in µg/ml reaction mixture)

Fig: 3.10

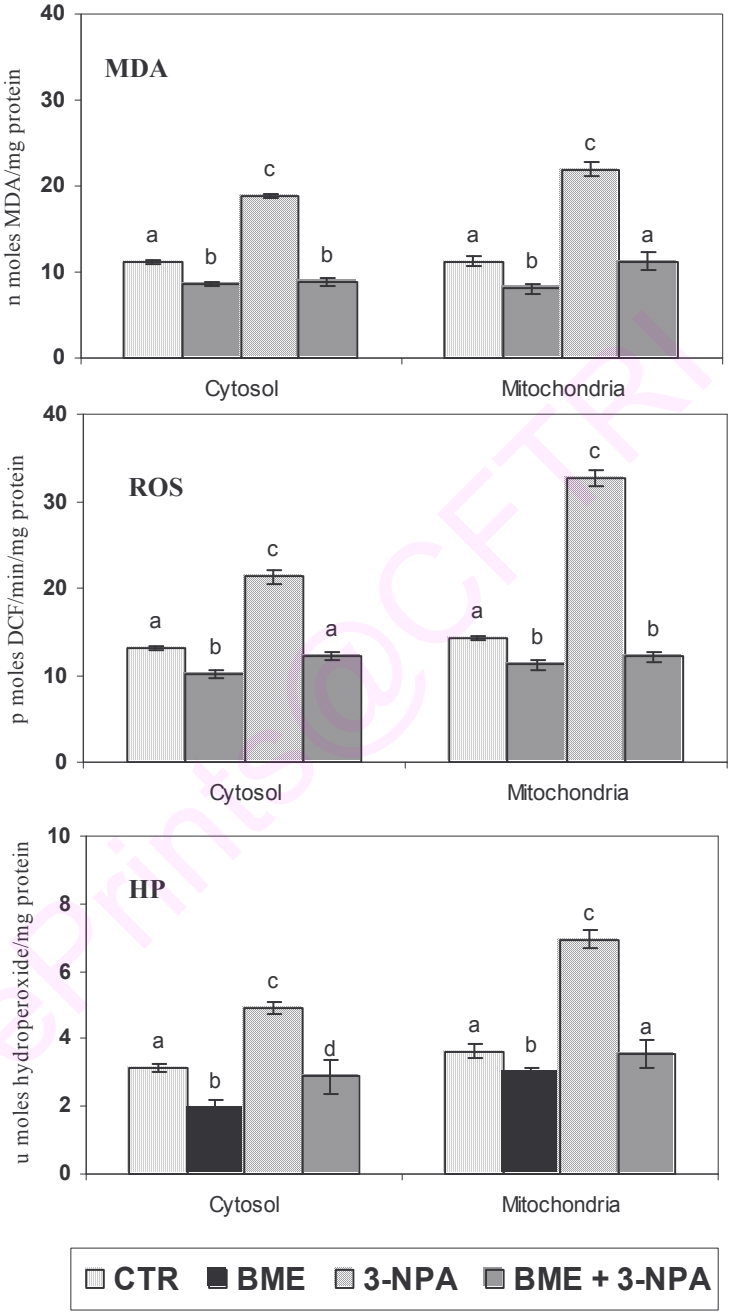
Attenuation of 3-NPA-induced oxidative stress *in vitro* by *Bacopa monnieri* ethanolic extract (BME) in brain region mitochondria of prepubertal male mice.



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (* P<0.05; **P<0.01; ***P<0.001; #- compared with 3-NPA 2mM, P<0.001)
MDA: Malondialdehyde; ROS: Reactive oxygen species; HP: hydroperoxides; 3-NPA: 3-nitropropionic acid
(The concentration of BM extract is in µg/ml reaction mixture)

Fig: 3.11

Modulatory effect of BM extract prophylaxis on 3-NPA-induced oxidative impairments in striatum of prepubertal male mice.

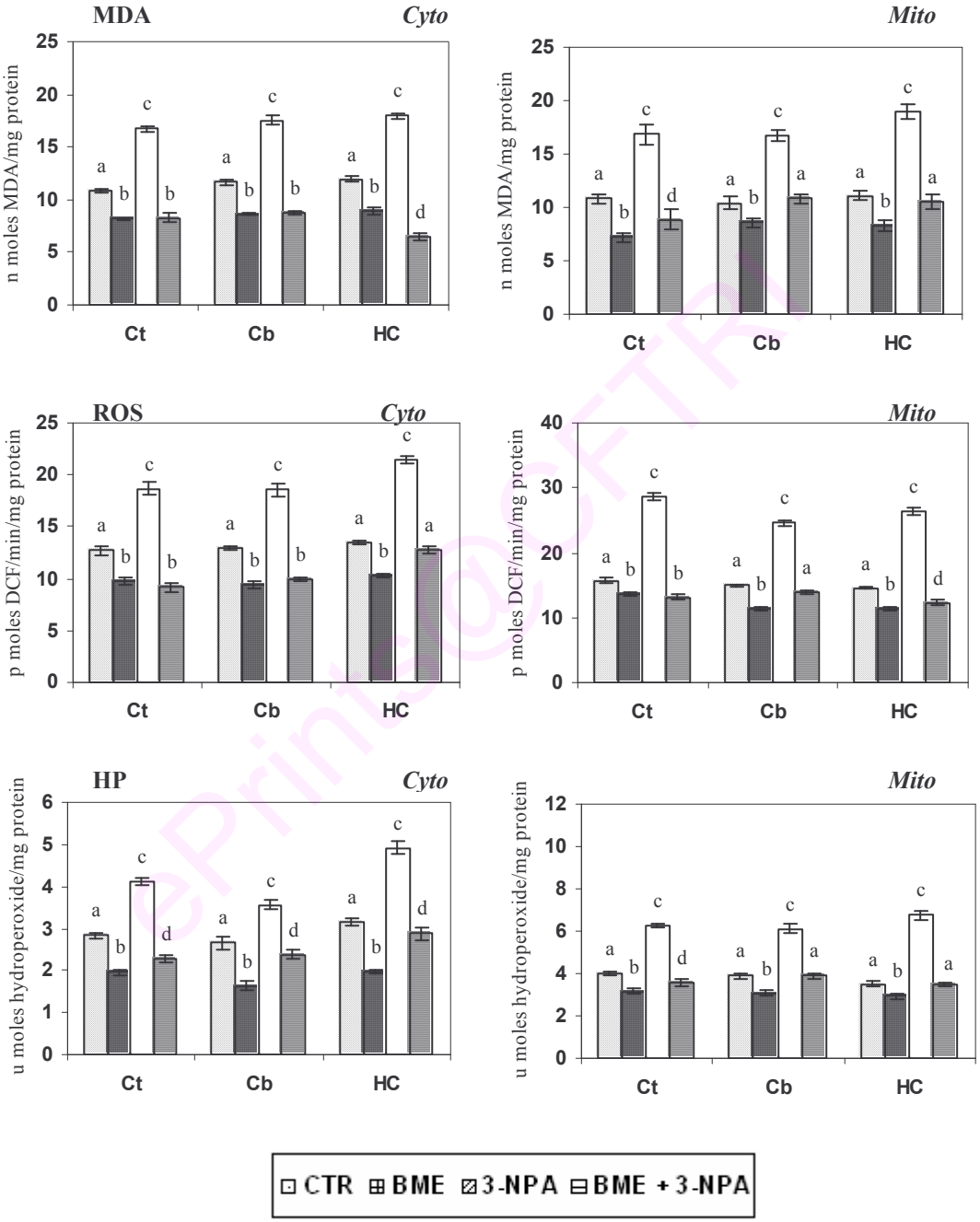


The values are mean ±SD (n=6). Data is analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kg bw/d for10d); 3-NPA: 3-nitropropionic acid (75mg/kgbw/d/2days); MDA: malondialdehyde; ROS: reactive oxygen species; HP: hydroperoxides

Fig: 3.12

Modulatory effects of BM extract prophylaxis on 3-NPA-induced oxidative impairments in cytosol and mitochondria of brain regions of prepubertal male mice.

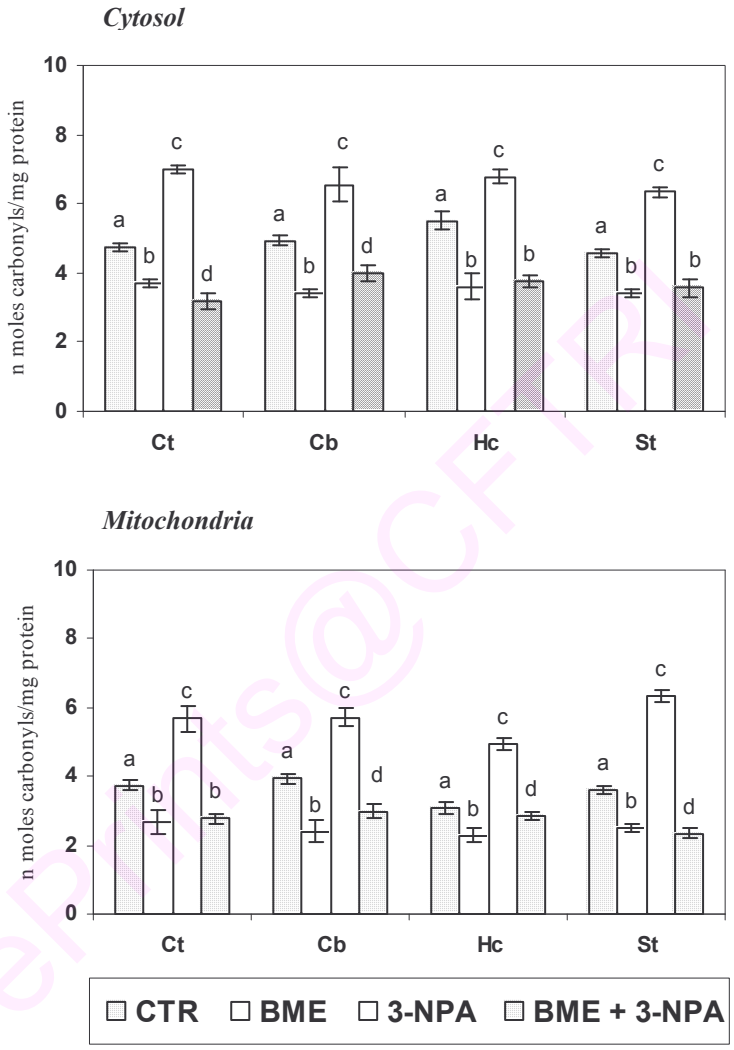


The values are mean ±SD (n=6).Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for10d); 3-NPA: 3-nitropropionic acid (75mg/kg bw/d for 2d); Cyto: cytosol; Mito: mitochondria; MDA-malondialdehyde; ROS-reactive oxygen species; HP-hydroperoxide

Fig: 3.13

Modulatory effects of BM extract prophylaxis on 3-NPA-induced protein carbonyls formation in cytosol and mitochondria of brain regions of prepubertal male mice.



The values are ±SD (n=6). Data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d);

3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

Table 3.2

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in antioxidant molecules in cytosol and mitochondria of striatum of prepubertal male mice.

Group	Striatum	
	Cytosol	Mitochondria
Reduced glutathione (GSH)[1]		
CTR	9.2±0.25[a]	10.1±0.23[a]
BME	11.5±0.13[b]	13.6 ±0.11[b]
3-NPA	7.1±0.51[c]	7.0±0.13[c]
BME+3-NPA	10.1±0.22[a]	9.9±0.23[a]
Oxidized glutathione (GSSG)[2]		
CTR	0.31 ±0.04[a]	0.87±0.45[a]
BME	0.28 ±0.03[b]	0.78±0.56[b]
3-NPA	0.45 ±0.01[c]	0.99±0.23[c]
BME+3-NPA	0.30 ±0.02[a]	0.85±0.11[a]
Total thiols (TSH)[3]		
CTR	8.6±0.13[a]	8.0±0.13[a]
BME	10.4±0.18[b]	10.2 ±0.23[b]
3-NPA	6.8±0.17[c]	5.1 ±0.45[c]
BME+3-NPA	8.8±0.23[a]	8.9±0.56[d]
Non-protein thiols (npSH)[3]		
CTR	0.97 ±0.05[a]	0.95 ±0.13[a]
BME	1.2±0.06[b]	1.2±0.11[b]
3-NPA	0.79 ±0.04[c]	0.50 ±0.13[c]
BME+3-NPA	0.99 ±0.04[a]	0.94 ±0.23[a]

The values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for10d)
3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

1-µg GSH/mg protein
2-µg GSSG/mg protein
3- ηmoles DTNB oxidized/mg protein

Table 3.3

Modulatory effect of BM extract prophylaxis on 3-NPA-induced perturbations in antioxidant molecules in cytosol of brain regions of prepubertal male mice.

Group	Brain regions		
	Cortex	Cerebellum	Hippocampus
Reduced glutathione (GSH)[1]			
CTR	9.1±0.13[a]	9.9±0.25[a]	8.9±0.14[a]
BME	11.9 ±0.11[b]	11.9±0.13[b]	10.6±0.35[b]
3-NPA	7.9±0.13[c]	7.6±0.51[c]	7.7±0.19[c]
BME+3-NPA	10.9±0.25[d]	10.9 ±0.22[d]	10.8±0.18[d]
Oxidized glutathione(GSSG)[2]			
CTR	0.32 ±0.03[a]	0.33 ±0.05[a]	0.31 ±0.04[a]
BME	0.29 ±0.01[b]	0.26 ±0.03[b]	0.27 ±0.05[b]
3-NPA	0.48 ±0.03[c]	0.44 ±0.01[c]	0.37 ±0.03[c]
BME+3-NPA	0.31 ±0.05[a]	0.29 ±0.02[a]	0.29 ±0.04[a]
Total thiols (TSH)[3]			
CTR	8.7±0.13[a]	7.9±0.25[a]	8.5±0.14[a]
BME	10.7±0.15[b]	9.4±0.13[b]	10.6±0.35[b]
NPA	7.8±0.16[c]	6.8±0.46[c]	7.5±0.17[c]
BME+3-NPA	8.9±0.15[a]	8.6±0.54[d]	8.8±0.19[a]
Non-protein thiols (npSH)[3]			
CTR	0.93 ±0.05[a]	0.99 ±0.02[a]	0.97 ±0.03[a]
BME	1.01 ±0.009[b]	1.22 ±0.06[b]	1.14 ±0.05[b]
3-NPA	0.67 ±0.02[c]	0.76 ±0.04[c]	0.68 ±0.07[c]
BME+3-NPA	0.91 ±0.03[a]	1.06 ±0.05[a]	0.98 ±0.03[a]

The values are ±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d)
3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

1-µg GSH/mg protein
2-µg GSSG/mg protein
3-ηmoles DTNB oxidized/mg protein

Table 3.4

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in antioxidant molecules in mitochondria of brain regions of prepubertal male mice.

Group	Brain regions		
	Cortex	Cerebellum	Hippocampus
Reduced glutathione (GSH)[1]			
CTR	10.08 ±0.23[a]	10.11 ±0.25[a]	10.01 ±0.34[a]
BME	13.51 ±0.11[b]	13.13 ±0.13[b]	13.35 ±0.35[b]
3-NPA	8.15 ±0.13[c]	8.09 ±0.66[c]	8.70 ±0.17[c]
BME+3-NPA	10.85 ±0.23[a]	10.12 ±0.51[a]	10.92 ±0.29[a]
Oxidized glutathione(GSSG)[2]			
CTR	0.89±0.45[a]	0.88±0.22[a]	0.86±0.28[a]
BME	0.80±0.56[b]	0.79±0.31[b]	0.78 ±0.17[b]
3-NPA	0.98±0.45[c]	0.98±0.13[c]	0.97±0.18[c]
BME+3-NPA	0.86 ±0.35[a]	0.89±0.31[a]	0.84±0.20[a]
Total thiols (TSH)[3]			
CTR	8.26 ±0.31[a]	8.45 ±0.41[a]	8.31 ±0.12[a]
BME	10.17 ±0.67[b]	10.56 ±0.37[b]	10.39 ±0.33[b]
3-NPA	6.17 ±0.45[c]	6.99 ±0.19[c]	6.83 ±0.13[c]
BME+3-NPA	9.07 ±0.12[d]	9.42 ±0.10[d]	9.28 ±0.11[d]
Non-protein thiols (npSH)[3]			
CTR	0.97 ±0.10[a]	0.87 ±0.15[a]	0.91 ±0.14[a]
BME	1.16 ±0.11[b]	1.07 ±0.13[b]	1.13 ±0.15[b]
3-NPA	0.41 ±0.13[c]	0.57 ±0.16[c]	0.77 ±0.10[c]
BME+3-NPA	1.08 ±0.11[d]	1.01 ±0.21[b]	0.95 ±0.21[a]

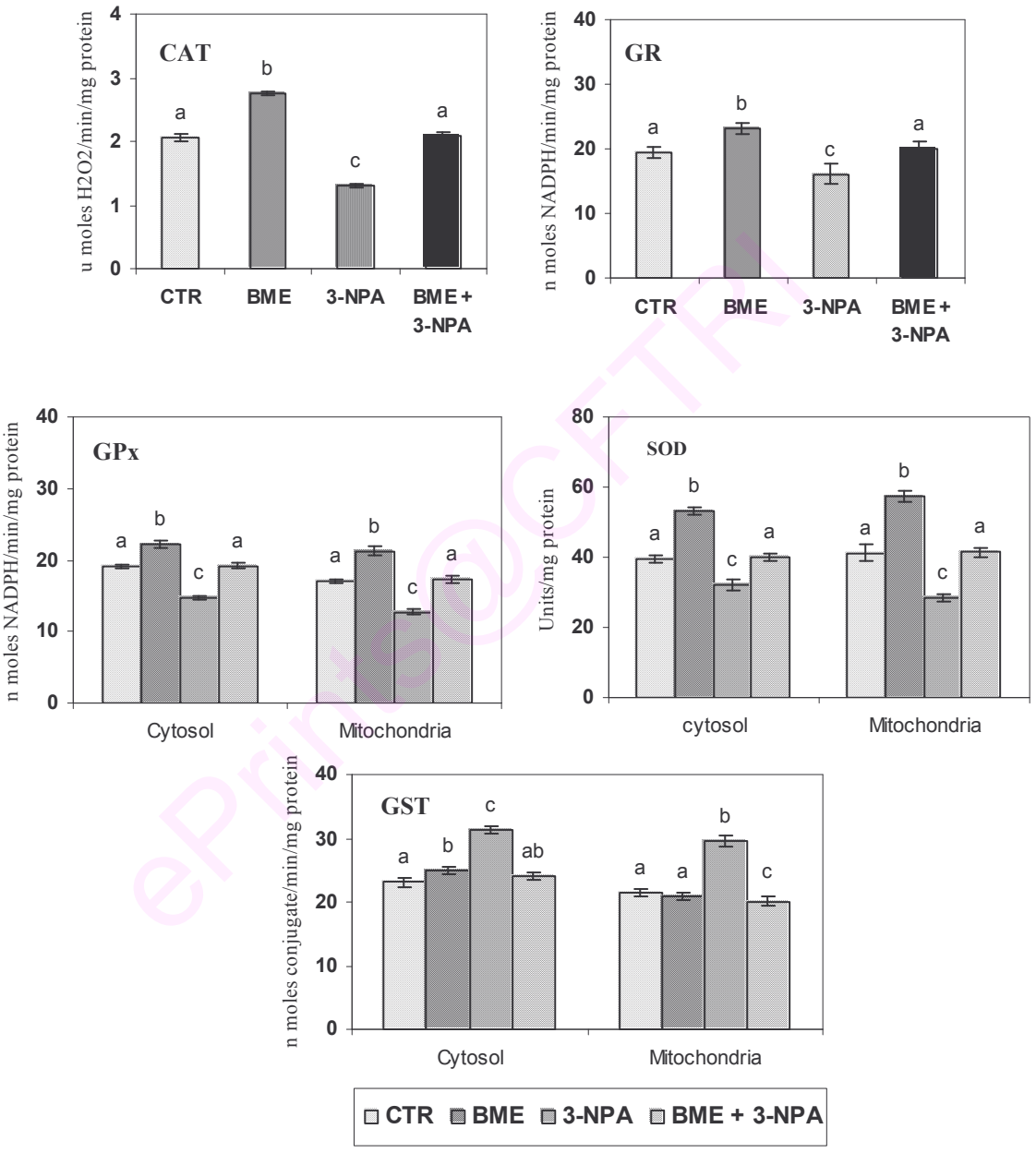
Values are mean ±SD (n=6).
Data is analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d/10d)
3-NPA: 3-nitropropionic acid (75mg/kg bw/d/2d)

1-µg GSH/mg protein
2-µg GSSG/mg protein
3-ηmoles DTNB oxidized/mg protein

Fig.3.14

Modulatory effects of BM extract prophylaxis on 3-NPA-induced alterations in antioxidant enzymes in cytosol and mitochondria of striatum of prepubertal male mice.



The values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/10days); 3-NPA: 3-nitropropionic acid (75mg/kg bw/2days); CAT-Catalase; GR-glutathione reductase; GPx-Glutathione peroxidase; SOD-Superoxide dismutase; GST-Glutathione-S-Transferase

Table.3.5

Antioxidant enzymes in cytosol of brain regions of prepubertal mice given BME prophylaxis followed by 3-NPA administration.

Group	Brain Regions		
	Cortex	Cerebellum	Hippocampus
Catalase (CAT)[1]			
CTR	2.35± 0.06[a]	2.02±0.01[a]	2.15±0.05[a]
BME	2.95±0.03[b]	2.73±0.03[b]	2.42±0.04[b]
3-NPA	1.70±0.03[c]	1.62±0.02[c]	1.79±0.02[c]
BME+3-NPA	2.19±0.05[d]	1.97±0.03[a]	2.44±0.03[d]
Glutathione peroxidase (GPx[2)]			
CTR	19.72±0.12[a]	18.18±0.34[a]	18.89±0.22[a]
BME	23.24±0.13[b]	23.56±0.67[b]	20.12±0.31[b]
3-NPA	14.77±0.34[c]	13.45±0.54[c]	14.12±0.11[c]
BME+3-NPA	19.91±0.78[a]	18.56±0.91[a]	17.99±0.78[a]
Glutathione S transferase (GST[3)]			
CTR	23.12±1.56[a]	25.21±1.0[a]	25.45±1.11[a]
BME	25.12±0.98[b]	26.23±0.83[b]	26.67±0.78[b]
3-NPA	29.32±0.33[c]	29.01±0.45[c]	29.11±0.78[c]
BME+3-NPA	24.12±0.67[a]	24.87±0.54[a]	24.15±0.98[a]
Superoxide dismutase (SOD[4)]			
CTR	40.44±0.91[a]	38.23±0.67[a]	35.99±0.98[a]
BME	56.12±0.89[b]	50.45±1.12[b]	44.18±0.99[b]
3-NPA	30.12±1.56[c]	28.21±1.02[c]	28.45±1.11[c]
BME+3-NPA	42.12±0.98[a]	40.23±0.83[a]	36.67±0.78[a]

Values are mean ±SD (n=6).

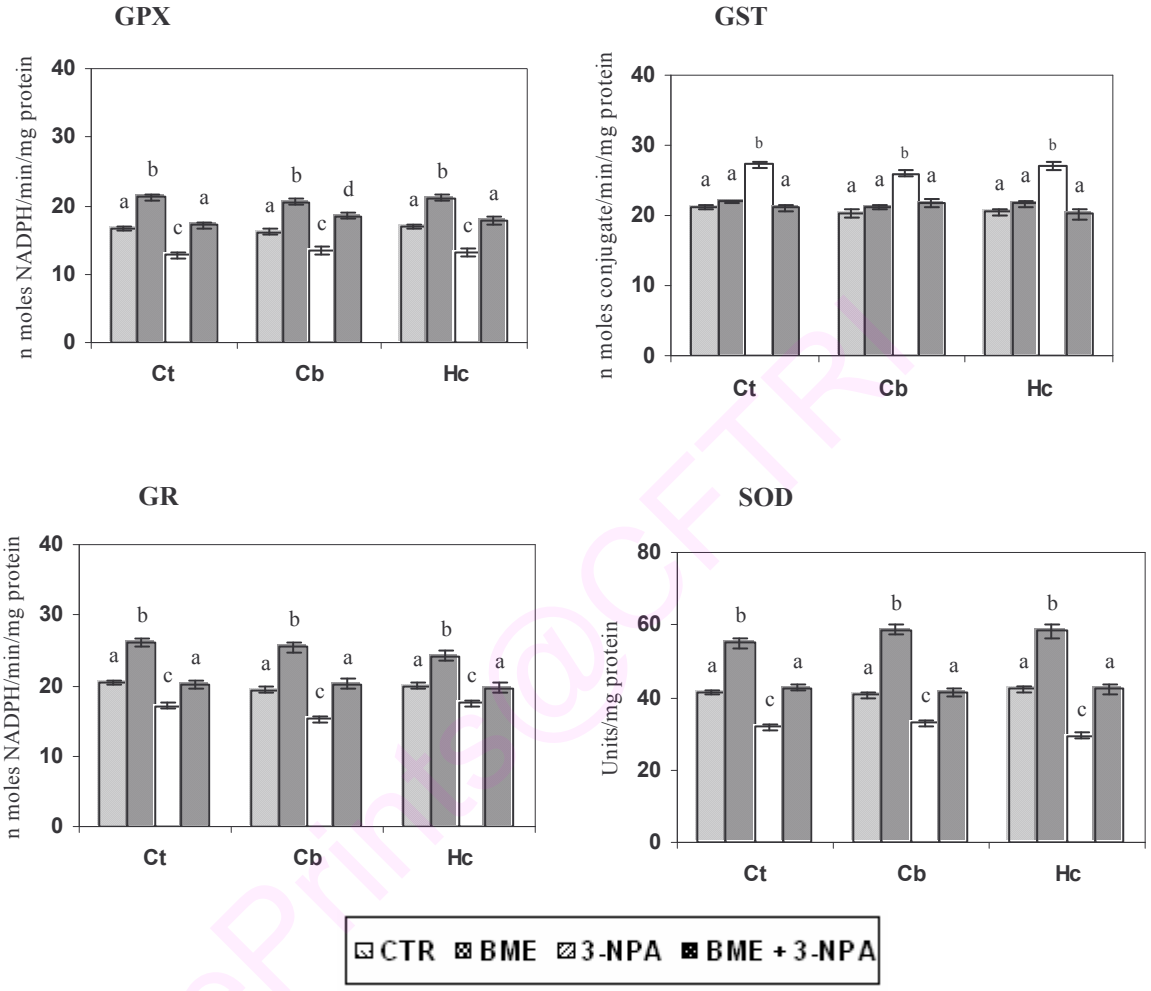
Data analyzed by one way ANOVA ($P<0.05$) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d)
3-NPA: 3-nitropropionic acid (75mg/kgbw/d/2d)

1-μ moles H_2O_2/min/mg protein; 2-η moles NADPH/min/mg protein
3-η moles conjugate/min/mg protein; 4-Units/mg protein

Fig: 3.15

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in antioxidant molecules in mitochondria of brain regions of prepubertal male mice.



The values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 10d)
3-NPA: 3-nitropropionic acid (75mg/kg bw/d for 2d)

GST-Glutathione-S-Transferase; GPx-Glutathione peroxidase;
GR-glutathione reductase; SOD-Superoxide dismutase;

Table 3.6

Effect of BM extract 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
Iron[1]				
CTR	2.31±0.25[a]	2.16±0.35[a]	1.80±0.30[a]	2.31±0.29[a]
BME	1.85±0.20[b]	1.86±0.21[b]	1.29±0.21[b]	1.67±0.21[b]
3-NPA	2.93±0.18[c]	3.18±0.35[c]	3.31±0.23[c]	3.23±0.23[c]
BME+3-NPA	1.75±0.21[d]	1.84±0.31[b]	1.91±0.25[d]	2.03±0.29[d]
Vitamin C[2]				
CTR	0.210±0.015[a]	0.155±0.005[a]	0.171±0.005[a]	0.186±0.006[a]
BME	0.208±0.010[a]	0.149±0.009[a]	0.166±0.008[a]	0.193±0.010[a]
3-NPA	0.156±0.011[b]	0.211±0.007[b]	0.207±0.009[b]	0.167±0.011[b]
BME+3-NPA	0.219±0.012[a]	0.235±0.009	0.180±0.004[a]	0.187±0.010[a]

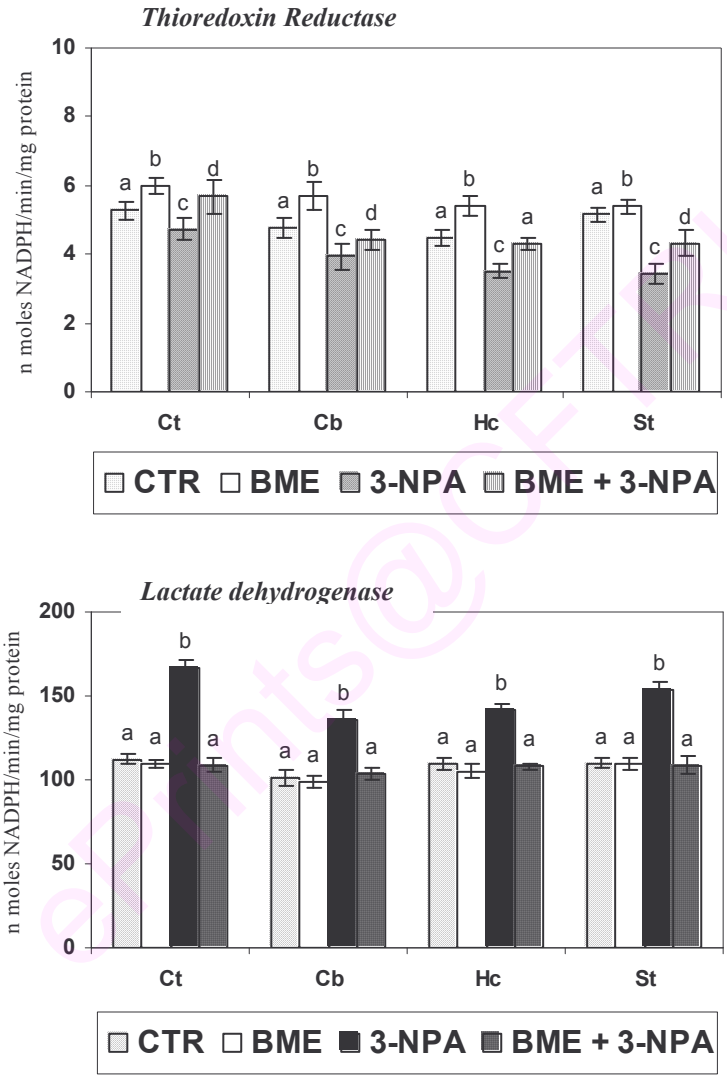
The values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d);
3-NPA: 3-nitropropionic acid (75mg/kgbw/2days)

1-OD/mg protein; 2-µg/mg tissue

Fig: 3.16

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbation in the activities of Thioredoxin reductase and Lactate dehydrogenase in brain regions of prepubertal male mice.



The values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) appropriate to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d)
3- NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

Table 3.7

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in TCA cycle enzymes in brain regions of prepubertal male mice.

Groups	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
MDH[1]				
CTR	587.2±10.31[a]	569.7±10.63[a]	548.2±11.68[a]	545.8±11.45[a]
BME	620.0±10.21[b]	601.8±12.55[b]	596.6±12.39[b]	598.3±11.23[b]
3-NPA	478.1±11.34[c]	468.1±11.36[c]	431.9±11.21[c]	409.4±10.78[c]
BME+3-NPA	590.0±12.21[a]	581.8±12.05[d]	566.4±11.34[d]	568.3±10.23[d]
SDH[2]				
CTR	5.06 ±0.34[a]	5.15 ±0.25[a]	4.29 ±0.21[a]	4.28 ±0.21[a]
BME	5.70 ±0.40[a]	5.30 ±0.24[a]	4.30 ±0.23[a]	4.76 ±0.22[a]
3-NPA	0.98 ±0.25[b]	1.23 ±0.12[b]	1.76 ±0.22[b]	0.72 ±0.19[b]
BME+3-NPA	1.82 ±0.16[c]	2.32 ±0.10[c]	2.45 ±0.21[c]	1.58 ±0.21[c]
ICDH[3]				
CTR	24.3±0.45[a]	23.8±0.52[a]	24.5±0.58[a]	23.3 ±0.45[a]
BME	25.0±0.56[a]	25.7±0.61[b]	26.4±0.57[b]	25.4 ±0.56[b]
3-NPA	17.7±0.45[b]	18.9±0.45[c]	19.5±1.18[c]	19.5 ±0.53[c]
BME+3-NPA	24.7±0.65[a]	23.4±0.53[a]	23.3±1.20[a]	23.3 ±1.11[a]
Fumarase[4]				
CTR	106.1±3.40[a]	110.2±3.20[a]	113.4±3.29[a]	109.3±3.40[a]
BME	112.2±2.43[a]	116.5±3.43[a]	119.5±2.42[a]	117.6±2.76[a]
3-NPA	88.2±3.48[b]	89.2±2.63[b]	92.3±2.23[b]	85.2±3.51[b]
BME+3-NPA	103.3±2.90[a]	109.2±3.70[a]	110.1±3.45[a]	105.3±3.33[a]
Citrate synthase[5]				
CTR	245.2±4.20[a]	223.3±4.43[a]	232.2±6.40[a]	222.3±5.45[a]
BME	253.7±3.41[b]	243.2±3.90[b]	245.2±3.49[b]	230.3±4.69[b]
3-NPA	175.2±6.30[c]	149.2±5.52[c]	164.2±5.61[c]	157.1±4.56[c]
BME+3-NPA	249.3±5.32[a]	230.3±4.56[a]	240.2±5.34[a]	238.9±4.42[b]

The values are mean ±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates.

Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d)

3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

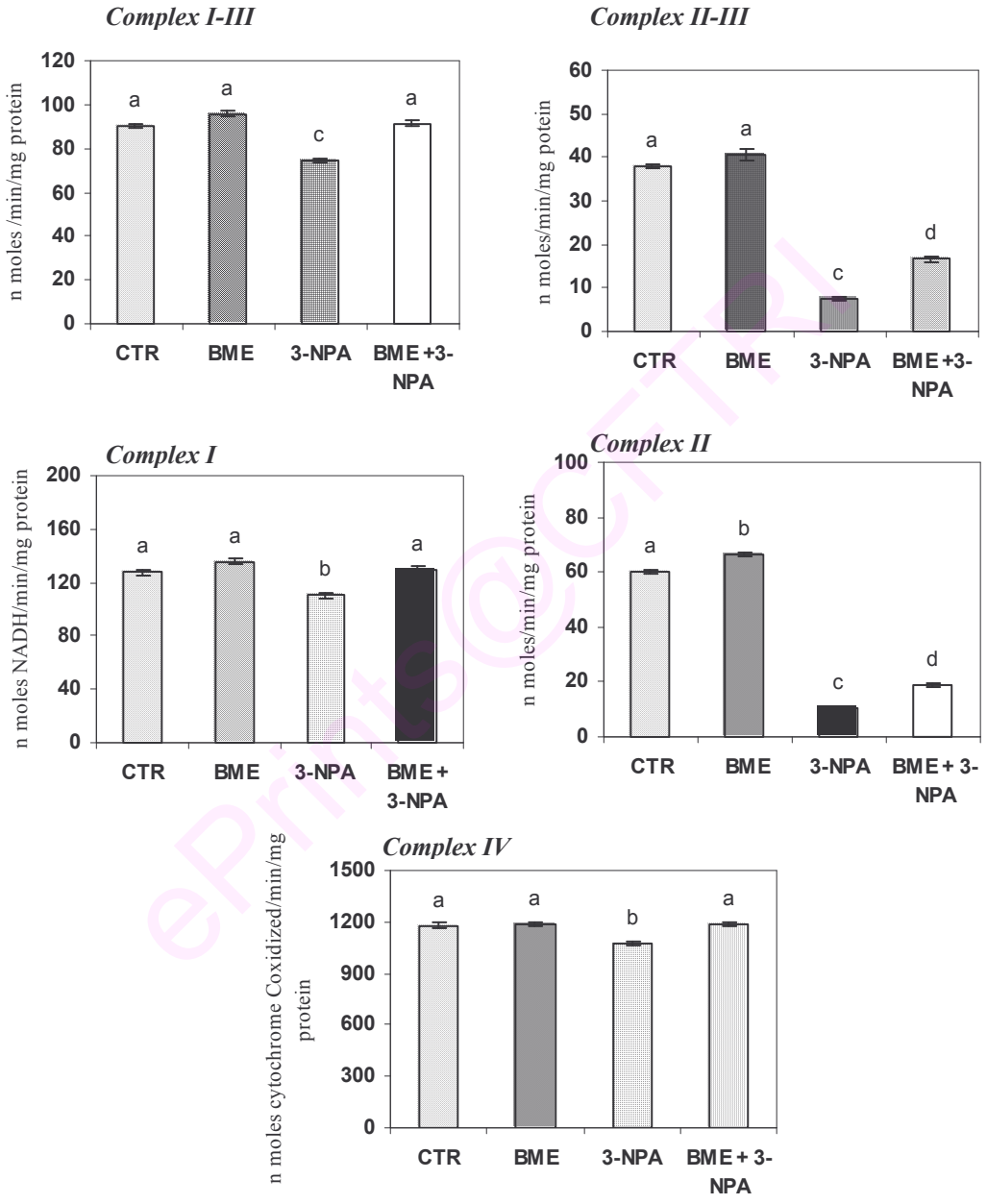
1-ηmoles NADH /min/mg protein; 2-OD at 490 nm/mg protein

3-η moles/min/mg protein; 4-ηmoles/min/mg protein

5-ηmoles /min/mg protein

Fig: 3.17

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in Electron Transport Chain enzymes in striatum of prepubertal male mice.



The values are mean ±SD (n=6). Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

BME: *Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 10d); 3-NPA: 3-nitropropionic acid (75mg/kg bw/d for 2d); Complex I-NADH ubiquinone oxidoreductase; Complex II-Succinate ubiquinone oxidoreductase; Complex-I-II-NADH cytochrome c reductae; Complex II-III-Succinate cytochrome c reductase; Complex IV-Cytochrome C oxidase.

Table 3.8

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbations in Electron Transport Chain enzymes in brain regions of prepubertal male mice.

Groups	Brain Regions		
	Cortex	Cerebellum	Hippocampus
Complex I[1]			
CTR	130.3±3.56[a]	122.3±2.34[a]	132.2±2.13[a]
BME	138.9±4.25[a]	134.5±1.25[a]	143.6±1.14[a]
3-NPA	115.3±2.35[c]	110.2±1.02[c]	112.2±1.02[c]
BME+3-NPA	132.2±4.50[a]	126.7±1.10[a]	130.2±1.10[a]
Complex II[2]			
CTR	62.3±1.02[a]	58.5±0.69[a]	57.9±0.49[a]
BME	65.3±0.97[a]	64.4±0.99[a]	63.5±0.97[a]
3-NPA	12.1±0.5[b]	10.1±0.28[b]	11.0±0.65[b]
BME+3-NPA	19.0±0.35[c]	18.1±0.59[c]	19.0±0.50[c]
Complex IV[3]			
CTR	1087.2±12.3[a]	1083.2±12.3[a]	1079.03±12.5[a]
BME	1078.2±13.5[a]	1089.0±10.9[a]	1087.45±13.5[a]
3-NPA	975.2±12.5[b]	970.2±10.5[b]	967.33±11.5[b]
BME+3-NPA	1089.0±12.9[a]	1086.4±11.6[a]	1080.23±12.3[a]
Complex I-III[4]			
CTR	89.2±1.2[a]	90.2±1.5[a]	88.9±1.2[a]
BME	94.6±1.6[a]	96.7±1.2[a]	93.4±1.3[a]
3-NPA	70.2±1.6[b]	78.9±1.3[b]	77.9±1.2[b]
BME+3-NPA	87.3±1.2[a]	85.3±1.2[c]	89.0±1.1[a]
Complex II-III[4]			
CTR	40.2±0.50[a]	37.9±1.23[a]	38.9±0.75[a]
BME	45.7±1.23[a]	41.2±1.02[a]	42.2±0.80[a]
3-NPA	10.1±0.67[b]	8.9±0.50[b]	8.6±0.45[b]
BME+3-NPA	17.9±0.65[c]	19.0±0.76[c]	16.3±0.38[c]

The values are mean ±SD (n=6). Data analyzed by one way ANOVA ($P<0.05$) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

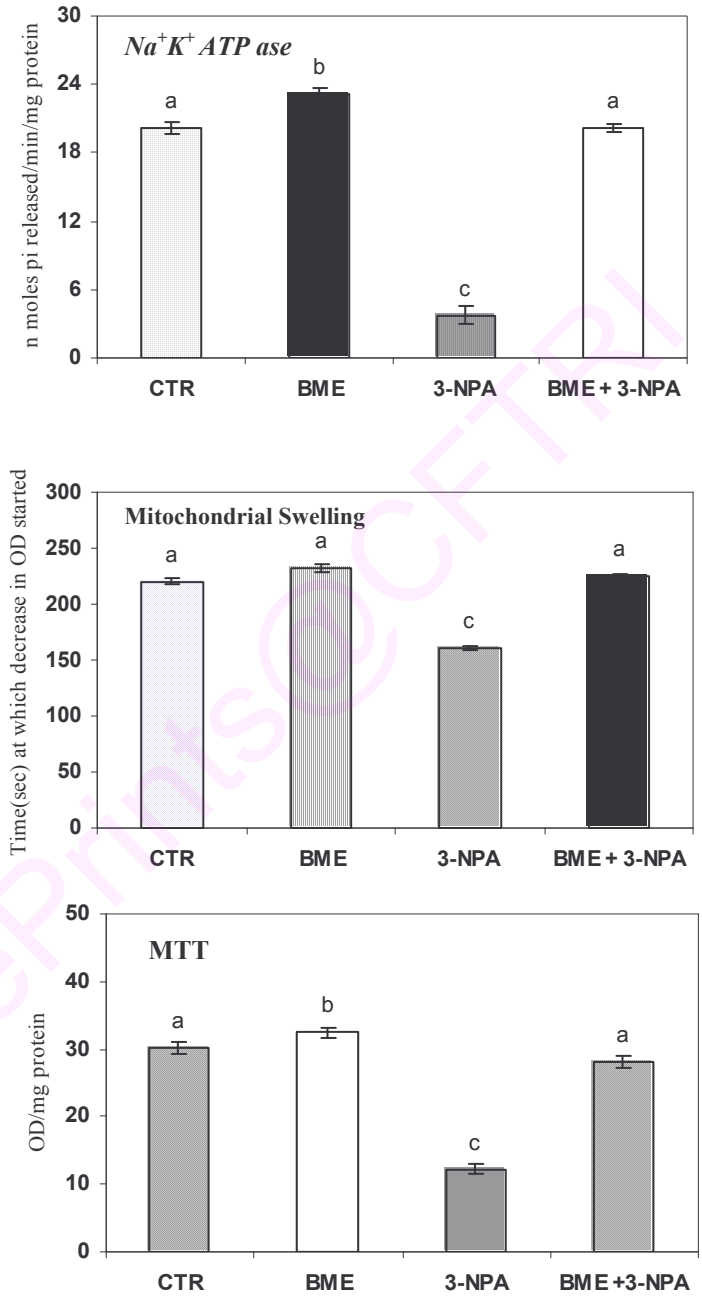
BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d)
3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d)

Complex I: NADH ubiquinone oxidoreductase; Complex II: Succinate ubiquinone oxido reductase
Complex-I-II: NADH cytochrome c reductase; Complex II-III: Succinate cytochrome c reductase
Complex IV: Cytochrome C oxidase

1-η moles NADH oxidized/min/mg protein; 2-η moles/min/mg protein
3-η moles/min/mg protein; 4-η moles/min/mg protein
5-η moles/min/mg protein

Fig: 3.18

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbation in Na^+ K^+ ATP ase, mitochondrial swelling, and MTT in striatum of prepubertal male mice.

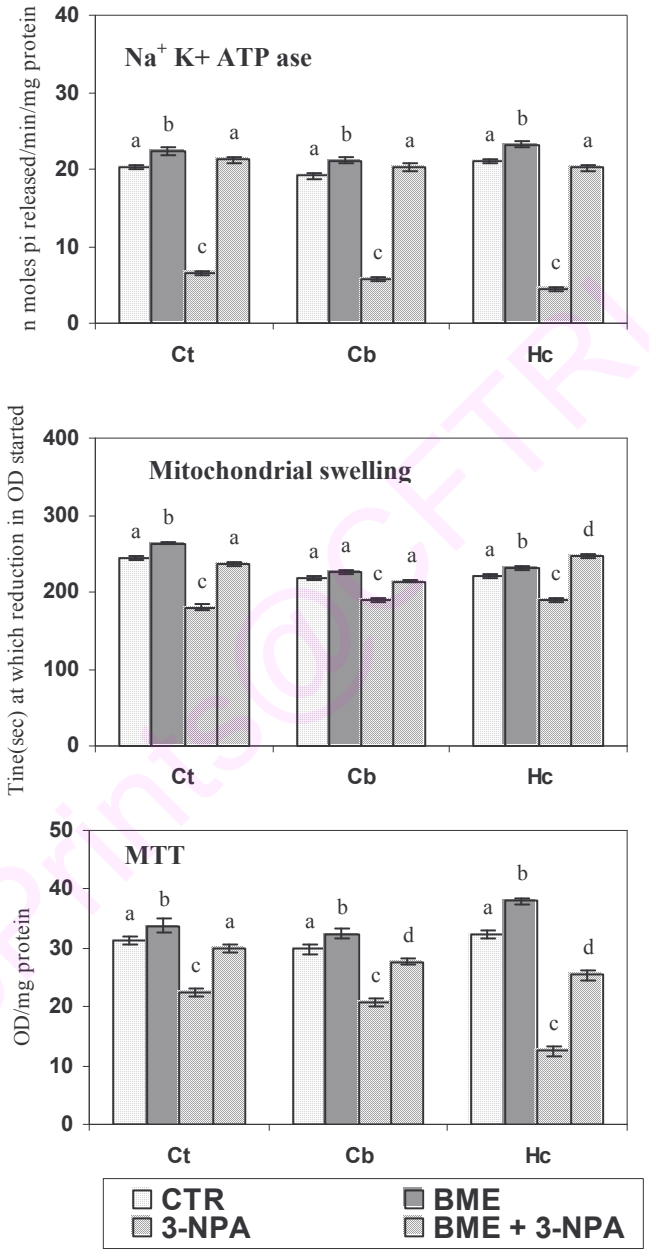


The values are mean ±SD (n=6).Data analyzed by one way ANOVA (*P<0.05) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for10d); NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d); Na^+ K^+ ATP ase: sodium potassium ATP ase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride

Fig.3.19

Modulatory effects of BM extract prophylaxis on 3-NPA-induced perturbation in Na^+ K^+ ATP ase, mitochondrial swelling, and MTT in brain regions of prepubertal male mice.

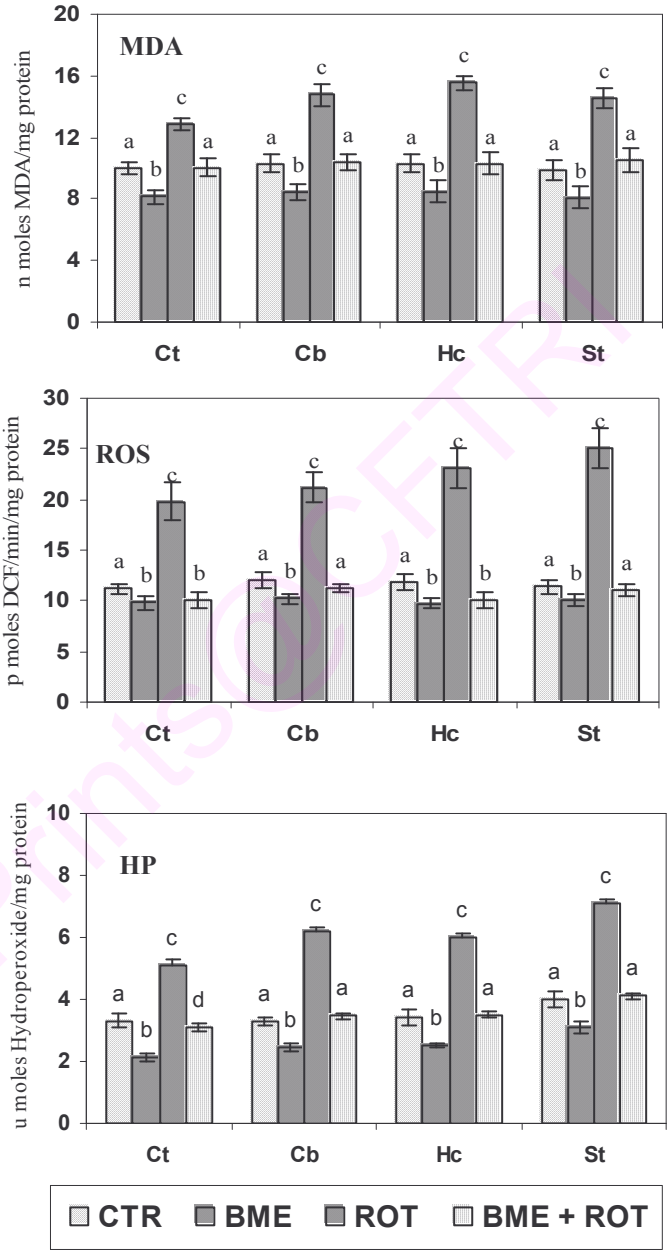


The values are mean ±SD (n=6). Data is analyzed by one way ANOVA ($P<0.05$) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 10d); 3-NPA: 3-nitropropionic acid (75mg/kgbw/d for 2d); Na^+ K^+ ATP ase: sodium potassium ATP ase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride

Fig: 3.20

Modulatory effect of BM extract on Rotenone-induced oxidative stress in cytosol of brain regions of prepubertal male mice.



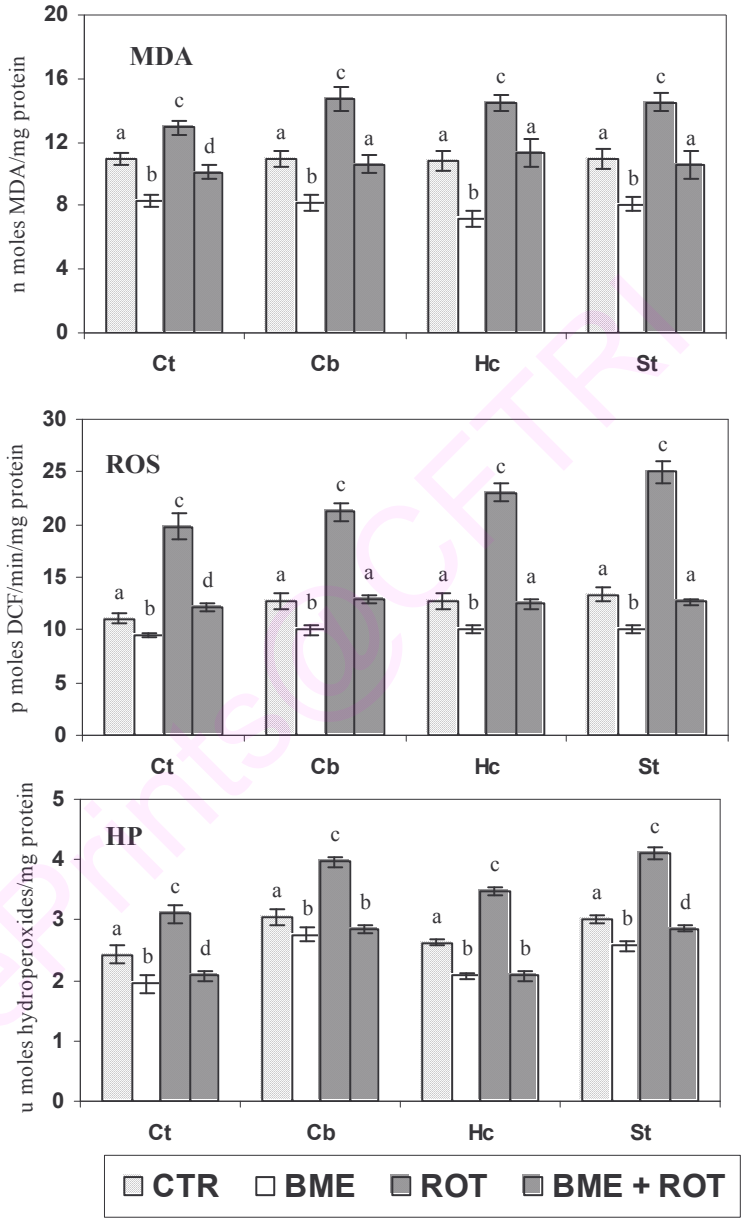
The values are mean ±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

BME- *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT-Rotenone (1mg/kgbw/d for 7d); MDA-Malondialdehyde; ROS-Reactive oxygen species; HP-Hydroperoxides

Fig: 3.21

Modulatory effect of BM extract on Rotenone-induced oxidative stress in mitochondria of brain regions of prepubertal male mice.



Values are mean±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT.

BME-*Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT-Rotenone (1mg/kgbw/d for 7d)
MDA-Malondialdehyde; ROS-Reactive oxygen species; HP-Hydroperoxides

Table 3.9

Modulatory effect of BM extract on Rotenone-induced protein carbonyls in brain regions of prepubertal male mice.

Group	η moles carbonyls/mg protein			
	Cortex	Cerebellum	Hippocampus	Striatum
Cytosol				
CTR	5.8 ±0.24[a]	5.9±0.13 [a]	6.47 ±0.26 [a]	6.43 ±0.26 [a]
BME	4.1±0.35 [b]	4.4±0.25 [b]	5.50±0.30 [b]	5.10±0.25 [b]
ROT	9.2±0.75 [c]	13.3±0.59 [c]	14.15±0.96 [c]	15.71±0.96 [c]
BME+ROT	5.1±0.45 [d]	5.5±0.55 [a]	6.50±0.70 [a]	6.90±0.75 [a]
Mitochondria				
CTR	3.4±0.24 [a]	3.8±0.13 [a]	3.61 ±0.26 [a]	3.46±0.26 [a]
BME	2.9±0.55 [b]	2.3±0.51 [b]	2.37±0.76 [b]	2.08±0.76 [b]
ROT	6.1±0.55 [c]	7.0±0.51 [c]	6.88±0.76 [c]	7.60±0.76 [c]
BME+ROT	3.6±0.40 [a]	3.9±0.45 [a]	3.50±0.54 [a]	3.75±0.45 [a]

Table 3.10

Modulatory effect of BM extract on Rotenone-induced perturbations in GSH in brain regions of prepubertal male mice.

Group	µg GSH/mg protein			
	Cortex	Cerebellum	Hippocampus	Striatum
Cytosol				
CTR	11.05±0.47 [a]	11.76±0.75 [a]	11.52±0.50 [a]	11.52±0.62 [a]
BME	13.30±0.70 [b]	13.76±0.70 [b]	13.52±0.32 [b]	13.87±0.50 [b]
ROT	9.80 ±0.88 [c]	10.50±0.54 [c]	10.31±0.60 [c]	9.91±0.63 [c]
BME+ROT	12.05±0.57 [d]	12.06±0.65 [a]	11.72±0.50 [a]	11.22±0.50 [a]
Mitochondria				
CTR	10.98±0.38 [a]	10.32±0.54 [a]	10.31±0.60 [a]	9.91±0.63 [a]
BME	12.21±0.56 [b]	11.45±0.60 [b]	12.01±0.75 [b]	11.98±0.45 [b]
ROT	7.90±0.40 [c]	7.76±0.75 [c]	6.52±0.50 [c]	5.52±0.62 [c]
BME+ROT	9.89±0.45 [a]	10.45±0.46 [a]	10.98±0.15 [a]	10.07±0.67 [a]

Values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT-Rotenone (1mg/kg bw/d for 7d)

Table 3.11

Modulatory effect of BM extract on Rotenone-induced alterations in antioxidant enzymes in cytosol of brain regions of prepubertal male mice.

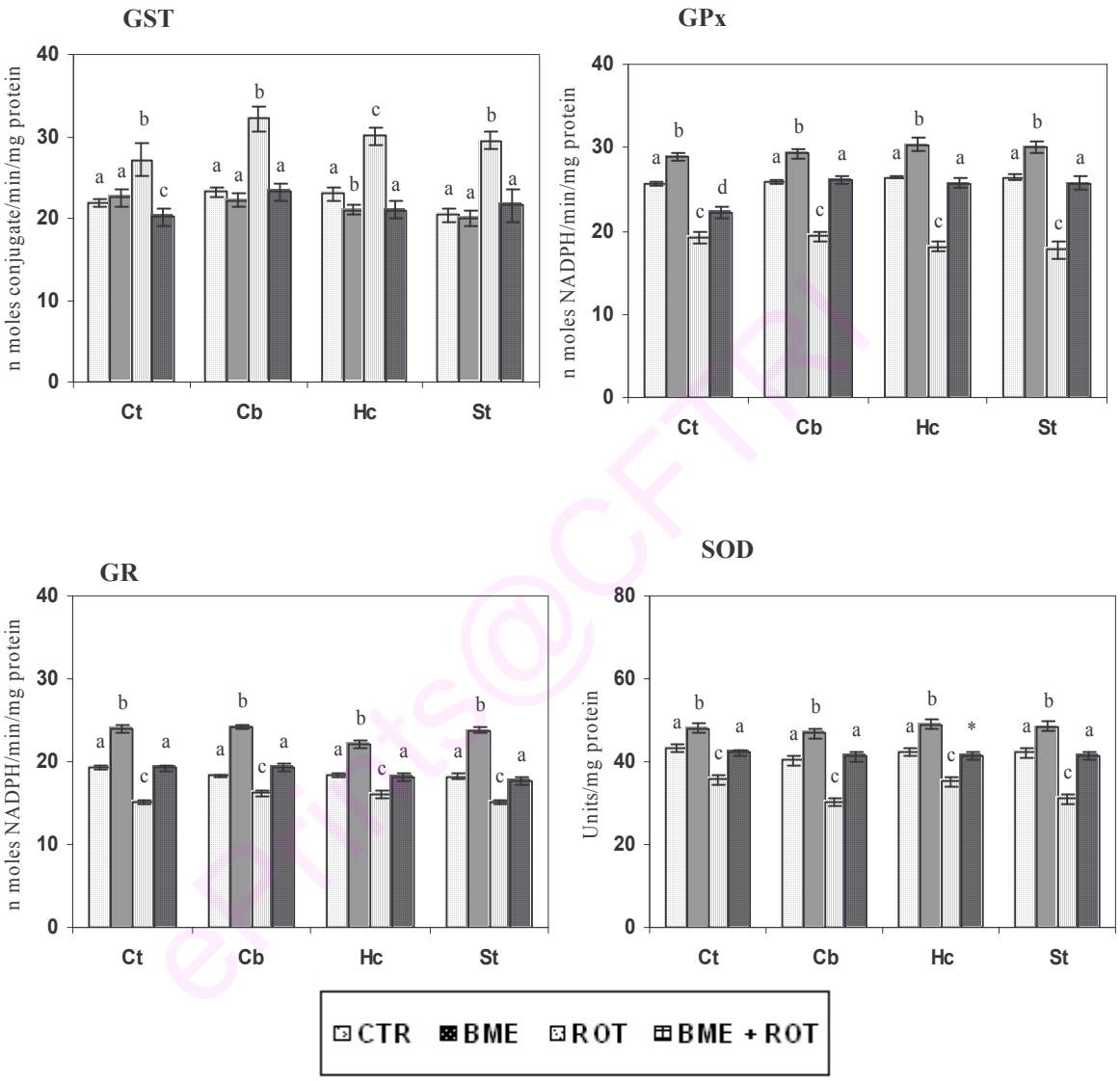
Groups	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
CAT[1]				
CTR	1.98±0.18[a]	1.82±0.10[a]	1.90±0.10[a]	1.91±0.08[a]
BME	2.25±0.09[b]	2.33±0.10[b]	2.45±0.08[b]	2.23±0.08[b]
ROT	0.90±0.40[c]	0.96±0.05[c]	0.82±0.10[c]	0.76±0.08[c]
BME+ROT	1.90±3.34[a]	1.73±0.08[a]	2.05±0.10[a]	1.98±0.07[a]
GPx[2]				
CTR	25.76±0.94[a]	25.86±0.13[a]	26.47±1.02[a]	26.43±1.06[a]
BME	29.50±0.55[b]	30.12±0.96[b]	32.15±1.06[b]	31.71±0.80[b]
ROT	19.20±0.75[c]	18.32±0.59[c]	17.15±0.96[c]	15.71±0.96[c]
BME+ROT	24.20±0.85[a]	28.32±0.79[a]	27.15±0.86[a]	25.70±0.86[a]
GST[3]				
CTR	21.17±0.50[a]	22.11±0.79[a]	21.79±0.78[a]	21.35±0.70[a]
BME	19.20±0.75[b]	21.32±0.59[b]	21.15±0.96[b]	21.71±0.96[b]
ROT	29.83±1.92[c]	31.21±1.54[c]	33.03±1.96[c]	35.03±1.96[c]
BME+ROT	19.75±0.65[b]	21.42±0.60[a]	21.15±0.66[a]	21.71±0.86[a]
GR[4]				
CTR	17.20±0.75[a]	18.12±0.50[a]	17.15±0.80[a]	17.71±0.50[a]
BME	19.20±0.65[b]	21.22±0.52[b]	20.15±0.86[b]	21.71±0.60[b]
ROT	14.20±0.49[c]	14.32±0.60[c]	13.15±0.66[c]	15.71±0.96[c]
BME+ROT	18.01±0.55[a]	18.32±0.66[a]	17.75±0.96[a]	17.80±0.49[a]
SOD[5]				
CTR	34.24±2.24[a]	35.15±1.67[a]	35.56±1.32[a]	34.15±1.24[a]
BME	38.90±3.34[b]	42.33±2.10[b]	39.05±2.50[b]	40.23±2.51[b]
ROT	58.90±3.34[c]	54.33±2.24[c]	59.05±2.54[c]	60.23±2.37[c]
BME+ROT	40.90±3.15[d]	34.33±2.35[c]	39.05±2.54[b]	40.70±2.36[b]

Values are mean ±SD (n=6).Data analyzed by one way ANOVA ($P<0.05$) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

BME: *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT: Rotenone(1 mg/kg bw/d for 7d)**;** CAT-Catalase; GPx-Glutathione peroxidase; GST-Glutathione–S-Transferase; GR- Glutathione reductase; SOD- Superoxide dismutase; 1-μ moles H_2O_2/min/mg protein; 2-ηmoles NADPH/min/mg protein;3-ηmoles conjugate/min/mg protein; 4-ηmoles NADPH/min/mg protein; 5-Units/mg protein

Fig: 3.22

Modulatory effect of BM extract on Rotenone-induced alterations in antioxidant enzymes in mitochondria of brain regions of prepubertal male mice.



The values are mean ±SD (n=6).

Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates.

Means followed by different letters differ significantly according to DMRT.

BME-*Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 7d); ROT-Rotenone (1mg/kg bw/d for 7d)

GPx-Glutathione peroxidase; GST-Glutathione –S-Transferase;
GR-Glutathione reductase; SOD-Superoxide dismutase

Table 3.12

Modulatory effect of BM extract on Rotenone-induced alterations LDH, MDH and TRR in brain regions of prepubertal male mice.

Group	Brain regions			
	Cortex	Cerebellum	Hippocampus	Striatum
LDH[1]				
CTR	120.3±4.5[a]	121.1±4.2[a]	119.2± 3.5[a]	118.1±3.8[a]
BME	111.4±4.8[a]	115.1±3.4[a]	115.2±4.2[a]	109.6±3.9[a]
ROT	182.2±5.5[b]	161.1±4.2[b]	179.2± 3.5[b]	190.1±5.5[b]
BME+ROT	114.3±5.6[c]	112.1±3.4[c]	109.2±4.3[c]	109.6±4.5[c]
MDH[2]				
CTR	622.8±10.5[a]	533.7±12.2[a]	532.3±11.2[a]	617.9±11.2[a]
BME	643.9±12.4[a]	556.8±12.3[a]	582.5±12.7[a]	629.9±11.1[a]
ROT	547.8±10.1[b]	398.5±10.4[b]	331.5±13.9[b]	464.1±11.1[b]
BME+ ROT	621.2±11.1[a]	541.2±11.2[a]	530.1±10.1[a]	610.1±12.1[a]
TRR[3]				
CTR	8.0±0.30[a]	6.3±0.50[a]	7.3±0.48[a]	6.9±0.53[a]
BME	9.2±0.51[b]	7.8±0.50[b]	8.9±0.62[b]	7.5±0.55[b]
ROT	3.9±0.42[c]	3.8±0.67[c]	4.5±0.70[c]	3.5±0.42[c]
BME+ROT	6.8±0.45[d]	5.9±0.56[d]	7.0±0.56[a]	6.5±0.54[a]

Values are mean ±SD (n=6).

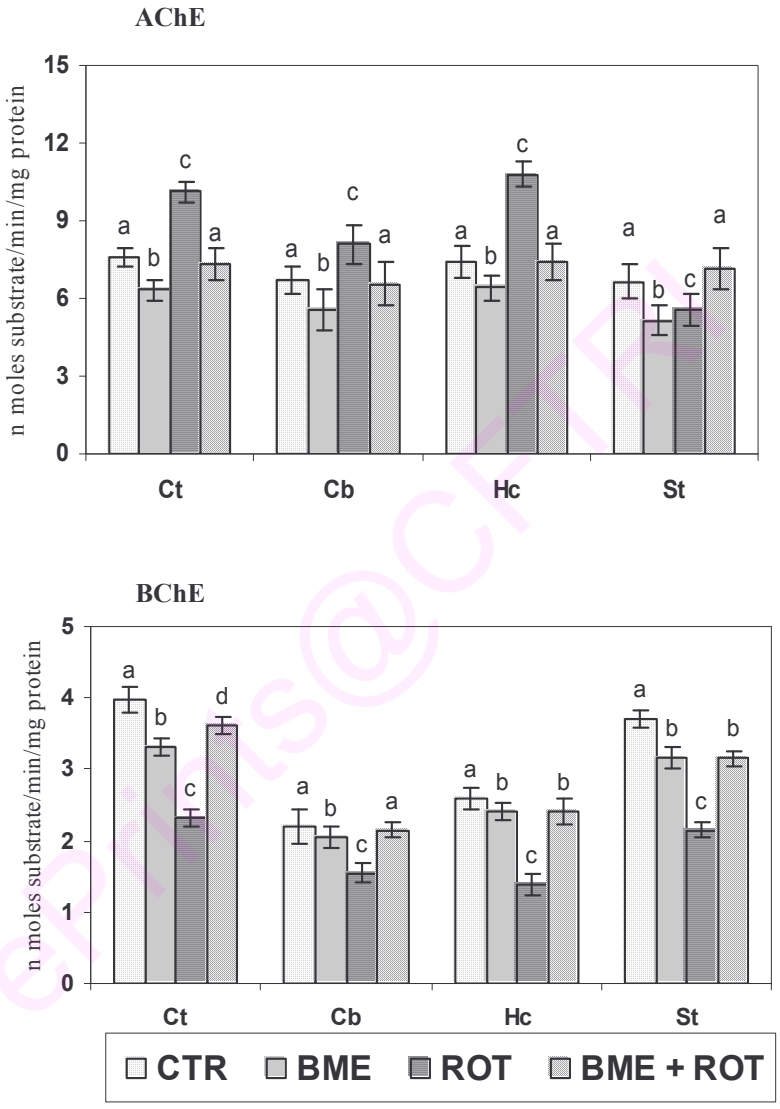
Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

BME- *Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT-Rotenone (1mg/kg bw/d for 7d)
LDH-Lactate dehydrogenase; MDH-Malate dehydrogenase; TRR-Thioredoxin reductase

1-n moles substrate/min/mg protein
2-n moles NADH oxidized/min/mg protein
3-n moles NADPH/min/mg protein

Fig: 3.23

Modulatory effect of BM extract on Rotenone-induced perturbations in cholinergic enzymes in brain regions of prepubertal male mice.



Values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT

BME -*Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 7d); ROT-Rotenone (1mg/kg bw/d for 7d)

AChE-Acetylcholinesterase; BChE-Butyrylcholinesterase

Table 3.13

Modulatory effect of BM extract on Rotenone-induced alterations in Electron transport chain enzymes in brain regions of prepubertal male mice.

Group	Brain Regions			
	Cortex	Cerebellum	Hippocampus	Striatum
***Complex I*[1]**				
CTR	143.24±3.18[a]	147.08 ±3.04[a]	124.40±3.69[a]	137.58±3.63[a]
BME	150.23±3.70[a]	159.12±3.75[a]	135.67±3.30[a]	147.12±4.52[a]
ROT	40.19±3.40[b]	47.18±4.75[b]	69.11±3.70[b]	45.23±3.62[b]
BME+ROT	96.52±3.45[c]	117.54±3.40[c]	91.68±4.16[c]	102.95±3.56[c]
***Complex I-III*[2]**				
CTR	76.52±2.35[a]	77.10±3.45[a]	63.35±3.12[a]	78.34±3.10[a]
BME	77.80±2.07[a]	78.02±2.70[a]	69.15±3.03[a]	80.30±4.01[a]
ROT	54.12±4.15[b]	60.71±3.45[b]	45.88±3.90[b]	41.89±4.21[b]
BME+ROT	70.39±3.14[c]	71.02±3.55[c]	60.19±2.37[c]	76.98±2.14[c]

Values are mean ±SD (n=6).

Data analyzed by one way ANOVA ($P<0.05$) to completely randomized design with replicates. Means followed by different letters differ significantly according to DMRT

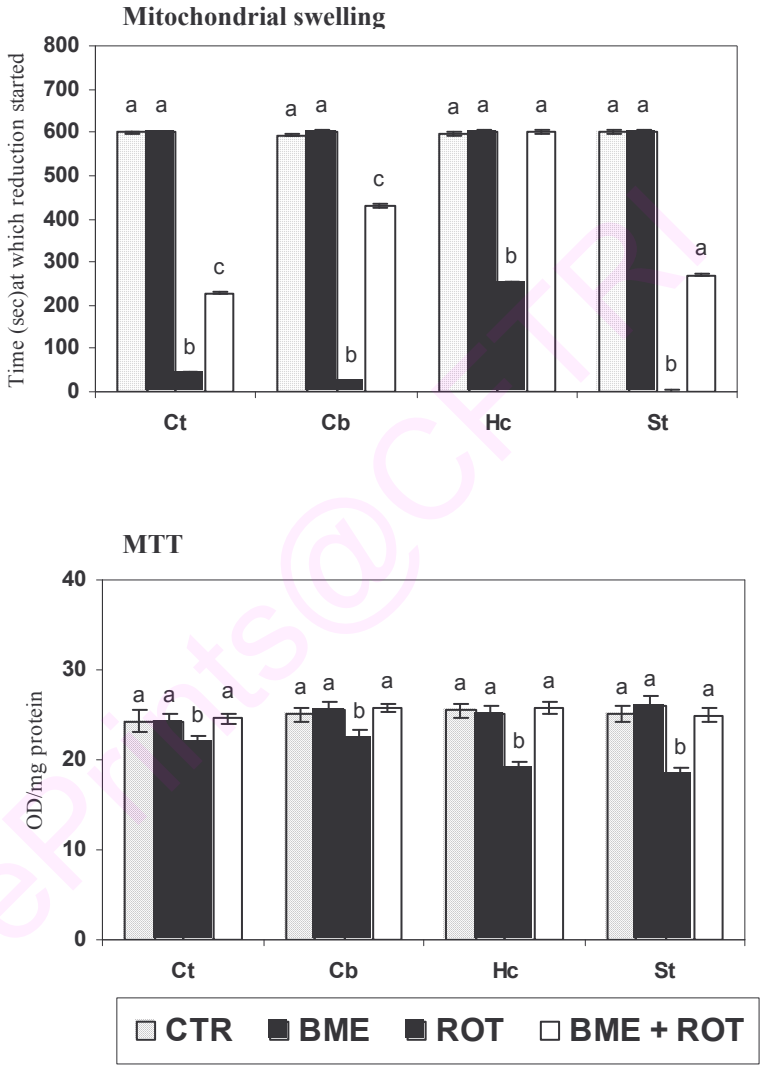
BME -*Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d); ROT-Rotenone(1mg/kgbw/d for 7d)

Complex I: NADH-ubiquinone oxidoreductase; Complex I-III: NADH-cytochrome C oxidoreductase

1-η moles NADH/min/mg protein; 2-η moles /min/mg protein

Fig: 3.24

Modulatory effect of BM extract on Rotenone-induced mitochondrial swelling and alterations in MTT reduction in brain regions of prepubertal male mice.

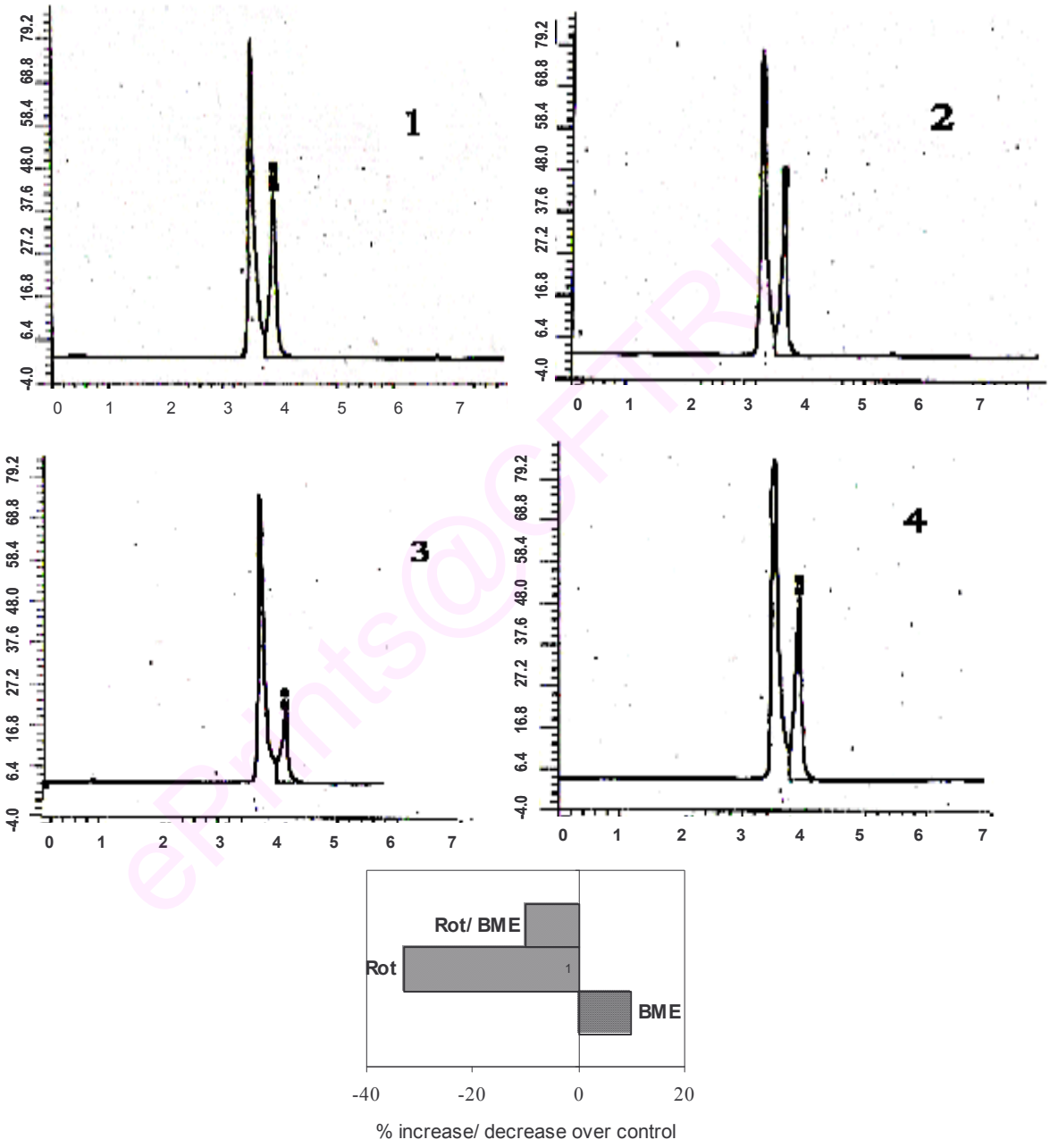


Values are mean ±SD (n=6).
Data analyzed by one way ANOVA (P<0.05) to completely randomized design with replicates.
Means followed by different letters differ significantly according to DMRT

BME.*Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 7d); ROT-Rotenone (1mg/kgbw/d for 7d)

Fig: 3.25

Modulatory effect of BM extract on Rotenone-induced alterations in dopamine levels in striatum of prepubertal male mice.



BME.*Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 7d); Rot-Rotenone (1mg/kgbw/d for 7d).
1-Control (Saline injected group) 2-Bacopa monnieri ethanolic extract (BME) group;
3-Rotenone group; 4-BME+ Rotenone group.

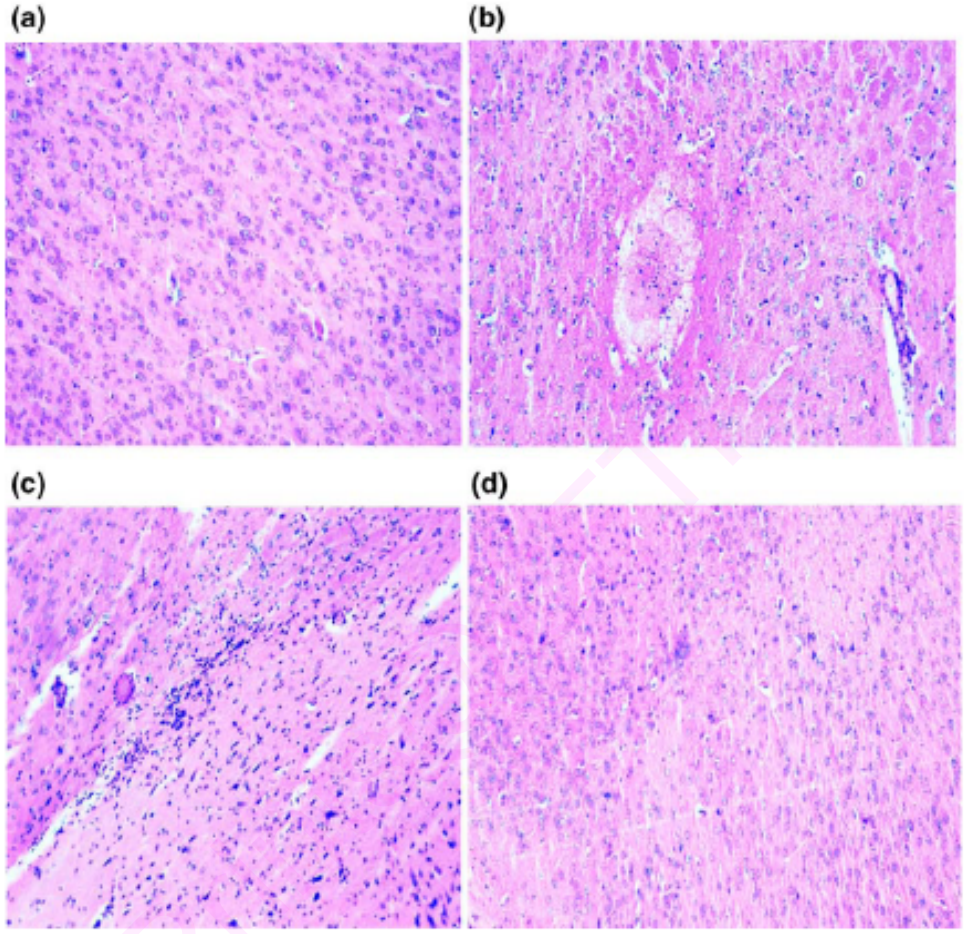


Plate 1: Photomicrograph of histopathological findings (X40)
Photomicrographs (a,b,c,d): Histopathological findings (x40) demonstrating striatal section of (a) Control mice (b) 3-NPA treated mice (c) 3-NPA + BM extract (d) BM extract alone.

Table.3.14a

Modulatory effect of BM extract on Rotenone- induced motor dysfunctions as determined by pole test* in prepubertal male mice.

Groups	Baseline$	Time taken in seconds						
		Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	6.66± 0.15	5.33± 0.15	5.33± 0.15	4.67± 0.10	5.0± 0.15	4.66± 0.13	4.33± 0.14	3.83± 0.15
Rot $_1$	6.33± 0.15	6.0± 0.25	8.17± 0.11*	8.0± 0.22*	8.83± 0.35*	9.5± 0.35*	15.4± 0.25*	7.75± 0.25*
Rot_1/BME	6.16± 0.15	5.33± 0.12	6.50± 0.15	5.5± 0.15	5.5± 0.25	5.17± 0.15*	5.01± 0.35*	4.66± 0.22*
Rot $_2$	6.25± 0.12	8.33± 0.19	¶					
Rot_2/BME	6.63± 0.09	6.66± 0.21	10.17± 0.25*	8.83± 0.28*	10.0± 0.45*	13.0± 0.35*	14.0± 0.15*	9.0± 0.21*

Table.3.14b

Modulatory effect of BME on Rotenone induced motor dysfunctions as determined by pole test* (T-turn) in prepubertal male mice.

Groups	Baseline$	Time taken in seconds						
		Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	1.83± 0.09	1.17± 0.13	1.17± 0.07	1.17± 0.06	1.17± 0.03	1.17± 0.07	1.17± 0.09	1.17± 0.07
Rot $_1$	1.9± 0.06	1.33± 0.05	1.833± 0.08*	1.67± 0.09*	1.67± 0.1*	1.67± 0.11*	2.0± 0.10*	3.0± 0.07*
Rot_1/BME	1.87± 0.07	1.83± 0.07	1.50± 0.10*	1.33± 0.12*	1.17± 0.13*	1.17± 0.12*	1.17± 0.15*	1.17± 0.16*
Rot $_2$	1.86± 0.08	1.93± 0.11*	¶				-	-
Rot_2/BME	1.87± 0.05	2.0± 0.12	2.16± 0.11*	2.0± 0.08*	2.0± 0.09*	2.0± 0.010*	1.87± 0.1*	2.33± 0.12*

Values are ±SD (n=12), data pooled from two independent experiments.
Data is analyzed by one way ANOVA (* P<0.05)

BME.*Bacopa monnieri* ethanolic extract (5mg/kgbw/d for 7d)
Rot_1-Rotenone, 1mg/kg bw/7days; Rot $_2$-Rotenone, 2mg/kg bw/7days

¶ - 100% mortality occurred on day 2 of post administration of rotenone
Pole test: T-total (Total time taken in seconds by the animal to reach ground)
T-turn: Time in seconds, taken to take a U- turn in the pole facing ground.
$ - day zero of the experiment

Table.3.15

Modulatory effect of BM extract on Rotenone induced motor dysfunctions as determined by rota rod * test in prepubertal mice.

Groups	Baseline$	Time taken in seconds						
		Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	120	120	120	120	120	120	120	120
Rot $_1$	120	100.3± 1.20	83± 1.45*	99± 1.56*	65.3± 2.34*	41.3± 1.34*	55.6± 2.12*	31± 1.22*
Rot$_1$/BM	120	115.5± 1.25	112± 1.5	113.0± 1.25	98.7± 1.20*	104.2± 2.05*	109.7± 1.45*	110± 1.12*
Rot $_2$	120	50 ± 1.10	30.25± 2.5*	¶				
Rot$_2$/BM	120	107.8± 1.67	95.17± 1.25*	55.7± 1.15*	71.8± 1.30*	24.7± 1.25*	20± 0.77*	10± 1.25*

The values are mean ±SD (n=6).Data analyzed by one way ANOVA, (* P<0.05).
BME- *Bacopa monnieri* ethanolic extract (5mg/kgbw/ d for 7d); Rot$_1$-Rotenone,1mg/kg bw/d for 7d);
Rot $_2$-Rotenone, 2mg/kg bw/d for 7d

¶ - 100% mortality occurred on day 2 of post administration of rotenone

* Rota rod: Time in seconds for which the animal was over the rotating rod
$ - day zero of the experiment

Table.3.16 a

Modulatory effect of BME on Rotenone induced motor dysfunctions as determined by stride length measurements (between fore limbs (cm) in prepubertal male mice

Groups	Stride length in centimeters							
	Baseline$	Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	4.83± 0.07	4.85± 0.10	4.87± 0.07	4.87± 0.06	4.87± 0.05	4.89± 0.08	4.87± 0.04	4.86± 0.04
Rot $_1$	4.89± 0.05	4.83± 0.10	4.88± 0.09	4.90± 0.06	4.98± 0.07	5.05± 0.05*	5.13± 0.05*	5.14± 0.07*
Rot$_1$/BM	4.86± 0.08	4.85± 0.08	4.86± 0.05	4.87± 0.05	4.86± 0.06	4.88± 0.05	4.87± 0.06	4.88± 0.10
Rot $_2$	4.90± 0.10	4.85± 0.07	5.09± 0.08	¶				
Rot$_2$/BM	4.85± 0.09	4.85± 0.08	4.86± 0.07	4.87± 0.08	4.87± 0.09	4.87± 0.10	4.86± 0.07	4.88± 0.08

The values are ±SD (n=6).Data is analyzed by one way ANOVA (* P<0.05)
BME-*Bacopa monnieri* ethanolic extract (5mg/kg bw/d for 7 d); Rot$_1$-Rotenone,1mg/kg bw/d for 7 d;
¶ - 100% mortality occurred on day 2 of post administration of rotenone

Rot $_2$-Rotenone, 2mg/kg bw/ d for 7d
$ - day zero of the experiment

Table.3.18b

Modulatory effect of BM extract on Rotenone induced motor dysfunctions as determined by stride length measurements (between hind limbs (cm) in prepubertal male mice.

Groups	Stride length in centimeters							
	Baseline[$]	Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	4.50± 0.06	4.52± 0.04	4.51± 0.07	4.50± 0.03	4.49± 0.07	4.50± 0.05	4.51± 0.07	4.50± 0.05
Rot 1	4.45± 0.08	4.48± 0.05	4.42± 0.05	4.39± 0.06	4.38± 0.05	4.32± 0.04*	4.32± 0.07*	4.31± 0.04*
Rot1/BM	4.45± 0.06	4.50± 0.08	4.50± 0.03	4.49± 0.04	4.47± 0.04	4.48± 0.03	4.48± 0.04	4.47± 0.07
Rot 2	4.47± 0.05	4.49± 0.06	4.32± 0.04	¶				
Rot2/BM	4.50± 0.07	4.49± 0.04	4.48± 0.05	4.48± 0.03	4.48± 0.03	4.47± 0.07	4.47± 0.05	4.46± 0.03

Table.3.18c

Modulatory effect of BM extract on Rotenone induced motor dysfunctions as determined by stride length measurements (between fore limbs and hind limbs (cm) in prepubertal male mice

Group	Baseline[$]	Stride length in centimeters						
		Measured on Days (post administration of rotenone)						
		1	2	3	4	5	6	7
CTR	0.335± 0.005	0.340± 0.007	0.340± 0.009	0.341± 0.005	0.339± 0.004	0.340± 0.005	0.339± 0.006	0.340± 0.004
Rot 1	0.340± 0.004	0.345± 0.005	0.360± 0.004	0.390± 0.006	0.398± 0.004	0.410± 0.006*	0.409± 0.007*	0.411± 0.005*
Rot 2	0.337± 0.007	0.336± 0.008	0.379± 0.007	--	--	--	--	--
Rot1/BM	0.338± 0.005	0.340± 0.009	0.341± 0.006	0.338± 0.007	0.339± 0.004	0.339± 0.006	0.339± 0.008	0.340± 0.003
Rot2/BM	0.337± 0.005	0.342± 0.004	0.340± 0.005	0.341± 0.004	0.340± 0.005	0.338± 0.007	0.338± 0.008	0.339± 0.008

The values are mean ±SD (n=6). Data analyzed by one way ANOVA (* P<0.05)

BME.*Bacopa monnieri* ethanolic extract (5mg/kgbw/7days)

¶ - 100% mortality occurred on day 2 of post administration of rotenone

Rot_1-Rotenone,1mg/kg bw/7 days; Rot_2-Rotenone, 2mg/kg bw/7 days

$ - day zero of the experiment

5.0 DISCUSSION

Bacopa monnieri (BM) is a medicinal herb, found throughout the Indian subcontinent in wet, damp and marshy areas (Kapoor, 1990). It is used in the Ayurvedic system of medicine for the treatment of anxiety and in improving intellect and memory (Singh and Dhawan, 1997). It is also claimed to be useful in the treatment of cardiac, respiratory and neuropharmacological (Russo, 2005) disorders like insomnia, insanity, depression, psychosis, epilepsy and stress. It is reported to possess anti-inflammatory, analgesic, antipyretic, sedative, free radical scavenging and anti-lipid peroxidative activities (Anbarasi et al., 2005). The pharmacological properties of *B. monnieri* have been attributed mainly due to the presence of characteristic saponins called as "bacosides." (Kishore and Singh, 2005). Despite the widespread usage of BM, data on the spectrum of neuroprotective effects in prepubertal mice are scarce. Hence, experiments were designed: (i) To assess the potential of BM leaf powder to modulate the endogenous oxidative markers in mitochondria/ cytosol of brain regions of mice (ii) To assess the antioxidant potency of BM (ethanolic extract) in chemical/ biological systems *in vitro,* (iii) To enumerate the prophylactic efficacy of BM extract against the neurotoxicant (3-NPA, Rotenone) - induced early oxidative stress and mitochondrial dysfunctions.

Dietary effects of Bacopa monnieri: Accumulation of free radicals, and consequent neurodegeneration in specific brain areas, has been proposed as the causal factor in AD, PD and other neurodegenerative diseases as well as aging (Glover and Sandler, 1993). This accumulation of oxidative free radicals is due to defective antioxidant defense mechanisms resulting from decreased function of the free radical scavenging enzymes (Harman, 1991). Potential therapy in these neurodegenerative conditions should, therefore, include agents capable of augmenting antioxidant defense systems (Maxwell, 1995). As such, an effective antioxidant agent should be capable of augmenting intracellular concentrations of antioxidant enzymes and reduce lipid peroxidation (Halliwell and Gutteridge, 1989). Further many lines of evidence suggest that mitochondria have a central role in ageing-related NDD (Petrozzi et al., 2007). In the present study, BM significantly reduced the levels of all measured oxidative markers (LPO, ROS and HP) in both mitochondria and cytosol of brain regions of prepubertal mice. This observation of lowered oxidative markers suggests the neuroprotective ability of BM even under normal conditions. Protein carbonyls, whose formation is considered a detectable marker of protein oxidation, are increased in AD and the resulting chemical modifications appear to be

involved in cellular metabolism. Oxidative damage can lead to loss in specific protein function and oxidized proteins are more prone to degradation by proteases (Stadtman, 1990). Hence a decrease in basal levels of protein oxidation proves to be a significant characteristic feature of BM which can be exploited further in therapeutics.

Further the oxidative damage to proteins is reflected by a decrease in the levels of protein thiols and free radicals are known to cause oxidation of thiol groups (Takenaka et al., 1991). It is reported that a decrease in GSH concentration may precede impairments of oxidative phosphorylation in PD (Dexter, 1994). Interestingly, depletion of mitochondrial GSH, but not cytosolic GSH in PC-12 cells resulted in the generation of ROS and inhibition of oxidative phosphorylation (Seyfield, 1999). In the present model significant increase in the levels of GSH, total thiols and non protein thiols in both mitochondria and cytosol of brain regions of mice fed BM is a clear suggestive of the potential of BM to enhance thiol antioxidants in brain.

To the best of our knowledge, there have been no studies which describe the effect of BM on antioxidant defenses in brain regions of prepubertal rodents. In the present study, BM significantly enhanced the activity levels of SOD, CAT and GSH-PX in all the brain regions. Earlier, sub-chronic administration of BM enhanced the oxidative free radical scavenging enzymes *viz.,* SOD, CAT and GSH-Px in brain regions of adult rats (Bhattacharya et al., 2000). The natural cellular antioxidant enzymes include SOD, which removes superoxide radicals by speeding their dismutation, CAT, a haeme enzyme which removes hydrogen peroxide and GSH-Px, a selenium-containing enzyme which removes hydrogen peroxide and other peroxides (Halliwell and Gutteridge, 1989). The radical scavenging activity of SOD is effective only when it is followed by actions of CAT and GSH-Px, since SOD generates hydrogen peroxide as a metabolite, which is more toxic than oxygen radicals and requires to be scavenged by CAT and/or GSH-Px (Harman, 1991). Interestingly, BM feeding also caused significant elevation in the activity of mitochondrial enzymes *viz.,* malate dehydrogenase and thioredoxin reductase which is indicative of the redox modulation and increased mitochondrial functions. Collectively the principle effects of BM *viz.,* enhanced activity of cytosolic antioxidant enzymes, mitochondrial enzymes coupled with the decrease in oxidative markers are likely to prove very useful to mitigate oxidative stress mediated disease conditions.

In the present study, dietary BM casused significant reduction in AChE activity in brain regions depicting the specific effect of BM on cholinergic systems. It is well-established that the cholinergic neurons are involved in several neuropsychic functions such as learning, memory, sleep etc. and acetylcholine (ACh) may play a vast role in modulating these functions (Mohapel et al., 2005). The central cholinergic deficit is strongly associated with many NDD such as AD and PD (Oda, 1999). Evidence of autism due to dysfunction of the cholinergic system has recently been reported (Lam et al., 2006). Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) are specific cholinergic marker proteins for the functional state of cholinergic neurons, both of which can play a key role in the maintenance of ACh levels at the cholinergic neurons (Eckenstein and Sofroniew, 1983). Inhibition of AChE by cholinesterase inhibitors, such as donepezil and rivastigmine, causes an increase of extracellular ACh levels in the brain. Treatment of AD patients with cholinesterase inhibitors causes symptomatic benefit and seems to delay disease progression for 6–12 months (Aarsland et al. 2004). Although speculative, BM could be exploited as a natural adjuvant therapeutic against AD.

Collectively our salient findings *viz..,* decreased basal oxidative markers, increased thiol related antioxidant molecules and antioxidant enzymes clearly depicts the potential of BM as a therapeutic antioxidant. Further, the present investigation may help in explaining, at least in part, the mechanism of nootropic action of BM demonstrated earlier both under experimental and clinical situations. More importantly, these first evidences in prepubertal mice clearly indicate the beneficial effects of BM and also provide the basis of memory enhancement reported in children.

Prophylactic efficacy of Bacopa monnieri: Currently no effective treatment exists for HD despite intense efforts to develop alternative therapeutic strategies. However, 3-NPA models provide valuable tools to evaluate neuroprotective strategies since they involve marked degeneration of striatum (Brouillet et al., 2005). So far, these models have allowed for the assessment of several neuroprotective approaches such as inhibition of glutamate signaling, supplementation with energetic substrates, inhibition of proteases and gene transfer of neurotrophic factors (Excartin, 2007). Nutraceutical antioxidants are dietary supplements that can exert positive pharmacological effects on specific human diseases by neutralizing the negative effects of ROS. Based on the evidences obtained in the dietary study, *ex vivo* experiments and more importantly data from in vitro chemical /biological systems, we have examined, whether an ethanolic extract of BM would render significant protection against 3-NPA

induced neuronal dysfunctions. The criteria for selection of 3-NPA as a model neurotoxicant has been adequately explained earlier (Chapter 1).

ROS are produced in the course of normal metabolism and they serve important physiological functions. However, because of their high reactivity, accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative degradation of critical molecules, such as the DNA, proteins, and lipids. Although cells possess an intricate network of defense mechanisms to neutralize excess ROS and reduce oxidative stress, some tissues (especially the brain) are much more vulnerable to oxidative stress because of their elevated consumption of oxygen and the consequent generation of large amounts of ROS. For the same reason, the mitochondrial DNA of brain cells is highly susceptible to structural alterations resulting in mitochondrial dysfunction. Several lines of evidence strongly suggest that these effects of ROS may be etiologically related to a number of NDD. In the present study, 3-NPA at the administered dosage resulted in significant increase in oxidative markers in both mitochondria and cytosol (in terms of LPO, ROS and HP levels) suggesting induction of early oxidative stress. BM prophylaxis resulted in marked protection against these perturbations clearly demonstrating its potential to offset 3-NPA-induced oxidative stress.

Oxidative alterations of proteins by ROS have been implicated in the progression of aging and age-related neurodegenerative disorders such as AD. Protein carbonyls, a marker of protein oxidation, are increased in AD brain, indicating that oxidative modification of proteins is relevant in AD. Oxidative damage can lead to several events such as loss in specific protein function, abnormal protein clearance, depletion of the cellular redox-balance and interference with the cell cycle and, ultimately, to neuronal death (Castegna et al., 2002). 3-NPA at the administered dose resulted significant increase in protein carbonyls in both mitochondria and cytosol of striatum and other brain regions. Mice which received BM prophylaxis showed no significant increase in protein carbonyl content indicating its neuroprotective efficacy.

Further in the current model, 3-NPA caused a significant decrease in GSH, total thiols and non protein thiols, while GSSG levels increased marginally. Interestingly, BM alone treated mice showed significant increase in GSH, thiol and non protein thiols in mitochondria and cytosol of brain regions. Earlier evidences indicate that depletion of GSH by BSO produces enlargement and degeneration of mitochondria in neonatal rats (Jain et al., 1991) and a decrease in the

activity of complexes I and IV in weaning rats (Heales et al., 1995) arguing for a sequence of primary disturbance of glutathione homeostasis and secondary inhibition of oxidative phosphorylation in the pathogenesis of PD. If alterations in glutathione metabolism play an important role in the pathogenesis of the neurodegenerative diseases, treatments that lead to enhanced synthesis of GSH or that inhibits its degradation may result in a slowing of disease progression. Because GSH itself penetrates the blood-brain barrier only poorly and cannot be taken up by neurons directly, treatments with GSH monoethyl ester or glutathione precursors or other glutathione analog have been used in patients or animal models. Hence, BM extract having the potential to enhance GSH/thiol related antioxidant molecules can be better exploited as a natural alternative therapeutic agent.

In the present study, 3-NPA administration in prepubertal mice resulted in marked increase in free iron levels in brain regions, while BM alone has resulted in significant reduction in free iron levels. Organisms overloaded by iron (eg: hemochromatosis, b-thalassemia, hemodialysis) contain higher amounts of “free available iron” and this can have deleterious effects. “Free-iron” is transported into an intermediate, labile iron pool (LIP), which represents a steady state exchangeable and readily chelatable iron compartment (Kakhlon and Cabantchik, under 3-NPA exposure (Guo et al., 2004). Interestingly, BM prophylaxis resulted in a marked reduction of 3-NPA mediated elevation in free Iron levels suggesting its iron chelating property *in vivo*. This observation is highly consistent with our previous *in vitro* findings. Hence we hypothesize that chelating potential may represent one of main mechanism/s by which BM renders neuroprotection against neurotoxicant-induced oxidative damage. Further the alterations in non-enzymatic antioxidant vitamin C levels and its modulation by BM extract is also indicative of the wide spectrum of protective propensity of BM.

BM prophylaxis also caused significant enhancement in the activity of antioxidant enzymes in both mitochondria and cytosol of brain regions of mice. The normalization of antioxidant enzymes among 3-NPA exposed BM prophylaxis group indicates the efficacy of BM to mitigate oxidative stress. Further there was a reduction in activities of TRR in mitochondria of brain regions of mice administered 3-NPA. The intracellular “redox homeostasis” or “redox buffering” capacity is substantiated primarily by GSH and thioredoxin (TRX) (Schafer and Buettner, 2001). The high ratios of reduced to oxidized GSH and TRX are maintained by the

activity of GSH reductase and TRX reductase, respectively. Both of these "redox buffering" thiol systems counteract intracellular oxidative stress; in addition to antioxidant functioning and scavenging free radicals in the cell, GSH and TRX are involved in cell signaling process (Droge, 2002).

3-NPA administration caused a significant increase in LDH activity in striatum and other brain regions of prepubertal mice suggesting increased membrane damage. Although no significant alterations were observed in BM alone group, BM prophylaxis markedly offset 3-NPA induced membrane damage. The decreases in TCA cycle enzymes following 3-NPA administration was also offset by BM treatment suggesting its specific protective effect in mitochondrial milieu. Although there were no significant changes in BM *per se* group, the activity of ETC complex enzymes (except Complex II) were significantly protected. However, only marginal protection was evident with complex II activity indicating that BM prophylaxis may not involve restoration of complex II activity, and is most probably related to the up regulation of mitochondrial antioxidant defense mechanisms.

Further the activity of Na^+ K^+ ATPase activity, (a membrane bound enzyme) was significantly reduced among 3-NPA administered mice. BM prophylaxis resulted in almost normalization of this membrane bound enzyme. Na^+, K^+ ATPase, a sulfhydryl-containing enzyme embedded in the cell membrane and responsible for the active transport of sodium and potassium ions in the nervous system could be sensitive to oxidizing agents (Folmer et al., 2004). This process regulates the cellular Na^+/K^+ concentrations and hence their gradients across the plasma membrane, which are required for vital functions such as membrane co-transports, cell volume regulation and membrane excitability (Doucet, 1988). This dimeric enzyme exists in several isoforms in brain and consumes the greater part of available ATP (Bertorello and Kats, 1995). Its inactivation leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events like excitotoxicity (Beal et al., 1993). Taking into account that maintenance of Na^+ K^+ATP-ase activity is critical for normal brain function and the reduction of its activity is related to selective neuronal damage (Lee, 1993), the protective effect of BM on this enzyme is highly relevant for neuroprotection.

In the present study, 3-NPA administration resulted in significant decrease in mPT, while BM prophylaxis nearly normalized the mPT among 3-NPA treated mice. Mitochondria, in addition to their role as energy producers, are also of fundamental importance in the apoptotic process

that depends on the release of specific factors localized in the mitochondrial inter-membrane space. Hence conditions controlling the permeability properties of mitochondrial membranes are critical for apoptosis to occur. The mitochondrial permeability transition can depend on a thiol–disulfide transition and the oxidation of pyridine nucleotides determines a significant decrease of mitochondrial thiols accompanied by a large swelling. Therefore, any alterations of the thiol-dependent systems linked to glutathione or thioredoxin can affect the permeability properties of the mitochondrial membranes and, consequently the apoptotic or necrotic process (Rigobello et al., 2005). Further under conditions of increased Ca^{2+} loading, especially when accompanied by oxidative stress and a fall in adenine nucleotides, mitochondria undergo permeability transition (mPT). Hence protection of mPT by BM prophylaxis indicates a total restoration of mitochondrial function to normalcy which is highly beneficial in neurodegenerative conditions. Interestingly, MTT reduction, which decreased with 3-NPA administration, was also brought to normalcy by BM prophylaxis. Collectively, these results suggest the potential of BM to significantly offset specific mitochondrial functions which may be due to its mitochondria targeted antioxidant activity.

BM treatment resulted in a significant decrease in AChE activity, while BChE activity was elevated in all the brain regions. BM prophylaxis markedly offset 3-NPA induced alterations in the activity of cholinergic enzymes. During the past 10 years, treatment of patients with AD has been based largely on a strategy of enhancing ACh-mediated transmission. Symptomatic benefits (cognitive, functional and behavioral) have been observed in multiple clinical trials with agents known to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which catalyze the breakdown of ACh (Francis et al., 2005). It is quite likely that BM extract may prove useful in the cholinergic therapies related to AD.

Neuroprotective effect of BM pretreatment against Rotenone induced oxidative dysfunctions: Among various animal models of PD, the rotenone model has recently drawn particular attention for two reasons: i) unlike other models it reproduces most of the movement disorder symptoms and the histopathological features of PD including Lewy bodies (ii) rotenone and other pesticides are powerful inhibitors of mitochondrial respiration and recent epidemiological studies suggest involvement of these toxic compounds in the higher incidence of sporadic Parkinsonism among the population of rural areas (Vanacore et al., 2002). The development of an effective neuroprotective therapy for PD is clearly a formidable task made even more difficult due to the variety of etiological and pathogenic factors that contribute to

dysfunction and death of substantia nigra (SN) dopamine (DA) neurons (Schapira, 2002). However, there is no single initiating factor for DA cell death in idiopathic PD and no consensus as to the mechanism(s) contributing to the final degeneration of DA neurons in this disease (Jellinger, 2000). Although studied extensively in adult animal models, the effect of rotenone on prepubertal mice brain is less investigated. The specific reasons for the selection of this model have been described in detail earlier (Chapter 1).

In the present model, rotenone at the administered dose resulted in significant increase in MDA, ROS and HP levels in both mitochondria and cytosol of brain regions of mice. These observations are consistent with recent findings in adult mice (Mao et al., 2007). ROS which are potentially very damaging to cells, lead to oxidation of essential cellular constituents including proteins, lipids, and DNA. The high content of degraded lipid products such as MDA may lead to further damage *via* oxidation of proteins and lipids, inhibition of mitochondrial transcription and opening of the mitochondrial permeability transition pore (Cassarino and Bennett, 1999). Rotenone could stimulate the release of H_2O_2 (Tada-Oikawa et al., 2003) which is a major source of ROS. As evidenced from the present study, BM pretreatment markedly offset rotenone induced oxidative dysfunctions. Oxidative damage to proteins results in the formation of protein carbonyls and marked increase in protein carbonyls has been observed in both mitochondria and cytosol of brain regions of mice. There have been reports of increased protein carbonyls on exposure to rotenone *in vitro* (Sherer et al., 2003). BM treatment resulted in complete protection against oxidative alterations in both mitochondria and cytosol of brain regions in all the brain regions.

In the present rotenone model, depletion of GSH levels was evident both in the mitochondrial and cytosolic compartments of brain regions. Diminution of GSH induced by rotenone is indicative of concomitant increase in ROS and early oxidative damage. BM treatment resulted in marked protection against GSH depletion, strongly campaigning as a possible therapeutic for oxidative stress mediated neuronal dysfunctions. In general, enzymatic defense against ROS is performed mainly by antioxidant enzymes such a CAT, GSH-Px, GR and SOD. In the present study, the depletion in GSH in rotenone mice was accompanied by decreased Catalase, GSH-Px and GR, while SOD activity was decreased in mitochondria and increased in cytosol. Further, there was an increase in GST activity in cytosol/ mitochondria of brain regions. GST are a ubiquitous group of detoxification enzymes involved in the metabolism of toxins and a glutathione isoform GST 1 expressed in the blood brain barrier may influence response to

neurotoxins which may explain the susceptibility of certain individuals to pesticides inducing Parkinsonian like symptoms (Schulz et al., 2000). Thus a disturbance in endogenous balance has occurred, while no such significant alterations were observed among BM treated mice suggesting the potential role of BM as a pharmacological tool in the treatment against PD.

In the present study, there was clear increase in LDH activity in all the brain regions among rotenone treated mice. It was reported that tissue acidosis enhances the levels of free iron through mobilization of iron from proteins. Increased activity of LDH is indicative of tissue acidosis and correlates with increased lactate level in rotenone administered animals (Mao et al., 2007). Moreover, increased LDH activity is indicative of mitochondrial dysfunction and impaired energy metabolism. Further, there was a decrease in the activity of mitochondrial TCA cycle enzymes (eg., MDH) which is indicative of altered mitochondrial dysfunctions. The redox enzymes like TRR were also decreased on rotenone administration indicating possible alteration in redox potential of cells particularly in the mitochondria. BM treatment resulted in marked protection against these critical alterations clearly suggesting the neuroprotective effects of BM against mitochondrial dysfunctions.

Further, most importantly, there was a decrease in the activity of ETC complex I in all the brain regions of rotenone exposed mice, while BM treatment resulted in significant protection against these perturbations. It has been suggested that inhibition of electron flow at Complexes I and II results from the damages to 4Fe-4S clusters. As a result, the intraprotein sites of $O_2^{\cdot}$ generation shift to more proximal sites, presumably flavines, and thus generation of ROS becomes independent of the mitochondrial metabolic state and result in oxidative damage. These changes lead to a higher probability of permeability transition of the brain mitochondria when challenged with Ca^{2+} and thus excitotoxic cell death (Panov et al., 2005). In this context the protection against complex I inhibition offered by BM extract opens windows for therapeutic interventions in PD and merits further investigations.

Rotenone has been shown to lead to selective dopaminergic cell death *in vivo*. In this context, earlier works reported selective nigrostriatal dopaminergic degeneration in rats infused intravenously or subcutaneously with rotenone, although rotenone also induced degeneration of non-dopaminergic neurons in both the basal ganglia and the brainstem (Betarbet et al., 2000; Sherer et al., 2005; Hirsch et al., 2003). On the other hand, in *in vitro* models, it was reported that

dopaminergic neurons were more sensitive to rotenone-induced toxicity than other neuronal cells and glial cells (Moon et al., 2005), but non-dopaminergic neurons were reduced by rotenone in primary mesencephalic culture (Sakka et al., 2003). Based on these evidences on striatal dopaminergic neuron loss, we quantified the dopamine levels in the striatal regions and observed significant (33%) decrease among rotenone administered mice. Interestingly BM treatment offered nearly 70% protection against this damage clearly suggesting the specific neuroprotective effect of BM against dopaminergic neuronal loss. Interestingly, BM treated mice showed considerable neuroprotection against rotenone induced motor dysfunctions determined during the course of the experiment .

There are three major mitochondrial functions that determine the performance and fate of the cell. These are: 1) oxidative phosphorylation that produces ATP for almost all cellular functions; 2) mitochondrial Ca $^{2+}$_dependent permeability transition (mPT) that may initiate apoptotic or necrotic death of a cell; and 3) generation of reactive oxygen species (ROS), a byproduct of normal aerobic metabolism. Compelling evidence exist that increased generation of ROS is responsible for the dysfunction and sensitization of the cell to death signals. Thus rotenone intoxication may cause damage to each of these major mitochondrial functions or all of them. To find methods of therapeutic intervention, understanding the early primary mechanisms of rotenone toxicity is a prerequisite(Panove et al., 2005). In the present study, since we have adopted a 7 day treatment schedule, we presume that various adaptive mechanisms have not yet shadowed the primary mechanisms of rotenone toxicity. Under these conditions, rotenone caused multiple mitochondrial dysfunctions including increased ROS, MDA, HP and protein carbonyls formation followed by decreased antioxidant enzymes, altered mitochondrial functions and cholinergic enzymes in all brain regions. More importantly, we established the potential of BM extract in ameliorating rotenone induced oxidative stress, mitochondrial dysfunctions and decreased dopamine levels. Because the mechanisms of cell death in PD are complex and involve multiple processes and may even differ across patients, screening potential neuroprotective agents for PD in more than one animal model and using multiple endpoints may better identify compounds with broad neuroprotective, neurorescue or neurorestorative capabilities that are likely to be effective in the clinic.

6.0 SUMMARY

1. Consumption of *B. monnieri* leaf powder in the diet (0.5 and1.0%) caused significant diminution in the levels of endogenous oxidative markers (MDA, ROS and HP levels) in both cytosol and mitochondria of different brain regions of prepubertal mice is suggestive of its ability to prevent peroxidation *in vivo.* Reduction in protein carbonyls levels in brain regions of BM mice indicated its potential to inhibit protein oxidation events.

2. *B. monnieri* fed mice exhibited enhanced GSH levels, total thiols and non-protein thiols in both cytosol and mitochondria of brain regions of mice and concomitant elevation in the activity of various enzymic antioxidant defenses indicating the potential of BM to augment the antioxidant machinery.

3. BM fed mice showed significant reduction in the activity of AChE in all brain regions indicating its ability to modulate cholinergic function.

4. *Results of the ex vivo* study clearly suggested that subsequent to BM consumption, the brain regions were more resistant to 3-NPA induced oxidative dysfunctions *in vitro*.

5. A standardized ethanolic extract of BM exhibited significant antioxidant activity in selected *in vitro* chemical systems and marked free radical scavenging activity in mitochondria, synaptosomes and slices under 3-NPA –induced oxidative stress *in vitro.*

6. BM extract prophylaxis markedly protected prepubertal mice against 3-NPA induced early oxidative stress in striatum and other brain regions. Further 3-NPA-induced depletion in GSH, thiols/non-protein thiols were also completely normalized by BM prophylaxis.

7. 3-NPA induced alterations in the activity of antioxidant enzymes striatum and other brain regions (cytosol and mitochondria) were significantly ameliorated by BM prophylaxis. Varying degree of protection in the activities of TCA cycle enzymes and thioredoxin reductase were also evident.
8. BM prophylaxis significantly attenuated 3-NPA induced elevations in the activity of LDH, ETC enzymes, iron levels and alterations in vitamin C levels. Further the perturbations in cholinergic enzymes AChE and BChE were also modulated.

9. BM prophylaxis significantly protected the 3-NPA induced mitochondrial dysfunctions such as reduction in mitochondrial respiration (MTT assay), increased mitochondrial swelling and decreased mitochondrial Na^+ K^+ activity in striatum and other brain regions.

10. BM extract treatment offered uniform and marked protection markedly protected prepubertal mice against rotenone-induced oxidative impairments in all brain regions of prepubertal mice. Further rotenone-induced depletion in GSH was completely normalized by BM treatment.

11. Rotenone- induced alterations in the activity of antioxidant enzymes striatum and other brain regions (cytosol and mitochondria) were significantly ameliorated by BM treatment. Varying degree of protection in the activities of TCA cycle enzymes and thioredoxin reductase were also evident.

12. BM extract treatment resulted in significant protection against increased activity of LDH and decreased ETC enzymes. Further significant ameliorative effect was also evident in the activities of cholinergic enzymes.

13. BM treatment resulted in amelioration of rotenone –induced reduced mitochondrial viability (MTT assay) and increased mitochondrial swelling in all brain regions indicating the mitochondria targeted protective effect of BM ethanolic extract.

14. BM treatment resulted in significant protection against rotenone –induced reduction in dopamine levels in striatum of prepubertal mice. Interestingly rotenone failed to induce any significant motor function deficits among mice given BM treatment which may be due to the normalization of dopamine levels in striatum. Histopathological examinations also confirmed the neuroprotective efficacy of BM extract against dopaminergic neurotoxicity.

1.0 INTRODUCTION

Dopaminergic (DA) neurons of the midbrain are the main source of dopamine in mammalian central nervous system and their loss is associated with one of the most prominent neurological disorders such as Parkinson's disease (Beal, 2003). Dopaminergic neurons are found in the harsh regions of brain, the substantia nigra pars compacta, which is dopamine rich and contain both redox available neuromelanin and a high iron content. Although their numbers are few, they play an important role in the control of multiple brain functions including voluntary movement and a broad array of behavioral processes such as mood, reward, addiction and stress. The selective degeneration of these dopaminergic neurons in the substantia nigra leads to PD, but the exact cause or this nigral cell loss is unknown (Chinta and Andersen, 2005). Dopaminergic neurons are particularly prone to oxidative stress due to their high rate of oxygen metabolism, low levels of antioxidants and high iron content (Halliwell, 1992).

Various cell lines including N27 cell lines have been employed to understand the mechanism/s related to neurodegenerative diseases and develop possible therapies. N27 (rat dopaminergic cell line 1RB3AN27 cells have been derived from embryonic rat mesencephalic neurons *via* SV40 large T antigen immortalization (Prasad et al., 1994; Adams et al., 2001). These cells possess all the physiological and biochemical properties of dopaminergic neurons and represent those cells that are lost during PD (Adams et al., 2001). N27 cells have been successfully tested for cell-transplantation in the rodent model of PD (Clarkson et al., 1998) and also have been tested in mitochondrial experiments associated with PD (Bharath and Andersen, 2005; Chinta and Andersen, 2006). Intact Complex I has been immunopurified from N27 mitochondria followed by successful mass spectrometric identification of all subunits suggesting the presence of healthy mitochondria (Schilling et al., 2005). Based on these features, N27 cell line has been considered as a superior cell model of PD compared to other dopaminergic cells such as rat adrenal pheochromocytoma (PC12) cell line (Vali et al., 2007).

The N27 cells expresses dopamine transporter (DAT) required for the uptake of MPP^+, tyrosine hydroxylase for dopamine synthesis and several other key features characteristic of dopamine neurons (Clarkson et al., 1998). N27 cell lines have been used to elucidate the mechanisms underlying GSH depletion in response to the Parkinsonian toxin, 1-Methyl-4-

phenylpyridinium (MPP+) (Drechsel et al, 2007) and for various studies related to Parkinson's disease (Chinta et al, 2006a, b; Bharath and Andersen, 2005). N27 cell line have also been used for studies on the pharmacological inhibition of neuronal NADPH oxidase against MPP+ induced oxidative stress and apoptosis (Anantharam et al, 2007) and to study the effect of curcumin on glutathione depletion (Jagatha et al., 2008). PC-12 cells have been used to study the effect of protocatechuic acid against MPP+ induced mitochondrial dysfunctions (Guan et al., 2006).

Accordingly, we have obtained mechanistic evidences regarding the protective effects of *Bacopa monnieri* against neurotoxicant induced oxidative stress and mitochondrial dysfunctions employing N27 cell lines. The model neurotoxicants *viz.,* 3-NPA and Rotenone were used for the studies. The neurotoxin 3-NPA is a Complex II inhibitor and is used as a toxin model to induce Huntington's disease. Oxidative stress has been suggested to play a role in 3-NPA toxicity; however, the underlying mechanism/s of oxidative damage are not fully understood (Schulz et al., 1996). The neurotoxin rotenone is a classical, high affinity inhibitor of complex I and is typically used to define the specific activity of the complex (Esposti, 1998). Because it is extremely lipophilic, it crosses biological membranes easily and independent of transporters and it gets into the brain very rapidly (Talpade et al., 2000). Several evidences (both *in vivo* and *in vitro*) suggest that this pesticide causes a delayed depletion of glutathione, which is mainly accompanied by oxidative damage to proteins and DNA (Sherer et al., 2002). It is believed that there is a site of electron leak upstream of the rotenone binding site in complex I. In the presence of rotenone, electrons may leak from the complex and then combine with molecular oxygen to form reactive oxygen species which in turn, reactive oxygen species attack proteins and DNA, causing specific modifications (Hensley et al., 1995)

The pharmacological properties of *Bacopa monnieri* has been attributed mainly to the presence of characteristic saponins called as 'bacosides' which are found to offer protective role in the synaptic functions of the nerves in hippocampus (Kishore and Singh, 2005). Ethanolic extract of *Bacopa monnieri* can alter components of oxidative stress cascade relevant to Alzheimer's disease neuropathology (Dhanasekaran et al., 2007). We explored the possible mechanism/s by which BM ethanolic extract abrogates 3-NPA and rotenone induced oxidative dysfunctions using N27 cell models.

2.0 OBJECTIVE

The primary focus of this investigation was to obtain mechanistic insights on the neuroprotective properties of *Bacopa monnieri* ethanolic extract. Since N27 cells are dopaminergic neurons, this study examined whether *Bacopa monnieri* could be a promising therapeutic agent for neurodegenerative diseases.

3.0 EXPERIMENTAL DESIGN

Cell lines and culture conditions

N27 (rat dopaminergic cell line 1RB3AN27 (N27) cells derived from embryonic rat mesencephalic neurons *via* SV40 large T antigen immortalization (Bharath and Andersen, 2005) were used. The cells were grown in RPMI medium 1640 containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were subcultured once a week *via* trypsin treatment.

Concentration of neurotoxicants and exposure

3-nitropropionic acid (3-NPA) was dissolved in saline (stock: 10mM) and suitable aliquots were added (2-10µl) to the cultures. For cytotoxicity studies the concentrations used ranged between 2-10mM. For studies to determine the effect of 3-NPA on oxidative markers and glutathione levels, concentrations of 1, 2 and 3 mM were used, while the highest concentration (3mM) was used for rest of the studies. Rotenone (Rot) was dissolved in DMSO: ethanol mixture (1:9, v/v) to obtain a concentration of 16mM. Suitable dilutions were made (100µM, 320µM) with ethanol and aliquots were added to the culture (2-10µl). DMSO concentration in the media was kept at 0.01% and parallel controls were run for the DMSO: ethanol mixture. Stock solutions were prepared fresh each time and diluted as per requirement.

Bacopa monnieri (BM) extract and exposure

Bacopa extract was dissolved in saline (1mg/ml) and suitable aliquots were added to cultures (2-10µl) after dilution to obtain the final concentrations (2-10µg/ml). In order to determine the non-lethal concentrations of BM extract, confluent cells were exposed (in triplicate) to varying concentrations (2-10µg/ml) of the extract for 24 hr and cell death was quantified by MTT assay.

Cytotoxicity investigations: determination of LC_{50} and LC_{75} concentrations

With an objective of determining the optimum dosage of the neurotoxicant, cells were grown in 96 well plates which were initially seeded with 5000 cells/well. 150µl of RPMI media was added to these cells and kept for 24 hours in a CO_2 incubator to achieve cell confluency. To the confluent cultures (in triplicate), various concentrations of neurotoxins (3-NPA, 1-10mM; and Rot, 2-20µM) was added and cultures were maintained for 24 hrs. Subsequently the incidence of cell death was assessed by an MTT assay (described in materials and methods). Based on percent lethality, LC_{50} and LC_{75} values were computed by following the graphical method of Wilcoxon (1945).

Modulation of cytotoxicity by BM extract

The modulatory potential of BM extract against neurotoxicant induced cell death was assessed by adopting two treatment regimes *viz.,* pretreatment and cotreatment. In the pretreatment regime, confluent cells (in 96 well plates) were exposed first to BM extract at various concentrations for 24hrs followed by exposure to either 3-NPA or rotenone (at LC_{50} and LC_{75}) for an additional 24 hrs. At the end of both treatment protocols, the modulatory potency of BM extract was assessed using two criteria viz., by MTT assay and LDH leakage into the media. In the co-treatment regime, confluent cells were exposed to BM extract and neurotoxicant simultaneously for 24 hrs only and the cell death was assessed by MTT assay.

Mechanistic studies to understand the neuroprotective property of BM extract

Effect of BM extract on neurotoxicant induced oxidative stress

In order to determine the effect of neurotoxicants on oxidative markers and the propensity of BM extract to protect against neurotoxicant–induced oxidative stress, only the pretreatment regime was followed. To the confluent cells (in triplicate), various concentrations of neurotoxins (3-NPA: 1, 2 and 3mM; Rotenone: 2, 4 and 6µM) and Bacopa extract (2, 4 and 6µg/ml) was added and maintained for another 24 hr. For modulatory studies, the higher concentration of 3-NPA (3mM) and rotenone (6µM) with BM extract at concentrations of 6µg/ml were employed. The supernatant of cell homogenates was used for ROS and HP assays as described under materials and methods.

Effect of BM extract on neurotoxicant induced perturbations on GSH and glutathione related enzymes in mitochondria

In order to determine the protective effect of BM extract on neurotoxicant-induced alterations in antioxidant status, the levels of GSH and GSSG in homogenates were measured. Further, the activity levels of glutathione related enzymes *viz.,* GPx, GST and GR was also determined. For these studies, the concentration of 3-NPA used was 3mM and rotenone - 6µM, while the BM extract concentration used was 6 µg/ml.

Modulatory effect on mitochondrial electron transport chain enzymes

Mitochondria isolated from cells were used for determining the effect of neurotoxicant on ETC complex as well to assess the propensity of BM extract to protect against neurotoxicant induced alterations in dopaminergic neurons. The activity of complex I and complex II-III were determined according to the methods described in materials and methods. For these studies, the concentration of 3-NPA used was 3mM and rotenone- 6µM, while the BM extract concentration used was 6µg/ml.

Determination of DNA damage: Single cell gel electrophoresis.

As a part of mechanistic study, comet assay was conducted to determine the extent of DNA damage in neurotoxicant exposed cells as well as to quantify the ameliorative effect of BM extract against single strand breaks and DNA damage. Comet assay was conducted as per the method described under materials and methods section. For this study, LC_{50} concentrations of 3-NPA and Rotenone were used, where as the concentration of BM extract used was 6µg/ml.

Alterations in Protein profile in homogenates and Mitochondria

In order to assess the effect of neurotoxicants on quantitative alterations in protein profile, whole cell homogenates and mitochondrial fractions were subjected to SDS-PAGE as per the methods described in materials and methods section. For homogenates, LC_{50} concentrations of 3-NPA and Rotenone were used where as the concentration of BM extract used was 6µg/ml. In case of mitochondria since LC_{50} concentrations resulted in loss of cells due to cell death to a large extent, lower concentrations of 3-NPA (3mM) and rotenone (6µM) were used, while the BM extract concentration used was 6µg/ml.

4.0 RESULTS

Cytotoxicity studies in N27 cells.

Cytotoxicity of Bacopa monnieri ethanolic (BME) extract

The cytotoxicity of BM extract was determined employing an MTT assay. The toxicity profile was a prerequisite for fixing the sub lethal concentrations to be used for further studies. On exposure to BM extract, there was no evidence of cell death up to a concentration of 6µg (Fig.4.1A). However, marginal but statistically significant (15-20%) cell death was evident at concentrations of 8 and 10µg. Hence further studies were conducted employing nonlethal concentration of 2, 4 and 6µg/ml.

Cytotoxicity of neurotoxicants: Determination of LC_{50} and LC_{75}

The cytotoxic response of N27 cells following exposure to varying concentrations of Rotenone (4-20µM) is depicted in Fig.4.1 B. Concentration -related lethality was observed among rotenone exposed cells and 50% lethality occurred at a concentration of 8 µM. The LC_{50} and LC_{75} values computed for rotenone were 8µM and 16µM respectively. The cytotoxic response of N27 cells following exposure to varying concentrations of 3-NPA (2-10mM) is depicted in Fig.4.1 C. Concentration-related lethality was observed among 3-NPA exposed cells and 50% lethality occurred at a concentration of 4mM. The LC_{50} and LC_{75} values computed for 3-NPA was 4mM and 6mM respectively.

Protective effects of Bacopa monnieri extract against neurotoxicant induced cell death

Modulation of 3-NPA mediated cytotoxicity

Two treatment protocols were followed to assess the modulatory effect of BM extract. The first protocol involved pretreatment of cells with BM extract (2, 4, 6µg) for 24 hrs followed by exposure to neurotoxin for additional 24hrs at concentrations equivalent to LC_{50} and LC_{75} values. Concentration-related protective effect was observed among BM treated cells against 3-NPA induced cell death as evidenced by the increased absorbance in MTT assay (Fig.4.2.A and B). The degree of protection conferred by BM extract varied between 10-35% against LC_{50} concentration while the percent protection ranged between 10-30% at LC_{75} concentration.

The second protocol followed was co-treatment of cells with BM extract (2, 4, 6µg) for 24 hrs with 3-NPA exposure at concentrations equivalent to LC_{50} and LC_{75} values. The percent

protection provided by BM extract was relatively higher against cell death induced by LC_{50} concentrations of 3-NPA (12-30%) compared to LC_{75} concentration of 3-NPA (8-20%) (Fig.4.3.and B).

Modulation of Rotenone induced cell death

In the pretreatment protocol, cells were exposed to BM extract (2, 4, 6µg) for 24 hrs followed by exposure to rotenone for additional 24hrs at concentrations equivalent to LC_{50} and LC_{75} values. Concentration-related protective effect was evident among BM treated cells against rotenone induced cell death as evidenced by the increased absorbance in MTT assay. The degree of protection conferred by BM extract varied between 6-36% against LC_{50} concentration, while the percent protection ranged between 10-20% at LC_{75} concentration (Fig.4.4.A and 4.4 B). However, no significant protection was observed at the lowest concentration of BM extract tested against rotenone induced cell death.

In the co-treatment protocol, cells were treated with BM extract (2, 4, 6µg) for 24 hrs and were simultaneously exposed to rotenone at concentrations equivalent to LC_{50} and LC_{75} values. Significant concentration-related protective effect was observed and the percent protection was 8-30% against LC_{50} concentration and 8-19% against LC_{75} concentration (Fig.4.5.A and B).

Ameliorative effect of BM extract on neurotoxin induced membrane damage

Only pretreatment protocol was followed for this study. Lactate dehydrogenase (LDH) released into the culture media was quantified to assess the plasma membrane damage induced by the neurotoxicants, 3-NPA and Rotenone (Fig.4.6 upper panel). In general there was a concentration-dependent increase in LDH leakage among 3-NPA (4mM, 6mM) treated cells. A marked increase (6- fold) in LDH leakage was observed at 4mM, while a more robust increase (8.7-fold) was evident at 6mM. On the other hand, pre-treatment with BM extract provided a concentration-related protection against 3-NPA induced LDH leakage. The lowest concentration of BM extract provided 73% protection, while a maximum of 90% protection was evident at the highest concentration among 3-NPA (4mM) exposed cells. However, the degree of protection averaged between 37-68% among BM extract pretreated cells exposed to higher concentration of 3-NPA (6mM).

In general, a concentration-dependent increase in LDH leakage was observed among rotenone (8µM, 16µM) exposed cells (Fig. 4.6 lower panel). A marked increase (5.6-fold) in LDH leakage was observed at 8µM, while a more robust increase (8.3-fold) was evident at 16µM. While the lowest concentration of BM extract failed to offer any protection, significant protective effect was discernible at higher levels. The degree of protection ranged between 44-65% against rotenone (LC_{50} conc), while the degree of protection was less robust (15-35%) at LC_{75} rotenone concentration.

Abrogation of neurotoxicant-induced Oxidative stress

Abrogation of neurotoxin induced ROS generation

Treatment of cells with BM extract alone resulted in significant (14-32%) diminution in ROS levels (Fig.4.7A). Both the neurotoxicants elevated ROS formation markedly (3-NPA: 130-257% increase; Rotenone: 68-181% increase) as reflected by higher fluorescence intensity compared to untreated control cells (Fig.4.7 B-C). Pretreatment of cells with BM extract rendered varying degrees of protection against neurotoxin induced ROS generation (Fig 4.8 A). The percent protection in case of 3-NPA exposure was 20-100%, while it ranged between 22-85% with rotenone (Fig.4.8 B).

Abrogation of neurotoxin induced hydroperoxides (HP)

Treatment of cells with BM extract alone resulted in significant (10-40%) reduction in the basal levels of HP (Fig. 4.9 A). Marked elevation was evident in the HP levels among cells exposed to the neurotoxicants. While 3-NPA caused 95-190% increase in HP levels, the elevations among rotenone exposed cells was less robust (24-81%). Interestingly, treatment of cells with BM extract resulted in lower levels of HP following neurotoxicant exposure. While the degree of protection was 32-80% with 3-NPA exposure, it ranged between 27-64% with rotenone (Fig.4.10 A-B).

Protective effect of BME against neurotoxin-induced Glutathione depletion

Cells treated with BM extract alone showed a concentration-dependent increase in reduced glutathione levels (39%), while the levels of oxidized glutathione (50%) levels were reduced (Fig.4.11A-B). However exposure to the neurotoxicants caused a significant decrease in reduced and oxidized glutathione levels. The reduction in glutathione levels on exposure to 3-NPA was concentration-dependent (35-47%) (Fig. 4.11 C-D), while rotenone caused reduction

that ranged between 18-49%. Likewise, the GSSG levels were also diminished in a concentration dependent manner in 3-NPA (35-41%) and Rotenone (32-40%) exposed cells (Fig.4.11E-F)

For modulation experiments, only one concentration of 3-NPA (3mM) and rotenone (6µM) was used. Varying degrees of protection was observed among BM extract treated cells exposed to the neurotoxicants (Fig.4.12A-D). BM extract treatment restored the 3-NPA induced depletion in reduced glutathione levels and the degree of protection ranged between 64-87%, while 50-80% protection was observed against GSSG depletion (Fig.4.12 A-B). Further with rotenone treatment, BM extract offered total protection (100%) and the reduced GSH was normalized at 6µg of BM extract, while no significant protection was offered against GSSG depletion among rotenone treated cells (Fig.4.12 A-B).

Protective effects against mitochondrial dysfunctions

Perturbations in glutathione related enzymes

Cells treated with BM (6µg) extract alone caused significant elevation in the activity of glutathione related enzymes (GSH-Px: 57%, GST: 28%, GR: 36%) (Fig.4.13). 3-NPA exposure resulted in significant decrease in the activities of GSH-Px (26%) and GR (35%), while the activity of GST was enhanced (35%). Further, rotenone exposure resulted in significant decrease in the activities of GPX (32%) and GR (47%), while the activity of GST was markedly increased (70%). On the other hand pretreatment of cells with BM extract resulted in varying degree of normalization of enzyme activities.

Protective effects against perturbations in TCA cycle enzymes

Treatment of cells with BM extract alone caused alterations in activities of MDH (42% increase) and SDH (11% increase). 3-NPA (3mM) exposure caused a robust (86%) reduction in the activity of SDH, while reduction in the activities of MDH and CS were marginal (15%). However, pretreatment with BM extract did not have an appreciable effect on 3-NPA-induced reduction in SDH activity. BM extract pretreatment significantly protected the activities of MDH and CS against 3-NPA-induced reduction.

Further, among rotenone exposed cells, there was a decrease in the activities MDH (10%) and CS (29%), while the activity of SDH was enhanced (24%). Significant (41%) degree of

protection was observed against reduction in CS activity, while no observable protection was evident against changes in MDH and SDH activity following BM extract pretreatment (Fig.4.14)

Amelioration of alterations in ETC enzymes

Treatment of cells with BM extract alone caused a marginal increase in the activities of Complex I (Rot-sensitive: 8% and Rot–insensitive: 17%) and Complex II-III (48%) enzymes. However, 3-NPA exposure resulted in decrease (15%) in activity of Rot-insensitive complex I while it caused a robust (47%) decrease in complex II-III activity. Interestingly pretreatment with BM extract resulted in complete restoration (100% protection) against 3-NPA-induced reduction in Rot- insensitive complex I activity, while the restoration was partial (66% protection) against complex II-III activity.

Rotenone exposure caused a significant decrease (35%) in the activity of Rot-sensitive complex I and an increase (34%) in Rot-insensitive complex I, while there was no effect on complex II-III activity. Pretreatment with BM extract resulted in partial restoration (75% protection) against reduction in activity of rotenone sensitive complex I (Fig.4.15.A-C).

Protective effect against neurotoxin induced DNA damage

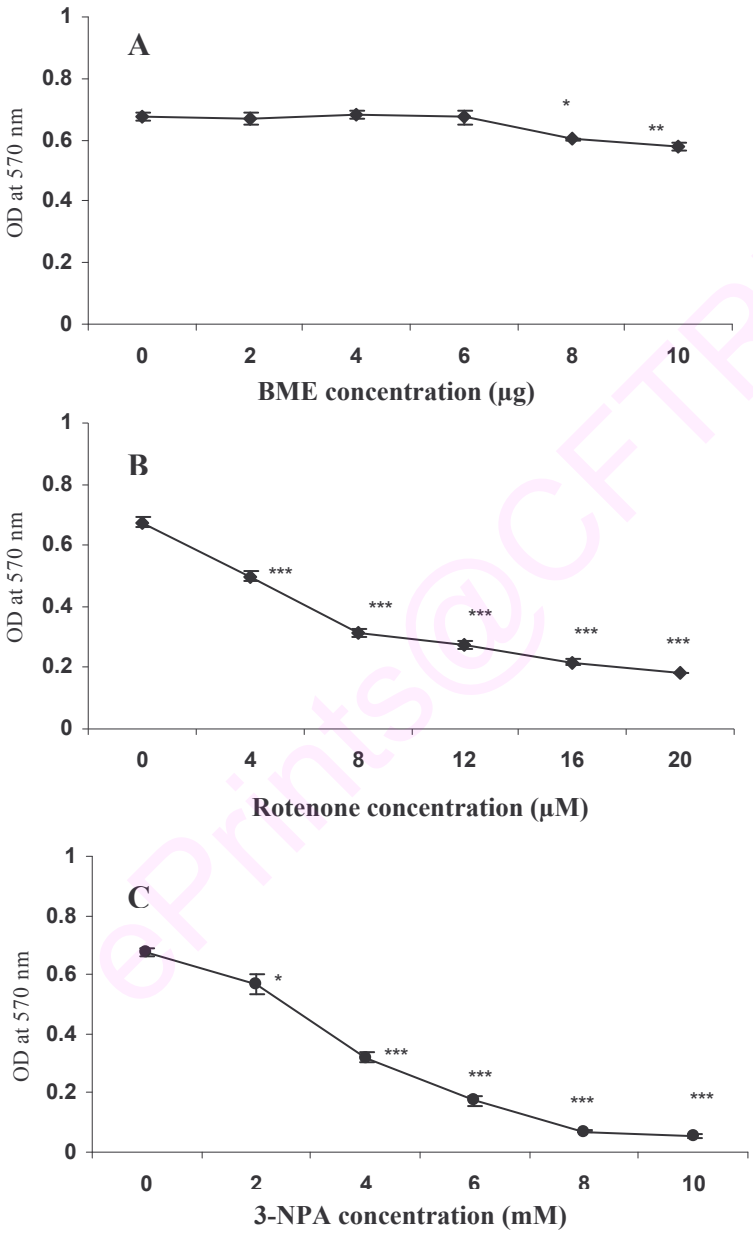
The background incidence of comets among control and BM extract alone treated cells was 5.4 and 3.4 % respectively (Table 4.1). However 3-NPA exposed cells stimulated marked increase (66%) in the frequency of comets, while rotenone exposed cells showed more robust (75%) incidence of comets. Interestingly, BM extract substantially reduced the incidence of comets and significant protection was evident against 3-NPA (88%) and Rotenone (84%) induced DNA damage.

Protection against decreased expression of proteins

There was a significant decrease in the essential proteins on exposure to 3-NPA and rotenone. SDS gel photographs of cell homogenates and mitochondria clearly depict decrease in amount of protein. The low molecular weight proteins were very less in mitochondria of cells exposed to 3-NPA and rotenone. The degree of reduction in protein expression was more prominent on rotenone exposure. 43000Da and 66000Da there is clear expression of new proteins in BME modulation groups of both 3-NPA and Rotenone exposed cells.

Fig: 4.1

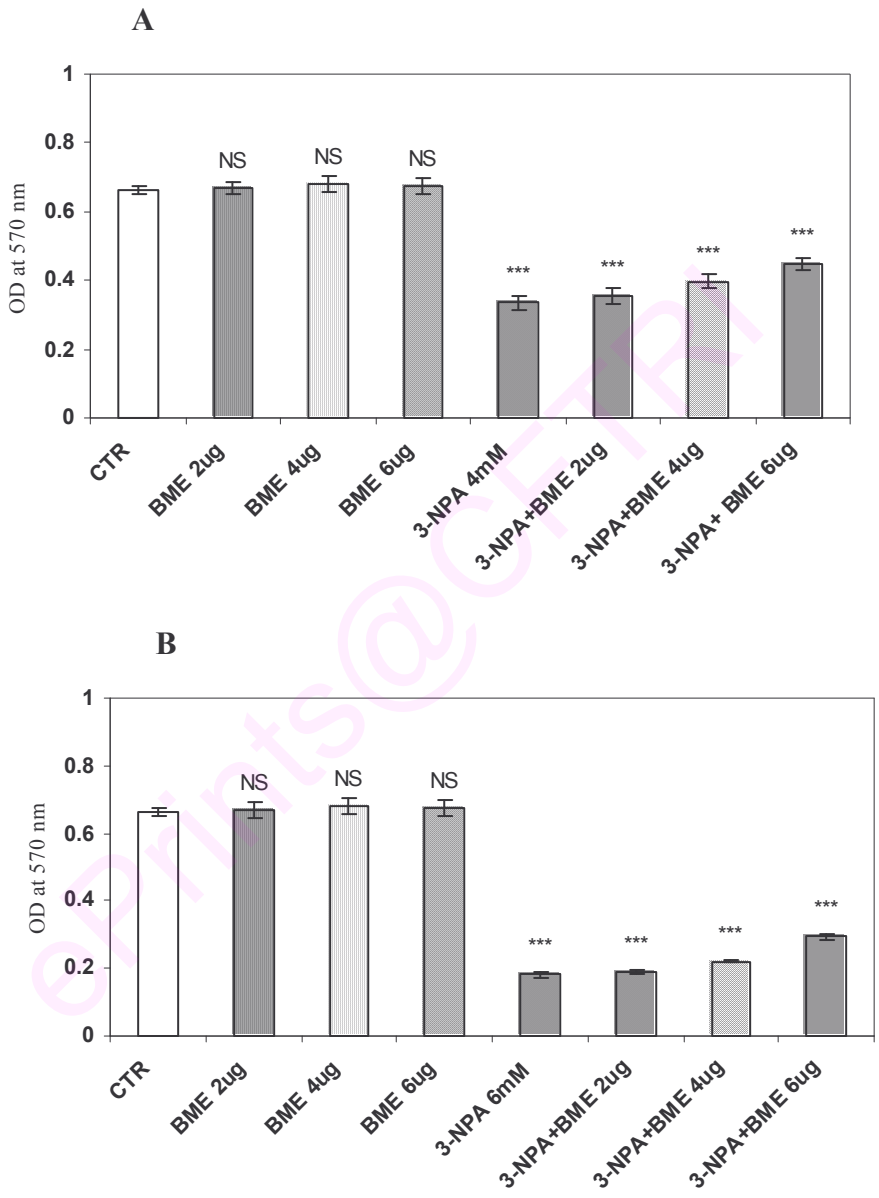
Response of N27 cells following exposure to *Bacopa monnieri* ethanolic extract, rotenone and 3-NPA



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001); BME-*Bacopa monnieri* ethanolic extract; 3-NPA: 3-nitropropionic acid

Fig: 4.2

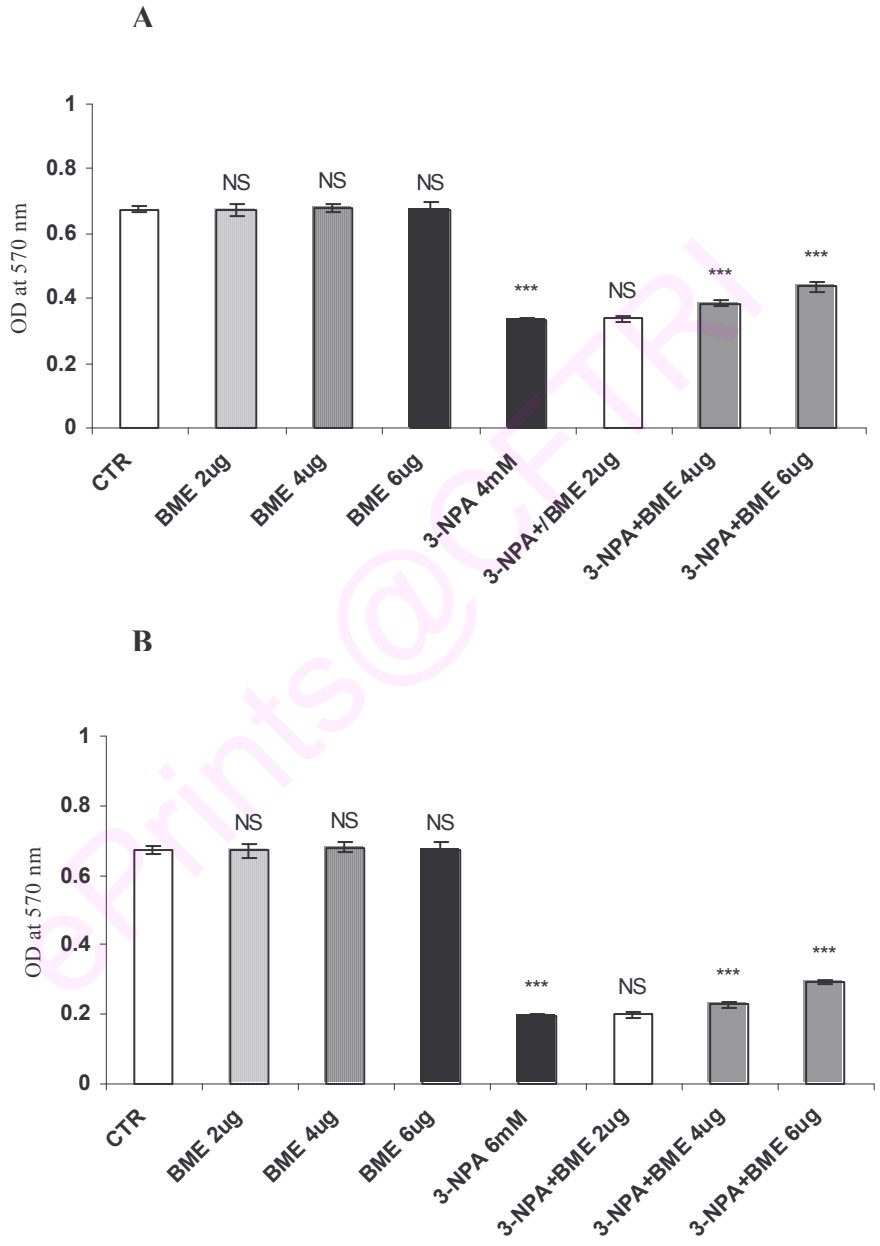
Modulation of 3-NPA induced cytotoxic response by *Bacopa monnieri* extract pretreatment in N27 cells



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001; NS-not significant).
BME-*Bacopa monnieri* ethanolic extract;
NPA: 3-nitropropionic acid

Fig: 4.3

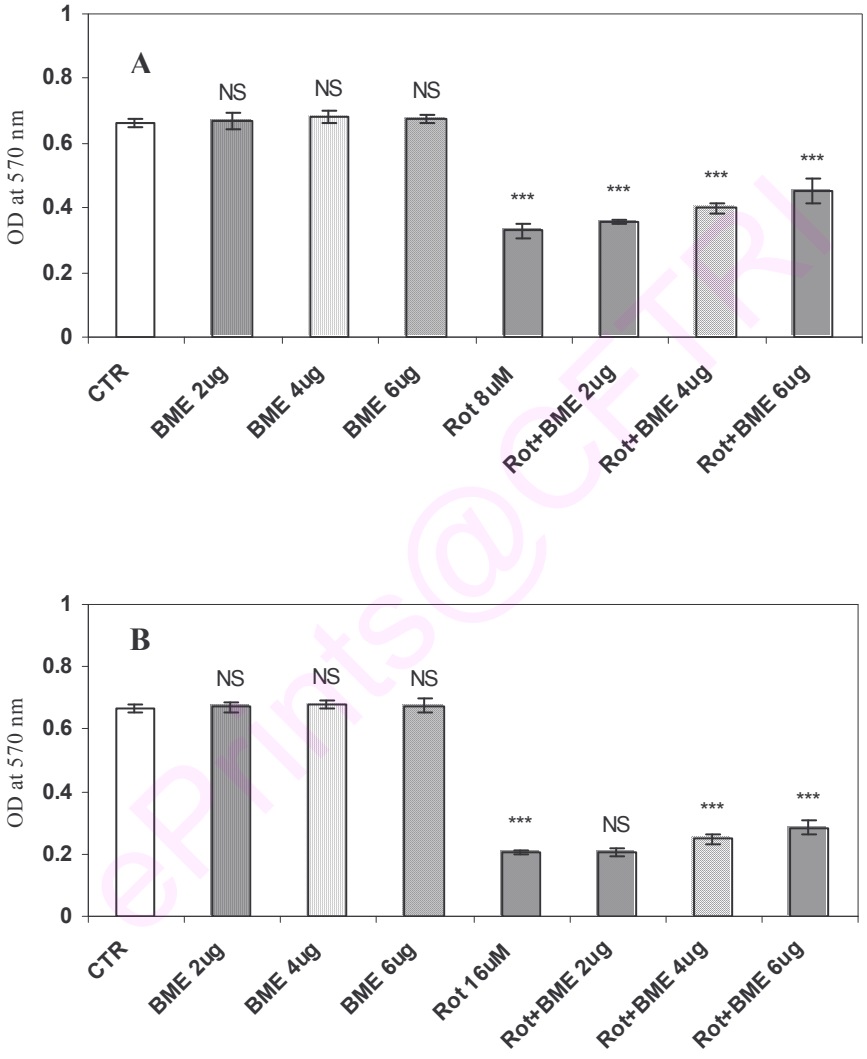
3-NPA induced cytotoxic response as modulated co-exposure of N27 cells with BM ethanolic extract



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant);
BME-*Bacopa monnieri* ethanolic extract;
NPA: 3-nitropropionic acid

Fig: 4.4

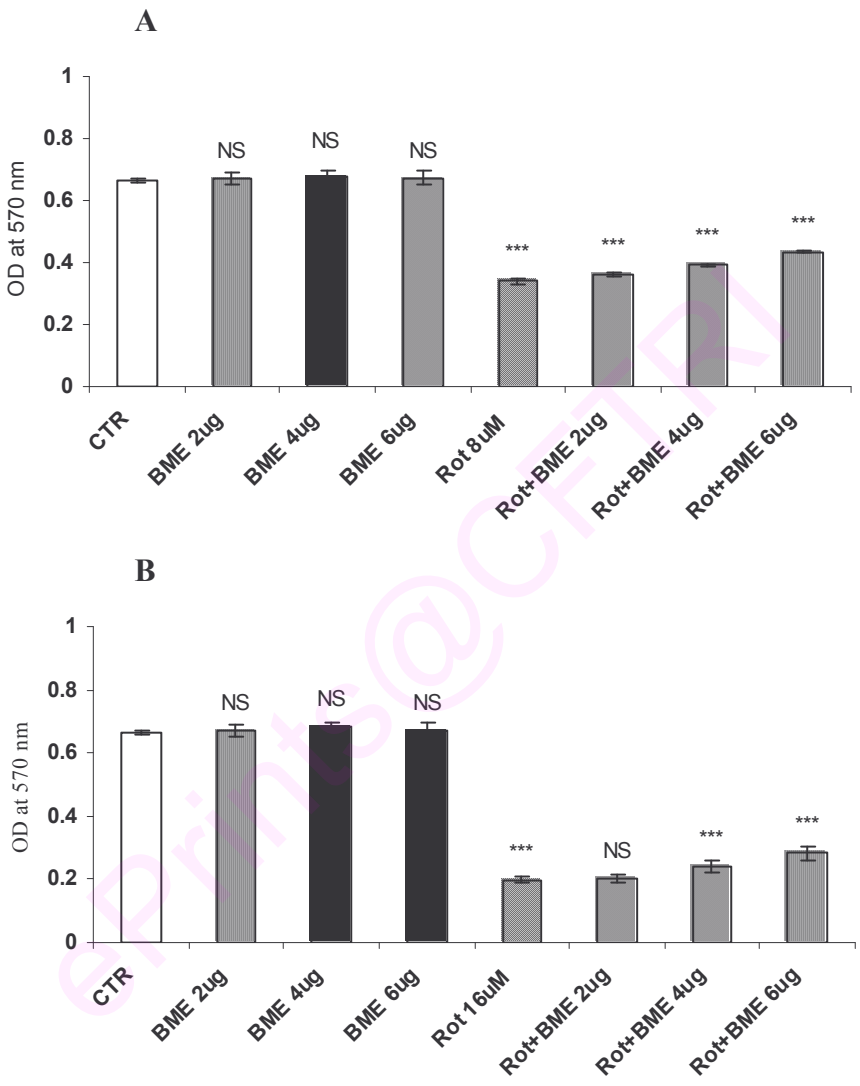
Modulation of Rotenone induced cytotoxic response in N27 cells by pretreatment with BM ethanolic extract.



Values are mean ± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001; NS-not significant). BME-*Bacopa monnieri* ethanolic extract; Rot-Rotenone

Fig: 4.5

Modulatory effect of *Bacopa monnieri* co-treatment on Rotenone (LC50 concentration-A; LC75 concentration-B) induced cytotoxic response in N27 cells: Rotenone

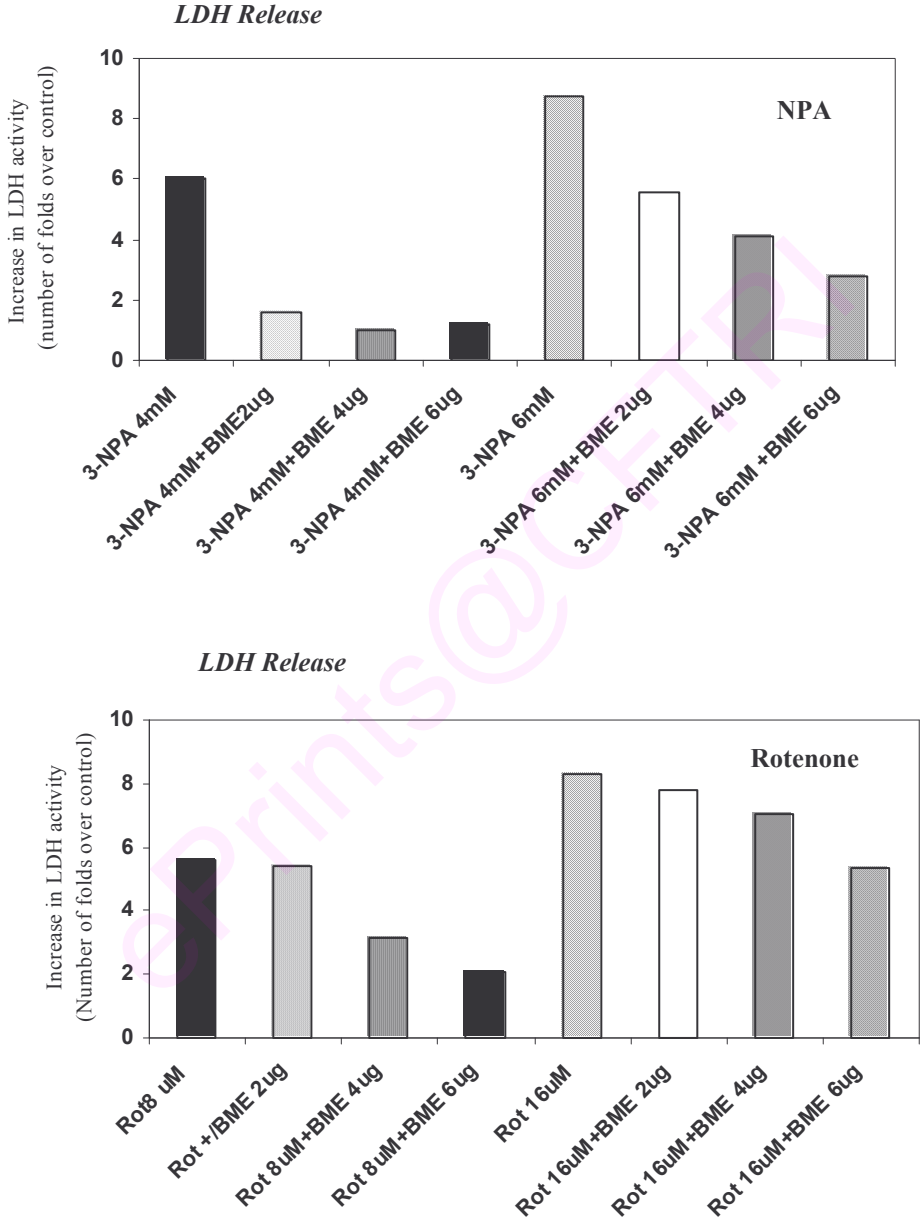


Values are mean± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001; NS-not significant).

BME-*Bacopa monnieri* ethanolic extract; Rot-Rotenone
A-Rotenone conc. LC_{50}
B- Rotenone conc LC_{75}

Fig: 4.6

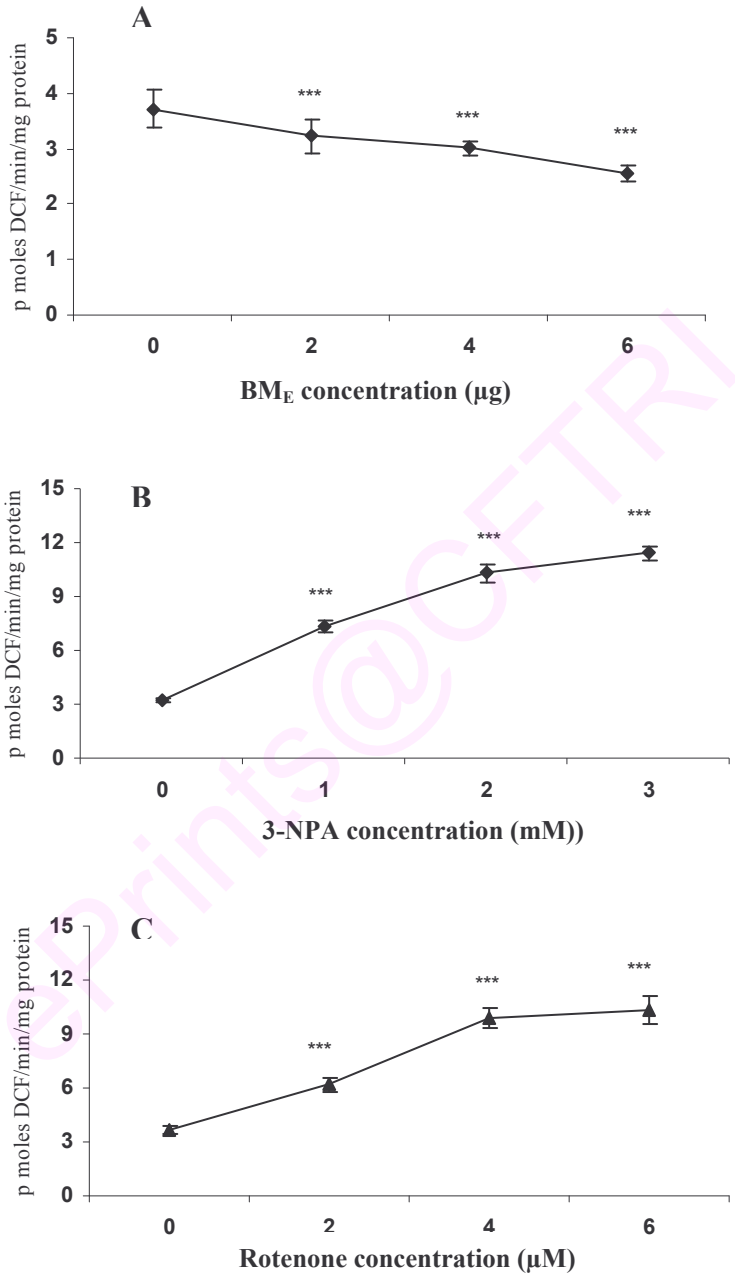
Release of LDH enzyme in to the media among N27 cells exposed to NPA, Rotenone (at LC $_{50}$/LC $_{75}$ conc) and its modulation by pretreatment with *B. monnieri* ethanolic extract (BME).



Values are ± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001; NS-not significant).
BME-*Bacopa monnieri* ethanolic extract; Rot-Rotenone
3-NPA concentration : 4 and 6 mM
Rotenone concentration: 8 and 16µM

Fig: 4.7

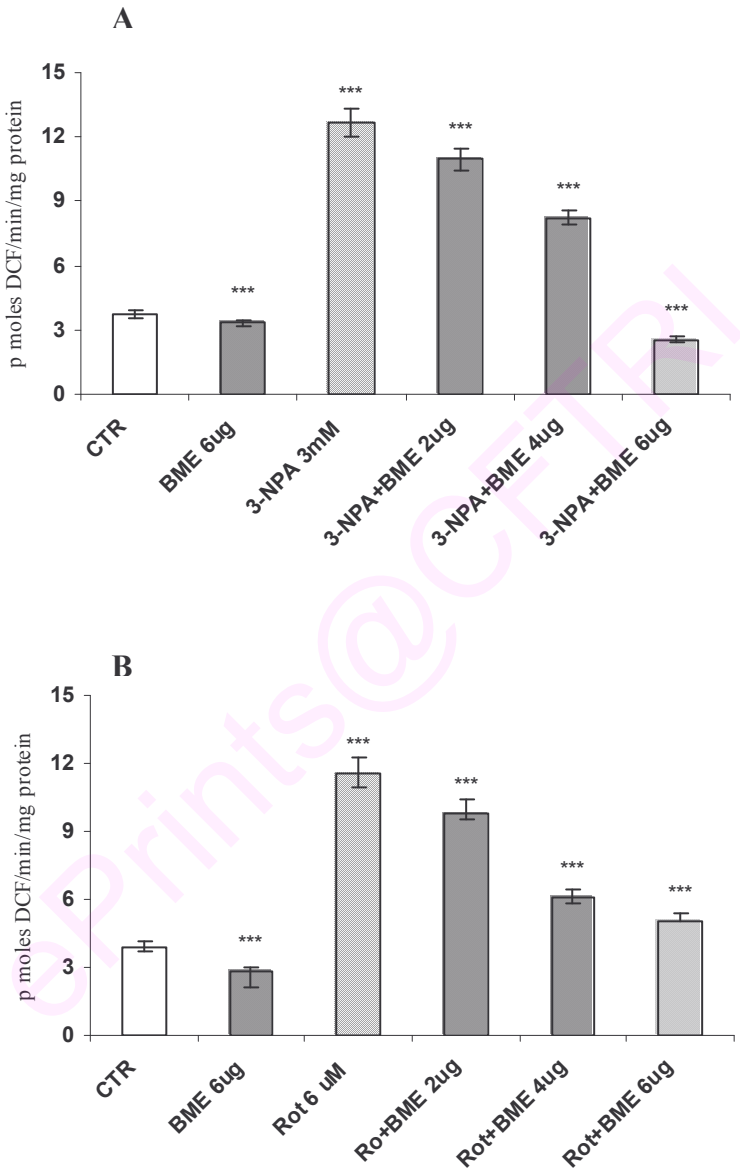
Cytosolic ROS levels in N27 cells treated with 3-NPA, Rotenone, and its modulation by pretreatment with *Bacopa monnieri* ethanolic extract (BME)



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
NPA: 3-nitropropionic acid; ROT-Rotenone; BME –*Bacopa monnieri* ethanolic extract

Fig: 4.8

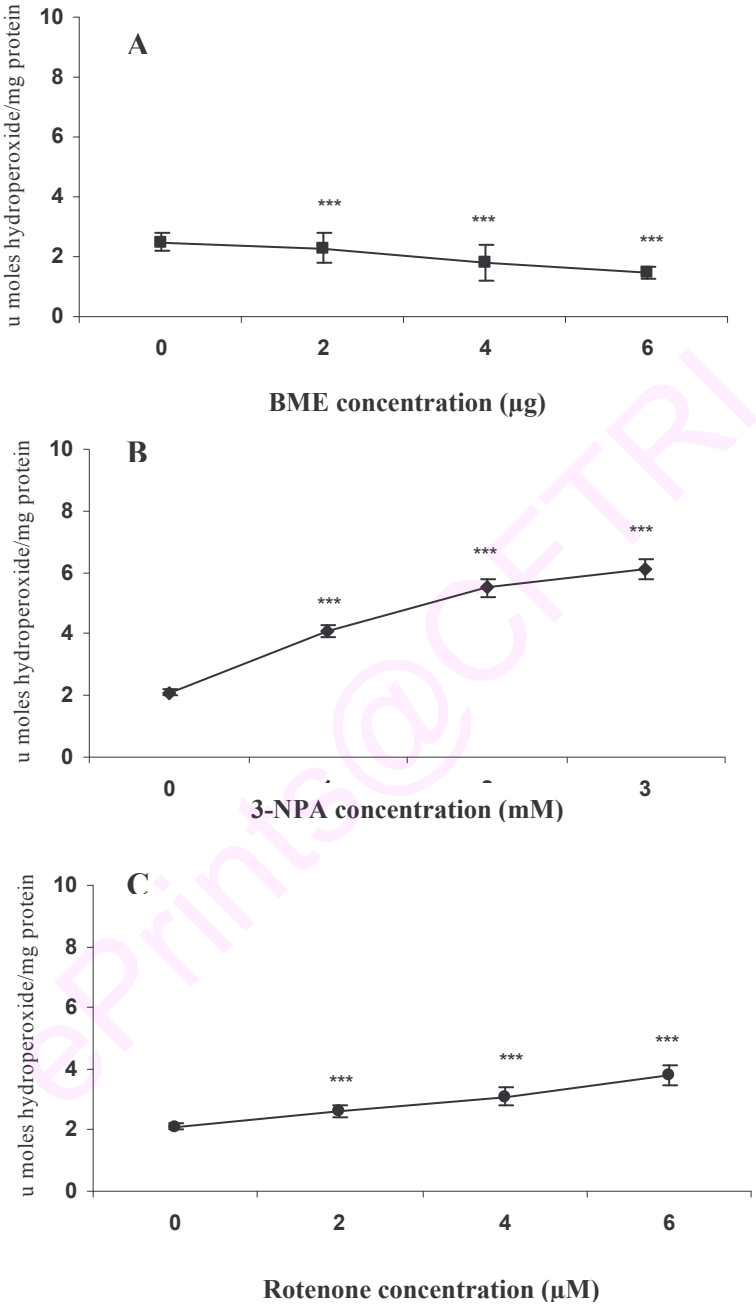
Modulatory effect of BM extract against 3-NPA and Rotenone–induced ROS generation in N27 cells.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract

Fig: 4.9

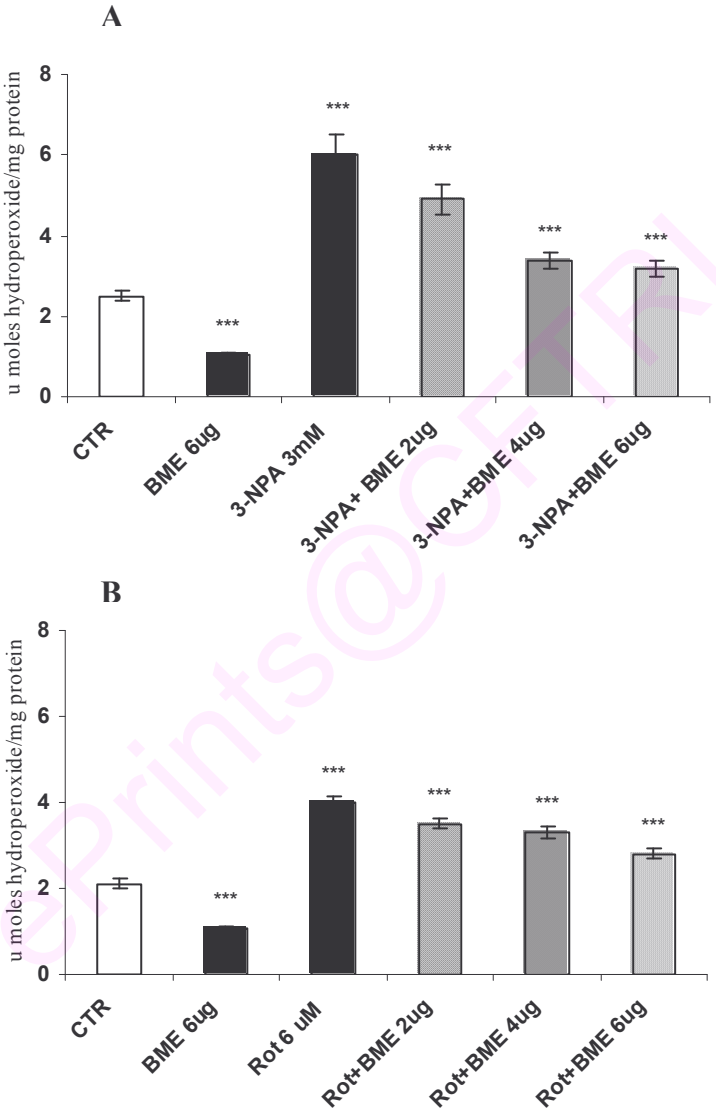
Hydroperoxide generation in N27 cells exposed to BM extract, 3-NPA and Rotenone.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT-Rotenone; BME –*Bacopa monnieri* ethanolic extract

Fig: 4.10

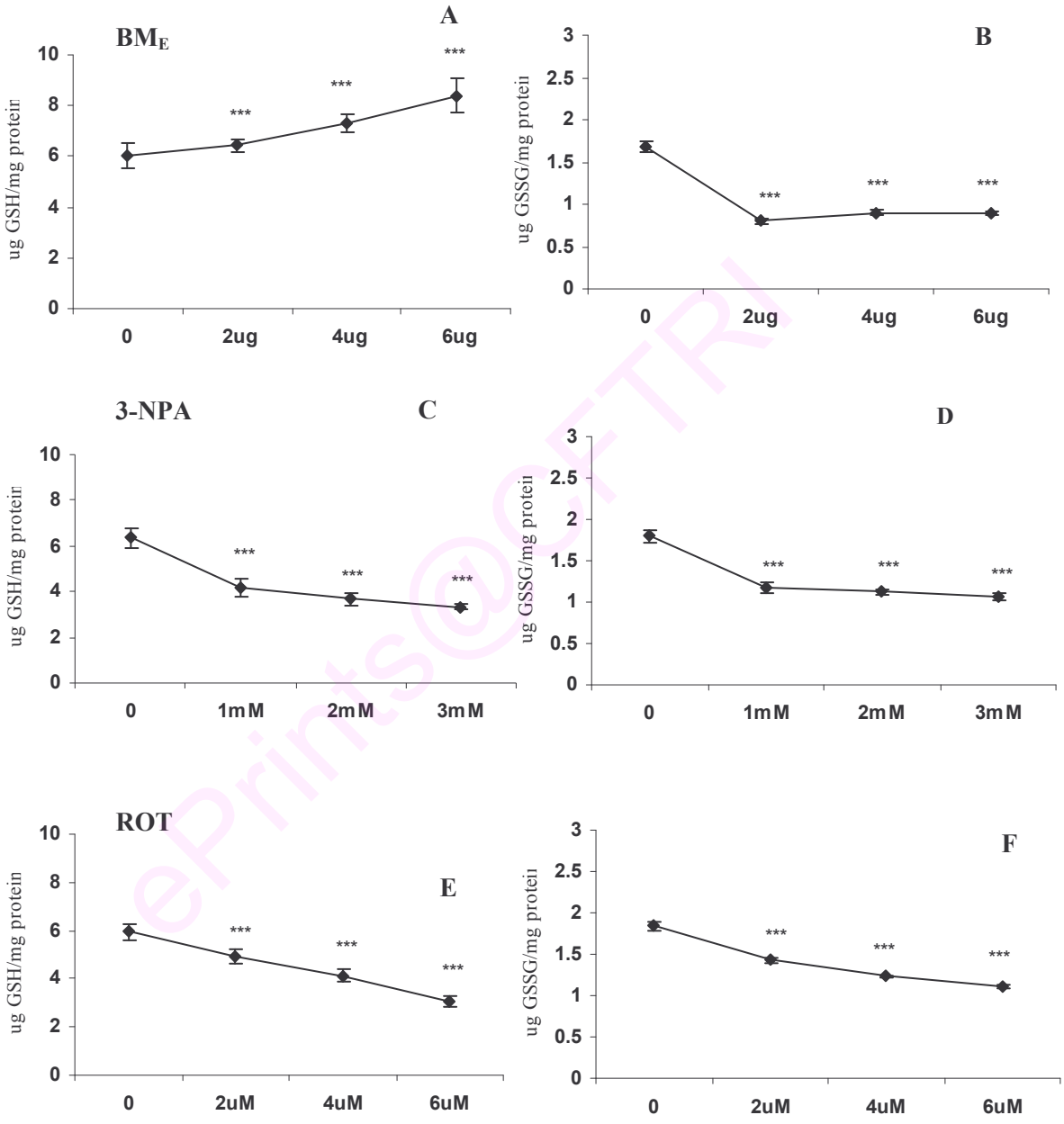
Modulatory effect of BM extract against 3-NPA-an Rotenone- induced hydroperoxide levels N27 cells.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract

Fig.4.11

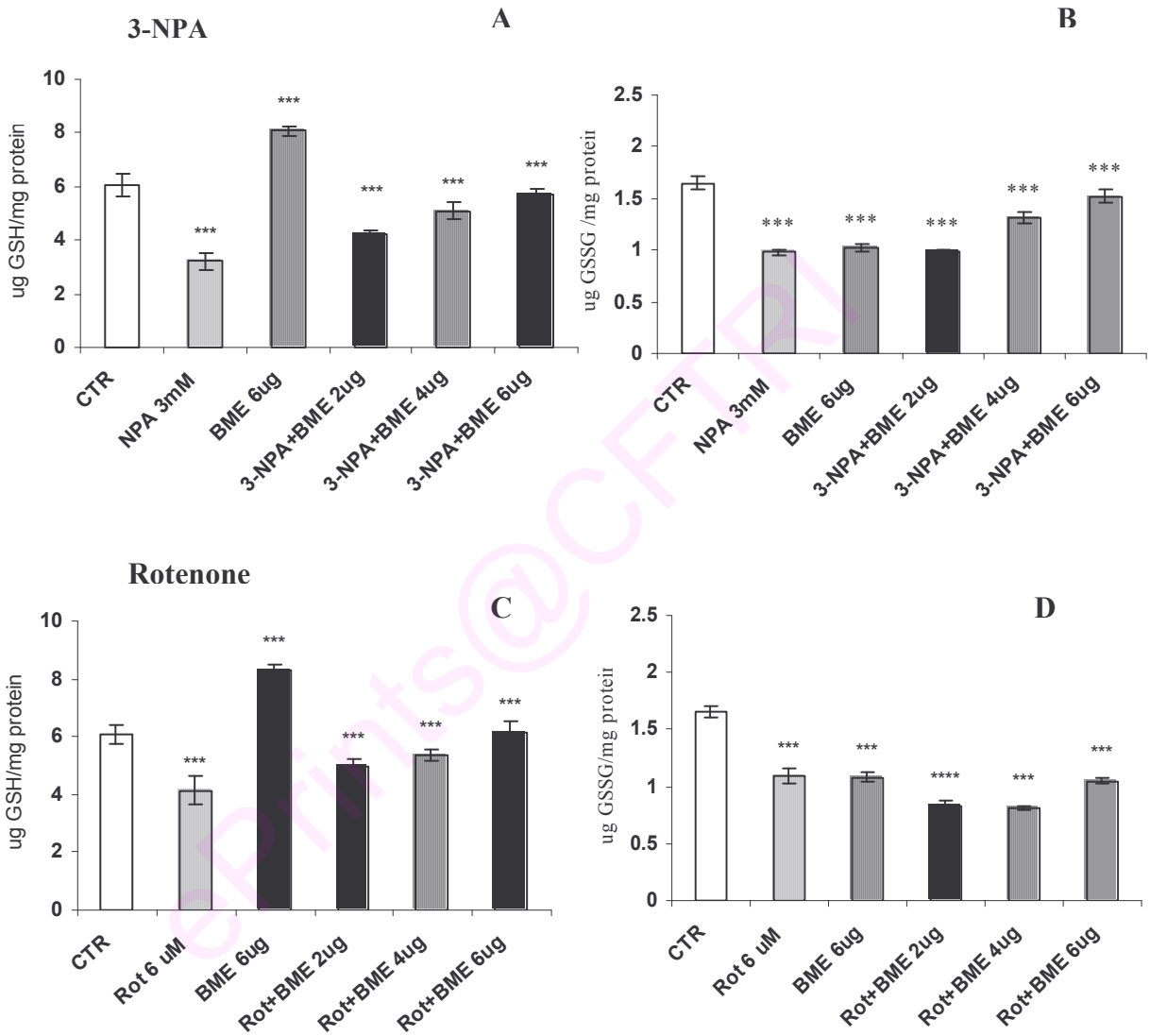
Alteration in glutathione levels in cytosol of N27 cells exposed to NPA, Rotenone and *Bacopa monnieri* ethanolic extract (BME)



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract

Fig: 4.12

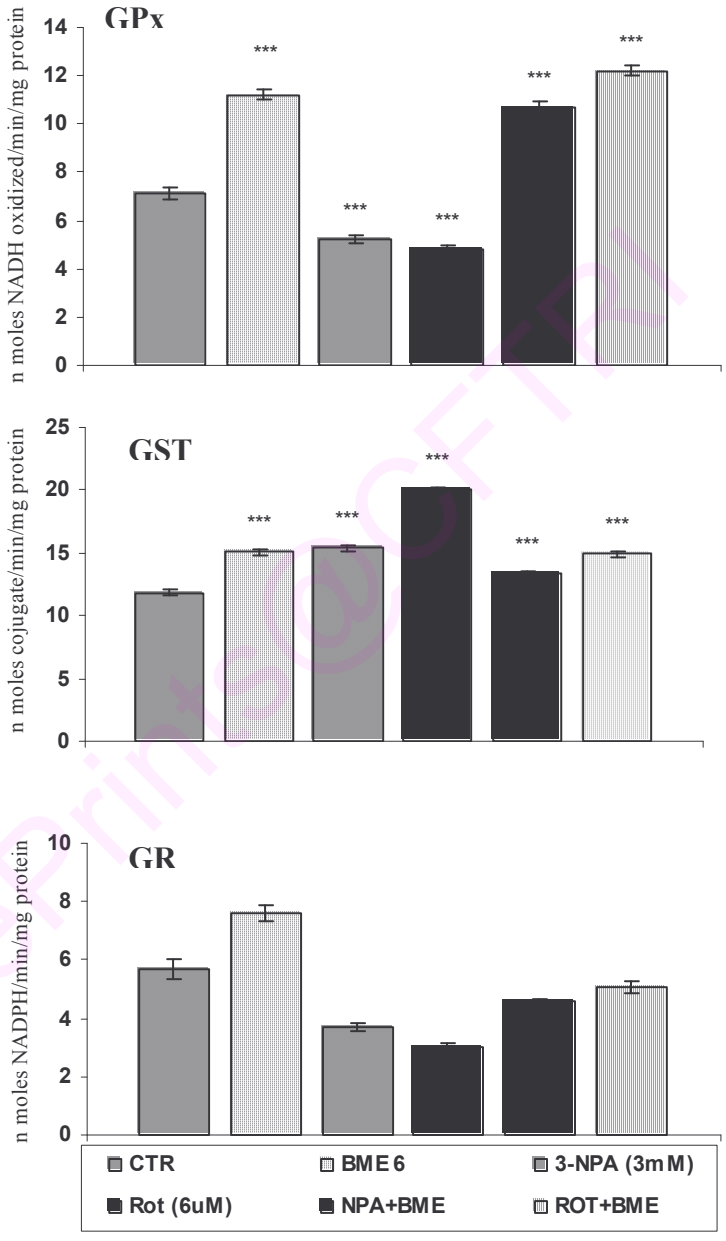
Effect of BM extract pretreatment on GSH and GSSG levels among 3-NPA and Rotenone exposed N27 cells.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract

Fig:4.13

Activities of glutathione related enzyme activities in mitochondria of N 27 cells treated with NPA and Rotenone and its modulation by pretreatment with *Bacopa monnieri* extract

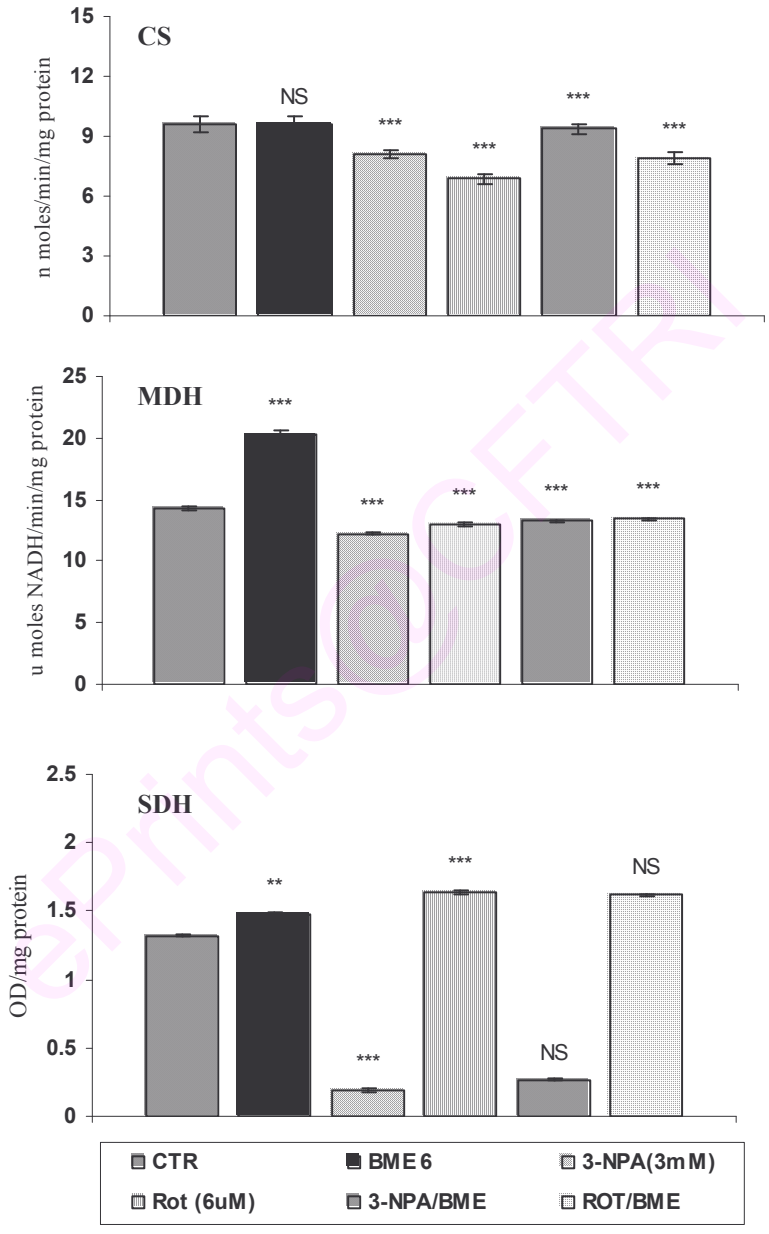


Values are ± S.D of six determinations each; data analyzed by one way ANOVA (*P<0.05; **P<0.01; ***P<0.001; NS-not significant)

3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME : *Bacopa monnieri* ethanolic extract
GPx-Glutathione peroxidase; GST-Glutathione –S-Transferase; GR-Glutathione reductase

Fig: 4.14

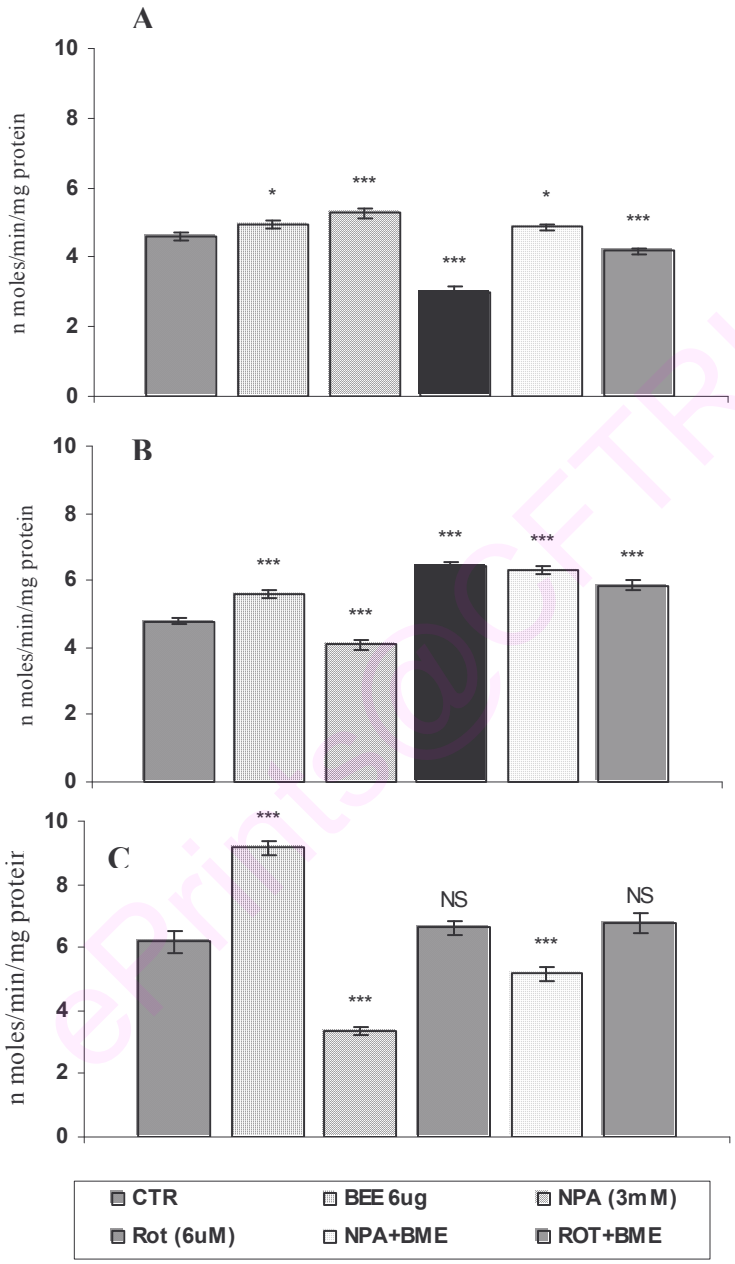
Activities of Citrate synthase (CS), MDH and SDH in mitochondria of N 27 cells treated with 3-NPA and Rotenone.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract
MDH-Malate dehydrogenase; SDH-Succinate dehydrogenase

Fig: 4.15

Modulation of BM extract on the activities of ETC enzymes in mitochondria of cells treated with 3-NPA and Rotenone.



Values are ± S.D of six determinations each; data analyzed by one way ANOVA
(*P<0.05; **P<0.01; ***P<0.001; NS-not significant)
3-NPA: 3-nitropropionic acid; ROT: Rotenone; BME: *Bacopa monnieri* ethanolic extract
A-Complex I rotenone sensitive
B-Complex I rotenone insensitive
C- Complex II-III, Succinate cytochrome c oxidase

Table 1

Modulatory effect of BM extract on the incidence of comets in 3-NPA and Rotenone treated N27 cells

Group	Total no:of cells	No: of comets
CTR	59 ±1.5	3.2 ±1.1
BME (6µg)	67 ±2.0	2.3 ±1.0**
3-NPA (4mM)	21 ±2.0	14 ±2.0***
ROT (8µM)	20 ±2.0	15 ±2.0***
3-NPA + BME	48 ±1.5	4 ±0.5***
ROT + BME	50 ±2.5	6 ±0.5**

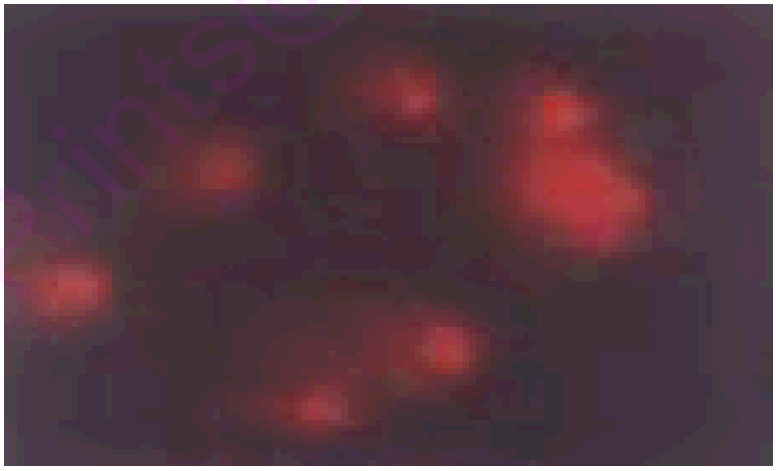


Plate 2: DNA single strand breaks (comet assay) in N27 cells exposed to rotenone (8µM). Fluorescent microscopic image after single cell gel electrophoresis. Typical comet images showing varying degree of DNA damage

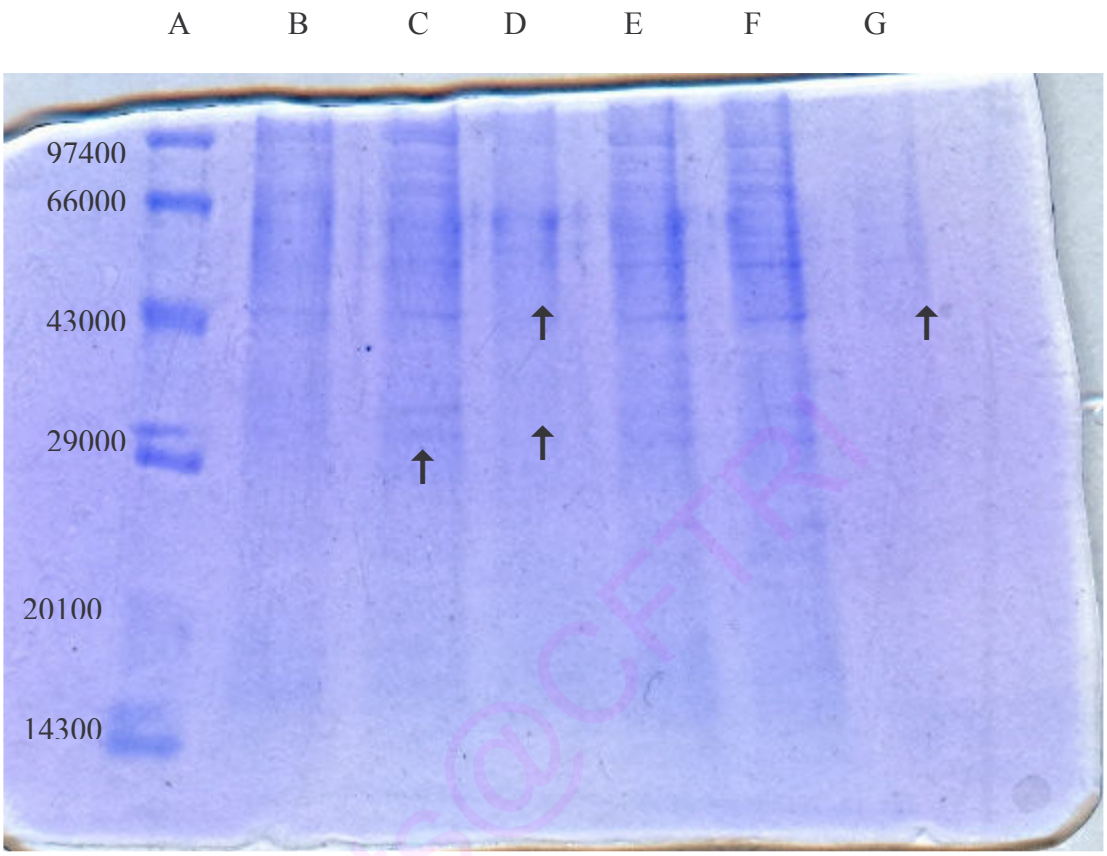


Plate 3: SDS gel photograph of mitochondrial proteins

Lane A-molecular markers (Da)
Lane B-Control
Lane C-BME (6ug)
Lane D -NPA (3mM)
Lane E-BME+NPA
Lane F-BME+Rot
Lane G-Rot (6 uM)

3-NPA: 3-nitropropionic acid; Rot-Rotenone;
BME –*Bacopa monnieri* ethanolic extract

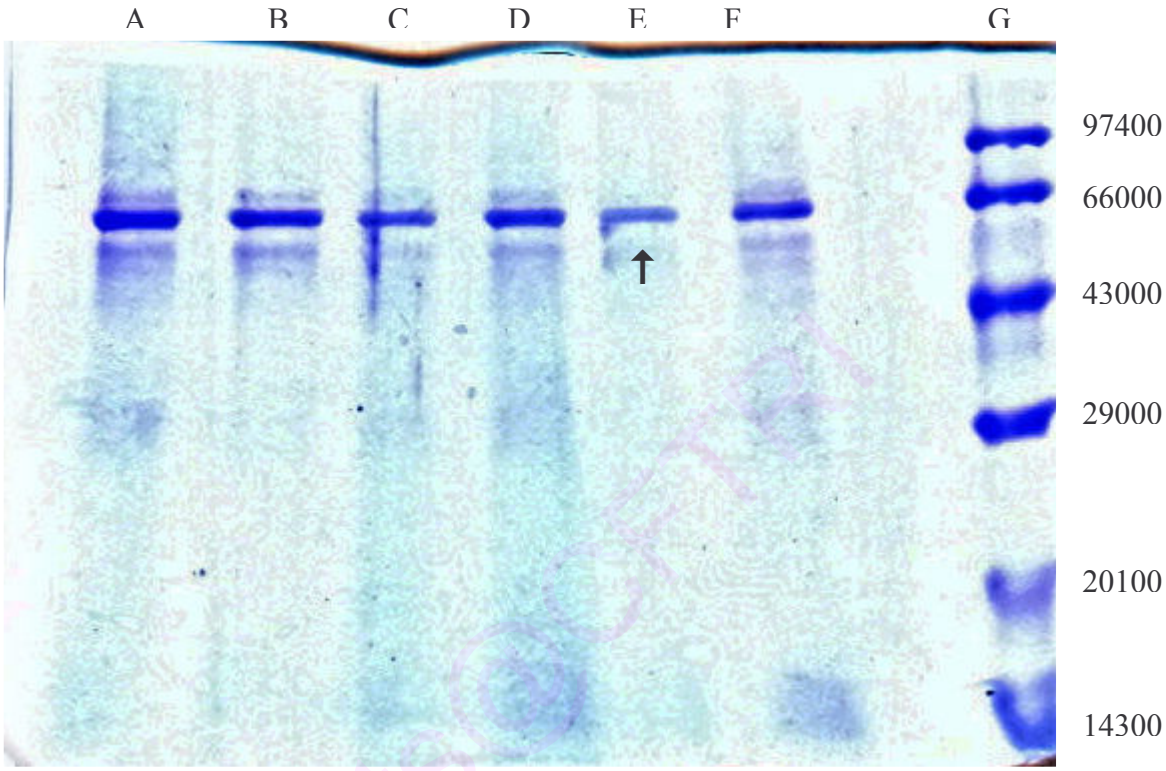


Plate 4: SDS gel photograph of cell homogenates

Lane A-control
Lane B-BM_E (6 ug)
Lane C-NPA (4mM)
Lane D-Rot (8uM)
Lane E-BME+ NPA
Lane F-BME+Rot
Lane G- molecular markers (Da)

3-NPA: 3-nitropropionic acid; Rot-Rotenone
BME –*Bacopa monnieri* ethanolic extract

5.0 DISCUSSION

Although dopaminergic (DA) neurons correspond to less than 1% of the total number of brain neurons, they play an important role in regulating several aspects of basic brain function. They are necessary for tasks specific to the brain regions which they innervate including motor behavior, motivation and working memory. Regulation of dopamine therefore plays a crucial role in both mental and physical health (Chinta and Andersen, 2005). DA neurons are believed to be particularly prone to oxidative stress due to their high rate of oxygen metabolism, low levels of antioxidants, and high iron content. Dopamine is thought to be capable of generating toxic ROS *via* both its enzymatic and non-enzymatic catabolism (Halliwell, 1992). Specifically, dopamine oxidation can occur either spontaneously in the presence of transition metal ions or *via* an enzyme-catalyzed reaction involving monoamine oxidase and generate a spectrum of toxic species including H_2O_2, oxygen radicals, semiquinones, and quinones (Graham et al., 1978).

One of the basic mechanisms proposed for nigrostriatal cell loss in PD is an oxidative stress/injury resulting from a production of ROS exceeding the cellular antioxidant capacity. Mitochondrial involvement in the pathogenesis of AD and its relation to oxidative stress is a recurrent hypothesis in the scientific and clinical literature (Cui et al., 2004). Several lines of evidences have demonstrated the involvement of mitochondrial dysfunctions in the pathogenesis of HD. Though many lines of evidence suggest that mitochondria have a central role in ageing related neurodegenerative diseases (Lin and Beal 2006), it is still largely debated whether oxidative stress and mitochondrial impairment are involved in the onset and progression of these disorders or are merely a consequence of neurodegeneration (Petrozzi et al., 2007). It is speculated that, investigations related to mitochondrial pathophysiology in neurodegeneration may provide a target for additional treatments with agents that improve mitochondrial function, target the MPT, or that exert antioxidant activity.

The two neurotoxicants *viz.,* rotenone and 3-NPA employed in this study are well known mitochondrial complex I and complex II inhibitors respectively. Both the neurotoxins have specific effects on striatum, particularly the DA neurons and are used as good chemical to models that mimic Huntington's disease (3-NPA) and Parkinson's disease (Rotenone). The toxins which act by inhibition of mitochondrial complexes and uncoupling mitochondrial respiratory chain cause a

series of downstream effects similar to those observed during human disease pathogenesis. Many alternative antioxidative approaches *viz.,* including free-radical scavengers, GSH, GSH enhancing agents, ion chelators and drugs that interfere with oxidative metabolism of DA have been attempted (Esposito et al., 2002). Though various synthetic and natural substances have been used to protect against the dysfunctions caused by these neurotoxins, not many studies have examined the efficacy of Ayurvedic medicinal plants under such conditions. Hence in the present study we have attempted to understand the mechanism/s by which *B. monnieri* ethanolic extract mitigates neurotoxicant induced oxidative stress and associated mitochondrial dysfunctions.

In the present study, N27 cells on exposure to 3-NPA or Rotenone exhibited marked increase in ROS levels which may be related to the inherent potential of these neurotoxicants to inhibit mitochondrial complex enzymes. It has been reported by several groups that complex I inhibition by rotenone may result in opening of the mitochondrial permeability transition pore and massive production of ROS (Pei et al., 2003; Panov et al., 2005). 3-NPA exposure is also known to produce ROS including H_2O_2 and there by oxidative stress (Wei et al., 2004). Interestingly, pretreatment as well as co-treatment with BM extract was found to protect significantly against rotenone and 3-NPA induced cell death of dopaminergic neurons, indicating its neuroprotective potential. Pretreatment with BM extract was found to attenuate LDH leakage into media, suggesting protection against membrane damage. Earlier studies have shown that polyphenols such as epicatechin, tea extracts and flavanoids possess considerable neuroprotective effects in *in vitro* and *in vivo* models (Esposito et al., 2002). It is likely that, BM extract which is known to contain several saponins might have exerted its protective action by the scavenging of free radicals and there by prevent the activation of JNK and apoptosis induced by hydrogen peroxide.

In the present study, there was significant increase in hydroperoxide levels in cells on exposure to 3-NPA and Rotenone clearly indicating the ability of the neurotoxicants to generate hydrogen peroxide. BM treatment alone was able to significantly reduce the basal levels of hydroperoxides in N-27 cells. Hydrogen peroxide though comparatively inactive molecule, unlike superoxide can easily cross cell membranes. Hydrogen peroxide and superoxide may undergo further transformations in the presence of transition metals (particularly iron and copper) to give rise to the highly reactive hydroxyl radicals, by the Haber–Weiss or Fenton reactions. This

property, combined with the membrane permeability of hydrogen peroxide, gives superoxide and hydrogen peroxide the ability to affect the integrity of distant molecules within the cell (Halliwell, 2006; Cui et al., 2004). In the present study, BM extract pretreatment resulted in marked protection against hydroperoxide generation indicative of its scavenging ability. Further BM extract having high iron chelation property may also attribute to its antioxidant property by preventing Haber–Weiss or Fenton reactions (as described earlier in Chapter 3).

In the present study, glutathione levels among BM *per se* treated cells were significantly elevated, while, the levels showed concentration dependent depletion on exposure to 3-NPA or rotenone. On the other hand, BM pretreatment resulted in significant protection against neurotoxicants induced depletion in GSH levels. GSH has been shown to play a role in the rescue of cells from apoptosis; depletion of GSH, which renders the cellular environment more oxidizing, was concomitant with the onset of apoptosis. Generally, a more reducing environment (maintained by elevated levels of glutathione and thioredoxin) of the cell stimulates proliferation and a slight shift towards a mildly oxidizing environment initiates cell differentiation. A further shift towards a more oxidizing environment in the cell leads to apoptosis and necrosis. While apoptosis is induced by moderate oxidizing stimuli, necrosis is induced by an intense oxidizing effect (Evens, 2004). It has already been reported that restoration of cell GSH levels in several pathologies have proved to be beneficial. Strategies to boost cell glutathione levels are of marked therapeutic significance. There are two ways of increasing cell GSH levels 1) direct delivery of exogenously synthesized GSH to the cell and/or 2) increased GSH synthesis. In the present study combined with the results that BM extract alone is able to increase the levels of GSH related enzymes like GSH-Px, GST and GR, it can be assumed that there is increased glutathione turn over in the cell which may be even due to the increase in glutathione synthesizing enzymes.

Further in the present study, it was observed that BM *per se* was able to increase the activity of glutathione –dependent enzymes *viz.,* GSH-PX, GST and GR in mitochondria. Among cells exposed to 3-NPA and Rotenone, there was a significant decrease in GSH-PX and GR activity, while GST activity was enhanced indicating a state of oxidative stress. Glutathione-associated metabolism is a major mechanism for cellular protection against agents 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 inter-dependence between separate component members. Glutathione participates in detoxification at several different levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, glutathione provides the cell with multiple defenses not only against ROS but also against their toxic products as it is also involved in the ultimate removal of detoxified oxidation products from the cell. Most importantly, many of the GSH-dependent proteins are inducible and therefore represent a means whereby cells can adapt to oxidative stress (Hayes and Mclellan, 1999).

Complex I inhibition has several potentially damaging consequences. One possible result of complex I inhibition is increased formation of reactive oxygen species (ROS), creating oxidative damage within the cell. Oxidative stress has been implicated in PD (Zhang et al., 2000). Increased oxidative damage to lipids, DNA, and proteins has been observed in PD Substantia nigra pars compacta, along with decreased levels of reduced glutathione (Testa et al., 2005). Since neurotoxic effects of rotenone are at least in part mediated by free radicals and some antioxidants have been proved to rescue cells from Rotenone toxicity, therapeutic efforts aimed at removal of free radical or prevention of their formation may be beneficial in PD (Beal, 1995). 3-NPA, a complex II inhibitor, produces impairment in energy metabolism, formation of reactive oxygen species and nitrogen species and a decrease in intracellular ATP levels (Di Filippo et al., 2006). It is suggested that oxidative damage is a prerequisite for formation of striatal lesions and that antioxidant treatment may be a useful therapeutic strategy against 3-NPA neurotoxicity and perhaps against HD as well (La Fontaine et al., 2000). In the present study there was decrease in activity of complex I in rotenone and complex II among 3-NPA exposed cells. Though no significant protection was evident on dopaminergic neurons with BM pretreatment, significant protection was evident against complex t inhibition. This differential response may be related to elevated levels of the endogenous antioxidant glutathione which is demonstrated to be protective. This thinking is consistent with evidences which show that 3-NPA toxicity was significantly attenuated in rodents, following the administration of glutathione precursor, N-acetylcysteine (Pocernich et al., 2000).

In the present study, the protective effect of BM extract was also discernible in terms of marked protection against DNA damage induced by the neurotoxicants clearly demonstrating the

protective efficacy of BM extract against oxidative stress mediated DNA damage. Similar protective efficacy of BM extract against hydrogen peroxide DNA damage has been demonstrated in human fibroblasts (Russo et al., 2003). However, the present study is the first one to demonstrate protection against DNA damage in dopaminergic neuronal cells. Recent studies, have also demonstrated that BM ethanol extract significantly reduces beta-amyloid deposits in the brain of an Alzheimer's disease transgenic mouse model (PSAPP mice) suggesting its efficacy and mechanisms of action relevant to the treatment of Alzheimer's disease (Dhanasekaran et al., 2007).

In conclusion, data obtained in the present study clearly suggests that BM pretreatment abrogates neurotoxicants induced oxidative stress and offered protection against ROS generation, HP formation and glutathione depletion. Further, the levels of glutathione related enzymes were also increased. Taken together, BM provides significant protection against the oxidative stress and mitochondrial dysfunctions caused by Complex I and Complex II inhibitors. Nevertheless, it is desirable that future studies aimed at investigating the relationship between dietary antioxidant intake and the relative risk for NDD such as AD, PD, and ALS will throw more light on this very important aspect of public health (Esposito et al., 2002).

6.0 SUMMARY

1. Exposure of dopaminergic neurons (N27) to *B. monnieri* ethanolic extract did not induce any significant cytotoxicity upto 8µg as measured by MTT assay and LDH leakage studies. How ever both neurotoxicants (3-NPA and Rotenone) caused concentration dependent cytotoxicity and cell death. The toxicity profile for rotenone was LC 50: 8µM and LC75: 16µM and 3-NPA was LC 50: 4mM and LC75: 6mM.

2. On exposure to BM extract alone, N27 cells exhibited a concentration dependent decrease in ROS and HP levels, while neurotoxicant treated cells showed marked elevations in oxidative markers. Pretreatment of cells with BM extract prior to neurotoxicant exposure resulted in significant attenuation of oxidative markers suggesting protection offered.

3. A concentration-dependent increase in GSH levels and decrease in GSSG levels were evident in BM extract treated cells, while the levels were depleted following rotenone and 3-NPA treated cells. Pretreatment of cells with BM extract prior to neurotoxicant exposure caused significant restoration of GSH levels clearly suggesting the action of BM on GSH mediated protective efficacy.

4. Elevated activities of glutathione related antioxidant enzymes like GSH-Px, GST and GR were demonstrable in BM extract treated cells, while the levels were reduced significantly in rotenone and 3-NPA treated cells . On the other hand treatment of cells with BM extract resulted in varying degrees of normalization of enzyme activities.

5. Reductions in the activities of mitochondrial enzymes like citrate synthase and malate dehydrogenase in rotenone and 3-NPA treated cells were significantly protected with BM pretreatment. However, no protection was noticeable n in SDH activity.

6. Significant protection was observed against DNA damage and protein profile alterations induced by rotenone and 3-NPA by BM extract treatment depicting its high modulatory potential.

CONCLUSIONS

1. The neurotoxicant, 3-nitropropionic acid (3-NPA) elicited a marked concentration-dependent oxidative stress response in mitochondria and synaptosomes prepared from different brain regions of prepubertal mice. 3-NPA administration (i.p., 50 and 75 mg/kg bw/d for 2d) induced a marked reduction in the activity of SDH in brain and dose-dependent enhancement in various markers of oxidative stress, compromised antioxidant enzyme activities and depleted levels of glutathione in cytosol and mitochondria suggesting a state of oxidative stress in vivo. The induction levels in striatum were more robust as evidenced by elevated lipid peroxidation, protein oxidation, activities of antioxidant enzymes and reduced glutathione.

2. In prepubertal male mice, Rotenone exposure (i.p. 0.5 and 1.0 mg /kg bw/d for 7 d) resulted in a marked dose dependent elevation in lipid peroxidation, ROS generation, protein carbonyls, and alterations in enzymic antioxidants in both cytosol and mitochondria of all brain regions. Depletion of glutathione levels was a common feature in cytosol as well as mitochondria of brain regions. Mitochondrial dysfunctions in cortex and striatum among rotenone administered mice comprised of decreased mitochondrial viability, reduction in the activities of complex I and complex I-III. Significant increase in the activities of cholinergic enzymes, AChE and BChE were also evident among brain regions of rotenone exposed mice.

3. Growing male mice fed Khesari dhal (30% in diet) exhibited significant elevation in markers of oxidative stress as evidenced by enhanced MDA levels, ROS generation with concomitant alterations in the activity of antioxidant enzymes and increased protein carbonyl levels in brain regions. However no such alterations were noticeable in mice fed detoxified KD. KD fed mice showed marginal depletion of GSH levels in all brain regions, while detoxified KD fed mice showed normal levels. Among KD fed mice, both mitochondria and microsomal fractions of brain regions were also subjected to significant oxidative stress induced dysfunctions. KD fed mice showed significant inhibition of AChE activity in brain regions, clearly suggesting cholinergic dysfunctions, while no significant alterations were evident in DKD fed mice brain regions.

4. *Centella asiatica* leaf powder fed to prepubertal male mice (0.5, 1.0% in diet) caused significant diminution in the levels of oxidative markers (MDA, ROS and HP) and reduced levels of protein carbonyls in both cytosol and mitochondria of different brain regions. Enhanced GSH levels, total thiols and non-protein thiols in cytosol/ mitochondria of brain regions of mice were accompanied by concomitant elevation in the activity of various antioxidant enzymes suggesting the potential of CA to augment the levels of antioxidant defense in brain regions. Increase in the activity of AChE enzyme was uniform in all the brain regions of mice fed CA clearly indicating its potential to modulate cholinergic functions *in vivo*

5. A standardized CA aqueous extract exhibited significant antioxidant activity in selected chemical systems and marked free radical scavenging activity in mitochondria, synaptosomes and slices under 3-NPA –induced oxidative stress *in vitro.*

6. Supplements of CA leaf powder was able to significantly attenuate Khesari dhal induced oxidative stress in mitochondria/cytosol of brain regions of mice as assessed by varying degree of protection against oxidative dysfunctions. Alterations in antioxidant enzymes were restored to near normalcy and considerable protection against alterations in TCA cycle enzymes, decreased Na^+ K^+ ATP-ase activity and ETC enzymes was evident among mice given CA supplements.

7. Prepubertal male mice given CA extract prophylaxis were markedly protected against 3-NPA-induced increased oxidative stress in both cytosol and mitochondria. There was complete protection against neurotoxicant–induced elevations in oxidative markers and alterations in enzymic antioxidant defenses in cytosol/mitochondria. CA prophylaxis significantly ameliorated 3-NPA induced depletion of GSH, total thiols and non-protein thiols in both mitochondria and cytosol of striatum and other brain regions suggesting its neuroprotective efficacy.

8. Mitochondrial impairments elicited by 3-NPA were prevented to a greater extent among mice given CA prophylaxis. These comprised of alterations in the activity of citrate synthase, TCA cycle enzymes, ETC complex enzymes, activity of Na^+K^+ ATP ase. 3-NPA-induced increase in mitochondrial swelling property as well as reduced viability of mitochondria (MTT assay) was also significantly modulated by CA prophylaxis.

9. Collectively these experimental evidences demonstrate that CA leaf powder possesses considerable neuromodulatory activity at low dietary levels in prepubertal male mice. Further the aqueous extract exhibited marked prophylactic efficacy as evidenced by abrogation of 3-NPA induced early oxidative stress and mitochondrial dysfunctions. It is hypothesized that the neuroprotective action of CA extract may be related to its multiple free radical scavenging potency, ability to enhance antioxidant defenses GSH levels and redox status.

10. Consumption of *B. monnieri* leaf powder in the diet (0.5 and 1.0%) caused significant diminution in the levels of endogenous oxidative markers (MDA, ROS and HP levels) in both cytosol and mitochondria of different brain regions of prepubertal mice which is suggestive of its ability to prevent peroxidation *in vivo.* Reduction in protein carbonyls levels in brain regions of BM mice indicated its potential to inhibit protein oxidation events.

11. *B. monnieri* fed mice exhibited enhanced GSH levels, total thiols and non-protein thiols in both cytosol/mitochondria of brain regions and concomitant elevation in the activity of various enzymic antioxidant defenses indicating the potential of BM to augment the antioxidant defence machinery uniformly in different brain regions . BM fed mice showed significant reduction in the activity of AChE activity in brain regions indicating its ability to modulate cholinergic function.

12. A standardized ethanolic extract of BM exhibited significant antioxidant activity in selected *in vitro* chemical systems and marked free radical scavenging activity in mitochondria, synaptosomes and slices under 3-NPA –induced oxidative stress *in vitro.*

13. BM extract prophylaxis markedly protected prepubertal mice against 3-NPA induced early oxidative stress in striatum and other brain regions. Further 3-NPA-induced depletion in GSH, thiols/non-protein thiols were also completely mitigated by BM prophylaxis. 3-NPA induced alterations in the activity of antioxidant enzymes striatum and other brain regions (cytosol and mitochondria) were significantly ameliorated by BM prophylaxis. Varying degree of protection in the activities of TCA cycle enzymes and thioredoxin reductase were also evident.

14. BM prophylaxis significantly attenuated 3-NPA induced elevations in the activity of LDH, ETC enzymes, iron levels and alterations in vitamin C levels. Further the perturbations in cholinergic enzymes AChE and BchE were also modulated.

15. BM prophylaxis significantly protected the 3-NPA induced mitochondrial dysfunctions such as reduction in mitochondrial respiration (MTT assay), increased mitochondrial swelling and decreased mitochondrial Na^+ K^+ activity in striatum and other brain regions.

16. BM extract treatment offered uniform and marked protection to prepubertal mice against rotenone-induced oxidative impairments in all brain regions. Further rotenone-induced depletion in GSH was completely normalized by BM treatment. Rotenone- induced alterations in the activity of antioxidant enzymes in striatum and other brain regions were significantly ameliorated by BM treatment. Varying degree of protection in the activities of TCA cycle enzymes and thioredoxin reductase were also evident.

17. BM extract treatment resulted in significant protection against increased activity of LDH and decreased ETC enzymes. Further significant ameliorative effect was also evident in the activities of cholinergic enzymes. BM treatment resulted in amelioration of rotenone –induced reduced mitochondrial viability (MTT assay) and increased mitochondrial swelling in all brain regions indicating the mitochondria targeted protective effect of BM ethanolic extract.

18. BM treatment resulted in significant protection against rotenone –induced reduction in dopamine levels in striatum of prepubertal mice. Interestingly rotenone failed to induce any significant motor function deficits among mice given BM treatment which may be due to the normalization of dopamine levels in striatum. Histopathological examinations also confirmed the neuroprotective efficacy of BM extract against dopaminergic neurotoxicity.

19. BM treatment markedly attenuated the rotenone induced motor dysfunctions among prepubertal mice as determined by three measurements viz., Pole test, Rota Rod and Stride length. Significant increase in the motor co-ordination was evident among mice given BM extract treatment.

20. Exposure of dopaminergic neuronal cells (N27cells) to neurotoxicants (3-NPA and Rotenone) caused concentration dependent cell death and the toxicity profile for rotenone was LC 50: 8µM and LC75: 16µM and 3-NPA was LC 50: 4mM and LC75: 6mM. However, exposure of N27 cells to *B. monnieri* ethanolic extract did not induce any significant cytotoxicity upto 8µg as measured by MTT assay and LDH leakage.

21. With BM extract treatment alone, N27 cells exhibited a concentration dependent decrease in ROS and HP levels suggesting its ability to modulate endogenous markers of oxidative stress. Neurotoxicant exposure caused marked elevations in oxidative markers, while pretreatment of cells with BM extract resulted in significant abrogation of oxidative markers suggesting its neuroprotective property.

22. BM extract treatment alone caused a concentration dependent increase in GSH levels and decrease in GSSG levels in N27 cells, while the levels were depleted following rotenone and 3-NPA exposure. Pretreatment of cells with BM extract prior to neurotoxicant exposure caused significant restoration of GSH levels clearly indicating that the neuroprotective action of BM may be partly attributable to upregulation of GSH and related mechanisms.

23. Further evidences were discernible in terms of enhanced activities of glutathione related antioxidant enzymes such as GSH-Px, GST and GR in BM extract treated cells, while the levels were reduced significantly in rotenone and 3-NPA treated cells. On the other hand treatment of cells with BM extract resulted in varying degree of normalization of enzyme activities.

24. Interestingly, the reduction in the activities of mitochondrial enzymes such as citrate synthase and malate dehydrogenase among rotenone and 3-NPA treated cells were significantly restored to normalcy with BM pretreatment. However, no protection was noticeable with SDH activity. BM treatment also offered significant protection against DNA damage and protein profile alterations induced by rotenone and 3-NPA exposure suggesting its high neuromodulatory potential.