Assessment of neuroprotective efficacy of phytochemicals against oxidative stress mediated neurodegeneration in *Drosophila melanogaster*

A thesis

Submitted to the

Faculty of Bioscience

University of Mysore

For the degree of

Doctor of Philosophy

By

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October 2010

Department of Biochemistry and Nutrition

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

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Declaration

I hereby declare that the thesis entitled "**Assessment of neuroprotective efficacy of phytochemicals against oxidative stress mediated neurodegeneration in *Drosophila melanogaster*"** submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy** in **Bioscience** 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 November 2005 – October 2010.

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

Ravikumar Hosamani

Place: Mysore
Date: October 2010

Certificate

This is to certify that the thesis entitled "**Assessment of neuroprotective efficacy of phytochemicals against oxidative stress mediated neurodegeneration in *Drosophila melanogaster*** submitted by Mr. Ravikumar Hosamani, to the University of Mysore, Mysore, for the degree of Doctor of Philosophy is the result of work carried out by him under my supervision in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore - 570 020 during November 2005 – October 2010.

Signature of the candidate

Signature of the Guide

Signature of the Chairperson/HOD with seal

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Certificate

I hereby certify that the thesis entitled "**Assessment of neuroprotective efficacy of phytochemicals against oxidative stress mediated neurodegeneration in *Drosophila melanogaster*"** submitted by Mr. Ravikumar Hosamani for the degree of Doctor Philosophy in Bioscience, University of Mysore is the result of research work carried out by him at the department of Biochemistry & Nutrition, CFTRI, Mysore under my guidance and supervision during the period of November 2005-October 2010.

Dr. Muralidhara
Guide

Place: Mysore
Date: October 2010

Dedicated to

My parents

Acknowledgements

Being a student of **Dr. Muralidhara** has been the most enriching experience of my academic life. He is the most inspiring teacher I ever came across post my school days. I learnt from him the indispensable roles of passion, rigour and honesty in doing good scientific research. It was his view expressed in numerous discussions in the lab that helped me to appreciate the relevance of NeuroToxicology. I thank him for introducing me to the wonderland of flies and also the academic freedom that he provided throughout my doctoral research. Working with him was a rare opportunity as he is well beyond just a research guide helping in the overall intellectual development of me as a student.

I am very much thankful to Dr. V.Prakash, Director, CFTRI for his support to carry out my research work in the institute.

It's my immense pleasure to be grateful to Prof. S. R. Ramesh Department of Studies in Zoology for allowing me to utilize his lab facilities for maintaining and conducting my entire fly work. I also convey my regards to National *Drosophila* Stock Centre for providing stocks of *Drosophila*.

I would take this opportunity to thank Dr. P.V. Salimath, Head, Department of B & N, for his support and encouragement throughout my stay at department.

I wish to express my heartfelt thanks to Dr. Rajini PS, Scientist, FPIC Department for her kind help, support and encouragement during my Doctoral programme.

I wish to express my sincere thanks to the dept. staff viz., Dr. K Srinivasn, Dr. GM Muralikrishna, Dr. YP Venkatesh, Dr. KS Jaganath Rao, Dr. Akiilender Naidu, Dr. Shylaja Dharmesh, Dr. UJS Prasad Rao, Dr. V Baskaran, Dr. S Muthukumar, Dr. Kalpana Platel, Dr. CD Nandini. Dr. Mahadevamma, Sri S Vishwanatha, Mr. Vijaykumar and all the former and present staff of the department, who have helped me directly and indirectly during my research period. I would also like to extend my sincere thanks to all the members of animal house.

It gives me immense pleasure to extend my sincere thanks to all my lab mates Dr. Rajeshkumar, Dr. B Shreelatha, Dr. MS Mahesh, Dr. K Doreswamy, Dr. BM Thyagaraju, Dr. GK Shinomol, Dr. KN Chandrashekar, Manjunath, Lalith, Sebastin, Denny, Girish chandran, Gokul, Sathyaji and Dr. Chayakumari who made my life enjoyable and memorable at CFTRI without their support and help I am sure it would not have been possible to achieve my goal.

I extend my special thanks to all my friends at the Zoology department, UOM viz., Shruthi, Dinesh, Ramakrishna, Preeti, Ranjita, Ranjini, Bijaya and Kumar (kitchen staff) for their timely help and kind support during my work at Gangothri.

My earnest appreciation to all my friends, Dr. Kisan, Dr. Vasu, Apoorva, Shashi, Raju, Dr. Guruprasad, Leelaja, Neymath, Diwakar, Raghavendra, Srikanta, Satish, Chandru, Madhu, Ramesh, Vasu, Harishbabu, Revanappa, Umesh, Girish, Kushal, Rajpreeth, Kavitha, Sangeetha, Aruna, Sindhuja, Padma, Chandu, Ashok, Ravi, Dr. Ivan, Kiran, Srikanth who were extremely cordial, helping during my stay at CFTRI.

I am fortunate enough to have a bunch of wonderful and ever-ready help of my UG/school friends Bellad, Sangamesh, Kotresh, Dr. Gotyal, Prabhu Nayak, Gururaj, Dileep, Umesh Patil, Prashanth, and Mallikarjun Sapali, who all stood with me in my entire thick and thins.

I am very much thankful to my HKB (Hisar Kannada Balaga) friends - Satish, Dr. Suresh, Dr. Subhash, Gangadhar, Venky, Malakari, Swamy, Shridhar, Annasab, Govindreddy, Mohan sir and Govindaraju sir, for their help and support.

I canot forget those moments of arguments and discussions with my mess friends –Sadhu sir (the veteran), Reddy, Surya, Malli, Darukesh, Raju, Desia, Dr. Kumaresan, Dr. Mohan Dhale, Dr. Ashwini Sharma, Dr. Vishwanath, Dr. Police goudru, Prashanth SJ, Dr. Anil, Dr. Gangadhar, Banergy, Harsha, Dr. Vidya, Dr. Venkat and Vinod.

I will be failing in my duties, if I do not surface my genuflect love and affectionate gratitude to my beloved parents Shri. Ramesh and Smt. Prema Hosamani, Babu (brother), Shylashree (sister) and brother-in-law Subhash who constantly inspired, educated, guided and moulded me into the present position and whose constant fountain of love and encouragement brings out of my best all my endeavors. Without their blessings this thesis would never have seen the light of the day.

I am thankful to M/s Himalayan drugs Pvt. Ltd, Bengaluru for their sample gift of *Bacopa monnieri* extract, which is heart of my thesis. I acknowledge the UGC-CSIR, New Delhi for the award of Junior/Senior Research Fellowships.

Finally, happiness lies in pursuit as much as in reaching the good and today I stand with the kernel of my endeavor. While pursuing it, many a known and unknown hands pushed me forward, learned souls put me on the right path with their knowledge and experience. No words could adequately express my feelings. I shall ever remain thankfully indebted to them all. My apologies to anyone whom i have failed to mention.

Ravikumar Hosamani

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

Rot	Rotenone	nm	Nano meter
AA	Acylamide	nmol	Nano molar
PQ	Paraquat	O^{-2}	Superoxide
BM	*Bacopa monnieri*	pmol	Pico molar
CA	*Centella asiatica*	ROS	Reactive oxygen species
WS	*Withania somnifera*		
bw	Body weight	v/v	Volume/volume
Ctr	Control	W/v	Weight/volume
ATP	Adenosine triphosphate	W/W	Weight/weight
CDNB	1-chloro-2,4-dinitrobenzene	µ	Micro
Cyto	cytosol	p	Pico
EDT	Ethylene diamine tetracetic acid	^{0}c	Degree Celsius
g	Gram		
GSH	Glutathione		
GST	Glutathione-S-transferase		
h	hours		
GST	Glutathione-S-transferase		
H_2O_2	Hydrogen peroxide		
i.p	Intraperitoneally		
HPLC	High performance liquid chromatography		
Kg	Kilogram		
LPO	Lipid peroxidation		
M	Molar		
MEC	Molar extinction co-efficient		
MDA	Malondialdehyde		
mg	Milli gram		
min	Minute		
mL	milli litre		
n	Number		
NADH	Nicotinamide Adenine Dinucleatide reduced		
NADP	Nicotinamide Adenine Dinucleatide phosphate		
NADPH	Nicotinamide Adenine Dinucleatide phosphate reduced		

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ABSTRACT

Currently, there has been an increased interest globally to identify antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine. As plants produce significant amount of antioxidants to prevent oxidative stress, they represent a potential source of new compounds of therapeutic use. Numerous clinical and experimental studies have also demonstrated the putative positive benefits of antioxidants in altering, reversing or forestalling the neuronal and behavioral dysfunctions. Involvement of oxidative stress is well accepted concept among pathophysiology of various neurodegenerative diseases (NDD).

Owing to the existence of rich background literature, coupled with easy culture methods and short lifespan, *Drosophila* have been extensively utilized in the recent past to understand human NDD. In general studies in *Drosophila* have translated well to mammalian systems. To the best of our knowledge, the *Drosophila* system has not been employed as a model to understand the neuroprotective effects of phytochemicals. Hence, it is hypothesized that *Drosophila* can be utilized as a rapid model system to test the efficacy of putative neuroprotective compounds.

Utilizing *Drosophila melanogaster*, screening of phytochemicals for their potency to modulate neurotoxicant (Rotenone, Paraquat and Acrylamide) induced lethality response revealed the higher potential of *Bacopa monnieri* (BM) extract. Chronic systemic exposure to rotenone, a specific mitochondrial complex I inhibitor and a prime risk factor in PD pathogenesis has been exploited to understand oxidative stress -mediated neurodegeneration. Using this model specific question/s related to the possible neuropharmacological properties of *BM* leaf powder against rotenone-induced oxidative stress and neurotoxicity have been addressed. Biochemical evidences revealed that dietary feeding of BM to *Drosophila* for a short duration has the propensity to attenuate rotenone induced oxidative stress owing to its antioxidative nature and its ability to modulate the activity of antioxidant defenses. Additional evidences viz., lower incidence of rotenone induced mortality and higher resistance to paraquat among flies given BM prophylaxis clearly support such a

mechanism/s. Further, its neuro-modulatory potency was ascertained by its ability to significantly improve the locomotor performance among rotenone exposed flies and restoration of Dopamine levels**.**

Several evidences related to the neuroprotective efficacy of BM prophylaxis was obtained in the paraquat (PQ) model in *Drosophila.* BM prophylaxis markedly attenuated the PQ induced lethality, oxidative stress and mitochondrial functions determined in terms of the activities of TCA cycle and ETC enzymes. These neuroprotective effects of BM in the *Drosophila* model were validated in a mice model employing both acute and chronic paradigms of PQ. Interestingly prepubertal mice given BM prophylaxis (200 mg/kg bw /d, 4 weeks) were more resistant to acute PQ induced oxidative stress and mitochondrial damage. Differential protective effects were evident in different brain regions. Further, BM treatment of mice in a 'Chronic PQ intoxication regimen' rendered marked protection to cerebellum and striatum in terms of oxidative impairments, cholinergic function, and mitochondrial dysfunction. The findings in the fly model were reproducible in the rodent model suggesting the utility value of Drosophila to screen the neuroprotective efficacy of phytochemicals.

Since several NDD involve multiple cellular mechanisms, a combination of two or more potential compounds may yield additive or synergistic neuroprotective effects. Such an attempt was made in the *Drosophila* model by testing a combination of BM extract and creatine, a well known ergogenic nutritional supplement. The neuroprotective efficacy of creatine-enriched diet in *Drosophila* system was clearly evidenced by its ability to modulate endogenous oxidative markers, its propensity to mitigate rotenone-induced mitochondrial oxidative stress, restoration of dopamine levels and attenuation of neurotoxicity. Interestingly, a combination of BM extract and creatine at lower concentrations appeared to completely offset rotenone-induced neurotoxic effects suggesting a clear synergistic effect. Collectively these evidences confirm the utility value of *Drosophila* as a primary tool to rapidly screen compounds suspected to possess neuropharmacological properties prior to their testing in mammalian models and further therapeutic use in humans.

PREFACE

In this part of the thesis, an attempt has been made to review the relevant literature under **four** separate sections.

In the **first section**, a brief account of current and general understanding on oxidative stress, general implications of free radicals and an overview of the antioxidant defense mechanism/s in the nervous system have been presented.

The **second section** focuses on i) neurodegenerative diseases (NDD) and the vital role of oxidative stress in their progression (ii) Current understanding on the role of mitochondria and biochemical targets of oxidative stress in NDD (ii) Insights into the reasons underlying the increased vulnerability of brain in general to oxidative stress (iv) Chemical models of neurotoxicity employed in understanding the pathophysiology of NDD.

In the **third section**, a *Drosophila* model system has been described by providing a bulk of information from history, life cycle to how this model has been exploited to identify key pathways involved in various neurological diseases. Information is also provided as to how this model has been used for screening research and newly emerging areas like Drosophotoxicology.

In the **fourth section**, a comprehensive account on the importance of "neuroprotective" pharmacological therapies are provided with emphasis on various dietary antioxidants. In the end, a detailed literature on the medicinal plant - *Bacopa monnieri* (L) is presented.

1.0 FREE RADICALS AND OXIDATIVE STRESS

Free radicals and other reactive species

Oxygen radicals are involved in many biochemical activities of cells such as signal transduction and gene transcription. Human body produces oxygen free radicals and other reactive oxygen species as by-products through numerous physiological and biochemical processes. Free radicals are molecules with unpaired electron in their outer orbit. Unpaired electrons act as an electron acceptor and essentially "steal" electrons from other molecules, leading to their oxidation. The most common cellular free radicals are hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{\bullet -}$), and nitric oxide ($NO^{\bullet}$). Oxygen related free radicals (superoxide and hydroxyl radicals) and reactive species (hydrogen peroxide, nitric oxide, peroxynitrite and hypochlorous acid) are produced in the body primarily as a result of aerobic metabolism (Halliwell, 1994; 2006).

When the generation of oxidants exceeds the rate at which endogenous antioxidant defenses can scavenge oxidants; proteins, lipids, DNA and other macromolecules become targets for oxidative modification which leads to deterioration of cellular structural architecture, signaling and ultimately death. Also oxidized proteins present a threat to cell survival because apart from losing their functionality, they tend to form toxic aggregates termed lipofuscin. These compounds comprising lipids and a heterogeneous mix of proteins compromise the activity of lysosomes and could eventually lead to cell death (Yorimitsu and Klionsky, 2007).

Mitochondria- site of ROS generation

Generation of ROS is an inevitable outcome of oxygen dependent (aerobic) respiration. Reactive oxygen species (ROS) are defined as molecular entities that react with cellular components resulting in detrimental effects on their function. Most cellular ROS are generated during incomplete metabolic reduction of oxygen to water (Andersen, 2004). Mitochondria are considered a key source of reactive species. In eukaryotes, mitochondria generate energy in the form of ATP from macromolecules *via* Krebs cycle and electron transport

chain (ETC). The high energy end products of Krebs cycle, NADH and $FADH_2$ donate electrons to a series of electron carriers on ETC. This creates a proton gradient across the inner mitochondrial membrane which drives the oxidative phosphorylation of ADP to ATP by the ATP synthase. Therefore, functional compromise of this organelle has a large impact on oxidative homeostasis (Anthony et al., 2009).

Decreased complex I activity in the mitochondrial respiratory chain has been observed not only in SNpc of PD patients but also in platelets and cybrid cell lines (Swerdlow et al., 1996). This inhibition can lead to the generation of ROS which when produced in the near vicinity may target the respiratory chain leading to further inhibition with subsequent ROS production and mitochondrial damage (Zhang et al., 1990). Mitochondrial related energy failure may also disrupt the vesicular storage of DA leading to increased free cytosolic concentrations of the auto-oxidizable neurotransmitter. Importantly, dopaminergic neurons of the SNpc have been shown to be uniquely sensitive to complex I inhibition (Betarbet et al., 2000).

An overview of antioxidant defenses

Antioxidants are classified as exogenous (natural or synthetic) or endogenous compounds both responsible for removal of free radicals, scavenging ROS or their precursors, inhibiting formation of ROS and binding metal ions needed for catalysis of ROS generation. Natural antioxidant system is sorted in two major groups: enzymatic and non-enzymatic.

Enzymatic antioxidants are comprised of limited number of proteins such as catalase, glutathione peroxidase as well as superoxide dismutase (SOD) along with some supporting enzymes. Superoxide dismutase (SOD) is a ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress. It catalyzes $O_2^{\bullet-}$ radicals to H_2O_2. There are three forms of SOD. The copper-zinc SOD is located in the cytosol, the manganese SOD is primarily a mitochondrial enzyme and extracellular SOD is usually found on the outside of the plasma membrane (Folz et al., 1997). SOD speeds the conversion of superoxide to hydrogen peroxide.

Non enzymatic antioxidants are direct acting antioxidants, which are extremely important in defense against oxidative stress. Most of them include ascorbic and lipoic acid, polyphenols and carotenoids derived from dietary sources. The cell itself synthesizes a minority of these molecules. Indirectly acting antioxidants mostly include chelating agents and bind to redox metals to prevent free radical generation (Gilgun-Sherki et al., 2001).

Glutathione, the most abundant thiol-containing antioxidant of low molecular weight in cells is synthesized from glutamate, cysteine and glycine. As a major component of the cellular antioxidant system GSH has the following characteristics: 1) GSH effectively scavenges ROS (e.g., lipid peroxyl radical, peroxynitrite and H_2O_2) directly and indirectly through enzymatic reactions; 2) 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 3) GSH interacts with thiol proteins to regulate cellular redox homeostasis (Fang et al., 2001).

Vulnerability of central nervous system (CNS) to oxidative stress

The nervous system is composed of a wide variety of neurons and glial cells and has a very high metabolic rate that is almost exclusively dependent on aerobic glucose-dependent metabolism (Abbott et al., 1994). To support this high metabolic demand the brain receives approximately 15% of the total cardiac output and accounts for 20% of the oxygen consumption for the entire body, despite the fact that the brain accounts for only 1.5 to 2% of the total body weight. Thus, the nervous system is extremely sensitive to neurotoxicants that disrupt mitochondrial function and energy metabolism. High metabolic rate, elevated concentrations of polyunsaturated fatty acids, and low to moderate levels of antioxidant enzymes in the brain also predispose this organ to oxidative damage (Evans, 1993).

The major factors which render the brain more susceptible to oxidative stress can be summarized as follows:

High oxygen uptake: Brain is exposed to high oxygen concentrations, utilizing about one-fifth of the oxygen consumed by the body; compared to other tissues, the highest amount of oxygen to produce energy (Halliwell, 2006).

*Presence of iron***:** Iron, which accumulates in the brain as a function of age can be a potent catalyst for oxidative species formation and a powerful promoter of free radical damage in brain by catalyzing generation of highly reactive hydroxyl, alkoxyl/ peroxyl radicals from H_2O_2 and lipid peroxides respectively. There is increasing evidence that iron misregulation is involved in the mechanisms that underlie many NDD and as the brain ages, iron accumulates in regions that are affected by AD and PD (Thompson et al., 2001).

Enriched peroxidizable fatty acids: Brain is rich in polyunsaturated fatty acids that labile to peroxidation and oxidative modification. Double bonds of unsaturated fatty acids are hot spots for attack by free radicals that initiate cascade or chain reaction to damage neighboring unsaturated fatty acids (Butterfield and Lauderback, 2002).

Lower antioxidant defense: Brain is relatively deficient in antioxidant systems with lower activity of glutathione peroxidases and catalase compared to other organs (Mariani et al., 2005). Oxidative defenses extend beyond the classical antioxidant enzymes and low-molecular weight reductants. It is likely that oxidative stress arises early because antioxidant mechanisms undergo at least a transient compensatory increase that is apparently insufficient due to continued oxidative damage during disease progression.

Glutamate mediated excitotoxicity: Death of neuronal cells can cause massive excitotoxic glutamate release from brain extracellular fluids leading to excessive and prolonged increase in intracellular free Ca^{2+} and Na^{+}. Oxidative stress can damage neurons and release of excitatory amino acids (Mailly et al., 1999).

Enzymatic oxidative deamination of catecholamine: Autoxidation of catecholamine neurotransmitters like dopamine, serotonin and norepinephrine can react with O_2 to generate not only $O_2^{\bullet -}$ but also quinones/semiquinones that can deplete reduced glutathione (GSH) (Spencer et al., 1998). These catecholamines can spontaneously break down to free radicals or be metabolized to free radicals by endogenous enzymes such as monoamine oxidizes can render CNS prone to injury.

Metabolism in brain: Ongoing metabolism in brain generates H_2O_2 by many enzymes like monoamine oxidases A and B, flavoprotein enzymes located in the mitochondria leading to neuronal death (Halliwell, 2006).

Hemeproteins: Cytochromes P450 (CYPs), a family of heme proteins that metabolize xenobiotics in some brain regions are another potential source of oxidative stress. Isoform of cytochrome P450 (e.g., CYP2E1) readily releases electrons during its catalytic cycle producing more ROS (Gonzalez, 2005).

Hemoglobin may be neurotoxic: Protein hemoglobin may sometimes be neurotoxic. Isolated hemoglobin is degraded on exposure to excess H_2O_2 with release of prooxidant iron ions from the heme ring. In addition, hemoglobin reacts with H_2O_2 and other peroxides to form oxidizing species (heme ferryl) capable of stimulating lipid peroxidation (Gutteridge, 1986).

2.0 NEURODEGENERATIVE DISEASES

Neurodegenerative disorders (NDD) collectively are the leading cause of disability in the elderly, accounting for a significant increased proportion of morbidity and mortality in the developed world. As our population ages, an improved understanding of these diseases will be vital to develop more effective therapies and combating the staggering personal, social and economic costs of these diseases (Samii et al., 2004).Over the last two decades there has been enormous progress in understanding the initiating factors that trigger complex cascades that ultimately result in various NDD. Much of this progress has been the result of biochemical and histochemical characterization of proteins that accumulate within various inclusions in the diseased brain and genetic linkage studies identifying mutations in genes that cause NDD (Ross and Poirier, 2004).

Alzheimer's disease (AD): AD is a late-onset dementing illness with progressive loss of memory, task performance, speech and recognition of people and objects with degeneration of neurons (particularly in the basal forebrain and hippocampus) and altered neuronal connections (Selkoe, 2002). AD involves two major kinds of protein aggregates - Extracellular aggregates known as neuritic plaques and intracellular neurofibrillary tangles (NFTs).

Extracellular aggregates have Aβ peptide as their major constituent which is derived from proteolytic processing of the amyloid precursor protein (APP) (Serpell and Smith, 2002). Aβ is known to be toxic and plays a crucial role in AD pathogenesis and that toxicity is enhanced when the Aβ peptide becomes aggregated. Intracellular aggregates of the microtubule associated protein *tau* are called as neurofibrillary tangles (Esler and Wolfe, 2001).

Parkinson's disease (PD): PD is the second most common NDD and is progressive with a mean age at onset of ~55 and its incidence increases markedly with age. The primary hallmark of PD is the degeneration of the nigrostriatal dopaminergic pathway with depleting brain dopamine initiating aberrant motor activity such as tremor at rest, rigidity, slowness of voluntary movement and postural instability. Recessive early-onset PD can be caused by mutations in the genes encoding synuclein, parkin, DJ-1 or PINK1 presumably by a loss-of-function mechanism. A definitive diagnosis of PD can only be made at autopsy and it has customarily been based not only on the loss of nigrostriatal dopaminergic neurons but also on the presence of intraneuronal inclusions called Lewy bodies- the spherical eosinophilic cytoplasmic aggregates containing a variety of proteins of which α-synuclein is a major component and are found in every affected brain region (Vila and Przedborski 2004; Mizuno et al., 2008)

Huntington's disease (HD): HD is a progressive NDD characterized by progressive motor disorder (choreiform movements) associated with perseverative behaviour and impairment in cognition and plantification. It is caused by expansion of a CAG repeat coding for polyglutamine in the N-terminus of the huntingtin protein. The most striking neuropathological hallmark of HD is atrophy of the striatum and to a lesser extent the cerebral cortex (Vonsattel et al., 1998). Huntingtin aggregates contain fibers and appear to have β-SHEETS, as is a characteristic of amyloid. The complex II inhibitor like3-nitropropionic acid (3-NPA) can cause striatal lesions similar to HD (Norenberg and RamaRao, 2007).

Amyotrophic lateral sclerosis (ALS): ALS is a progressive fatal NDD characterized by progressive muscular paralysis, motor weakness, spasticityand atrophy reflecting degeneration of motor neurons in the primary motor cortex, corticospinal tracts, brainstem and spinal cord. The disease prevalence is ~5 people per 100,000 and the risk of ALS increases by one order of magnitude after age 60. Overall, there is a slight male prevalence (M:F ratio ~1.5:1). Symptoms of ALS are related to focal muscle weakness where the symptoms may start either distally or proximally in the upper limbs and lower limbs. Pathogenic mechanisms which may contribute to motor neuron injury and cell death in ALS are genetic factors, oxidative stress, protein aggregation, glutamatergic toxicity, mitochondrial dysfunction, impairment of axonal transport and inflammatory cascades (Rakhit, 2002).

Role of mitochondria in neurodegeneration

Mitochondria are key regulators of cell survival, death and a dysfunction of mitochondrial energy metabolism leading to reduced ATP production, impaired calcium buffering and increased generation of reactive oxygen species (ROS) (Beal, 2005). Several evidences suggest that mitochondria have a central role in ageing related NDD (Petrozzi et al., 2007).

In AD impaired energy metabolism and abnormalities of mitochondrial respiration are features of autopsied brain tissue affected by neurodegeneration but also of peripheral cells of AD patients (Small et al., 1995). Biochemical analysis in post-mortem AD brains has yielded evidence of/ impaired activities of three key enzymes: the pyruvate dehydrogenase complex (PDHC), the α-ketoglutarate dehydrogenase complex (KGDHC) of the tricarboxylic acid (TCA) cycle (Krebs' cycle) in the mitochondria. In PD mitochondrial involvement in the pathogenesis of PD is supported by post-mortem biochemical studies showing defect of the mitochondrial respiratory complex I, was found in samples of SNpc from patients who suffered from idiopathic PD. MPP^+ binds and inhibits complex I of the respiratory chain, leading to an inhibition of ATP synthesis and the generation of free radicals (Petrozzi et al., 2007).

In HD there is considerable evidence that one of the consequences of the gene expansion may be a mitochondrial metabolic defect resulting in

impaired energy metabolism which in turn can lead to an increased oxidative damage in HD. Biochemical studies of brain tissue from HD patients have demonstrated multiple defects in the caudate: decreased complex II activity and decreased complex II-III activity and no alteration of complex I or IV activities. In ALS, evidences from electron microscopy studies have shown abnormal mitochondrial morphology in skeletal muscle. Mitochondrial abnormalities were also detected in intramuscular nerves, proximal axons and anterior horns of the spinal cord of sALS patients. Deficits in the activities of mitochondrial respiratory chain complex I and complex IV have been identified in the skeletal muscle and in the spinal cord of sALS patients (Petrozzi et al., 2007).

Oxidative stress and mitochondrial dysfunction in Parkinson's disease

Although the cause of nigral cell death in PD remains unclear, several hypotheses have emerged based on data from animal/ cell models and genetic studies. The "oxidative stress hypothesis" postulates that a disruption in the balance of reactive oxygen (ROS) and reactive nitrogen species (RNS) contributes to oxidative damage of cellular macromolecules ultimately leading to cell death. The premise of this hypothesis is based on landmark studies demonstrating the potential for the generation of hydrogen peroxide and other ROS during the oxidative metabolism of DA (Graham, 1978), which exposes DAergic neurons of the SNpc to chronic oxidative stress compared to other regions of the brain. Other factors contributing to the increased oxidative stress include: i) high basal levels of aerobic activity, ii) autooxidation of DA, its precursors and metabolites, to form quinones and semiquinones capable of adducting protein sulfhydryl groups including glutathione and iii) increased iron levels in the SNpc contributing to Fenton chemistry (Jenner, 2003; Andersen, 2004). Analysis of postmortem brains from PD patients confirms the high level of oxidative stress in the SNpc marked by increased iron levels (Good et al., 1992), decreased GSH levels (Sian et al., 1994; Dexter, et al., 1994), increased lipid peroxidation and DNA and protein oxidation (Alam et al., 1997).

Mitochondrial dysfunction is also an intrinsic aspect of this hypothesis due to their major role in the production of cellular ROS (Figure 1). Decreased complex I activity in the mitochondrial respiratory chain was observed not only

in SNpc of PD patients (Schapira et al., 1990), but also in platelets (Parker et al., 1989) and cybrid cell lines (Swerdlow et al., 1996). This inhibition can lead to the generation of ROS which when produced in the near vicinity, may target the respiratory chain leading to further inhibition with subsequent ROS production, and mitochondrial damage (Zhang et al., 1990). Mitochondrial related energy failure may also disrupt the vesicular storage of DA, leading to increased free cytosolic concentrations of the auto-oxidizable neurotransmitter. Importantly, DAergic neurons of the SNpc have been shown to be uniquely sensitive to complex I inhibition (Betarbet et al., 2000). Inflammation can also contribute to ROS production and has been implicated in the pathogenesis of PD. Activation of microglia in response to injury is associated with an upregulation of inducible nitric oxide synthase (iNOS) resulting in increased production of NO. Increased immunostaining for iNOS has been detected in the SNpc of PD brains (Hunot et al., 1996), suggesting that RNS may also play a critical role in the disease (Good et al., 1998).

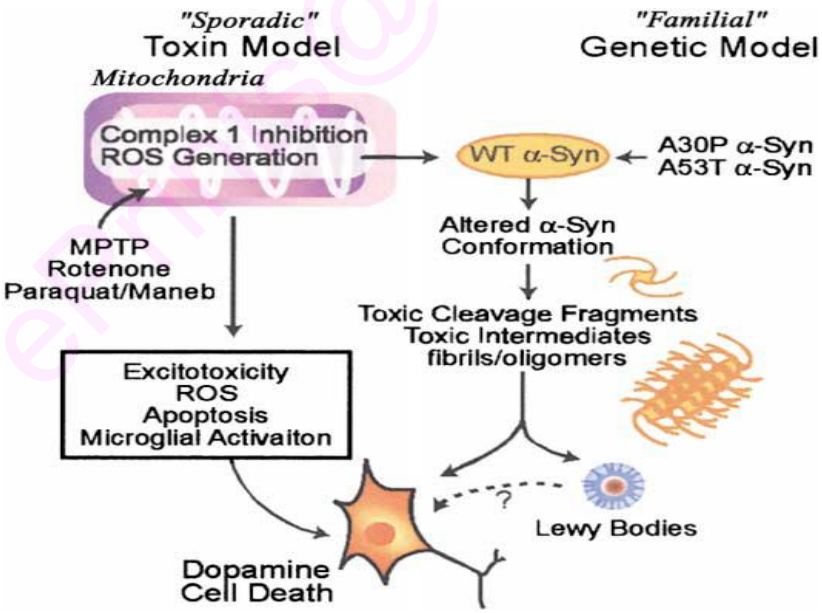


Figure 1

Toxin based model of Parkinson's disease

Numerous toxin based models have been developed, although only a few have been well characterized in regard to their mode of inducing neurodegeneration. 6-OHDA and MPTP have been the most thoroughly studied models whereas agricultural chemicals such as rotenone, paraquat dieldrin and maneb have received increased attention in recent years. While each of these toxicants contributes to the death of SNpc DAergic neurons in part through the induction of oxidative stress their similarities and differences have provided intriguing details on possible mechanisms of neurodegeneration relating to PD.

6-OHDA: the first catecholaminergic neurotoxin: 6-OHDA is a hydroxylated analogue of DA which uses the same transport system as DA and norepinephrine to produce specific degeneration of catecholaminergic neurons. Following stereotactic injection, 6-OHDA causes degeneration of DAergic neurons with dramatic loss of DA in the striatum. The toxic mechanism of this compound is dependent on its oxidation with concomitant production of ROS and para- and semiquinone products. This process appears to occur via a one-electron reduction of oxygen resulting in superoxide ($O_2^{\cdot -}$) and semiquinone radical intermediates, as superoxide dismutase (SOD), a potent scavenger of superoxide radicals, significantly inhibits the oxidation of 6-OHDA (Heikkila et al., 1973). Moreover, 6-OHDA toxicity is significantly inhibited in mice overexpressing cytosolic and mitochondrial forms of superoxide dismutase (SOD1 and SOD2, respectively) (Callio et al., 2005). Para- and semiquinone radicals produced during oxidation of 6-OHDA are capable of inducing cellular damage through reaction with nucelophiles such as protein and DNA, they do not appear to be primarily responsible for the toxic effects of 6-OHDA. Although 6-OHDA does not reproduce all the characteristics of sporadic PD, namely Lewy body formation, this acute model has been used in the efficacy testing of many pharmacological anti-Parkinsonian compounds.

MPTP: paving the way for the environmental hypothesis: In the early 1980s, MPTP was identified as the chemical agent responsible for producing a severe parkinsonian syndrome in a cohort of young adult drug users characterized by many clinical features of PD (Langston et al., 1983). Although

risk of human exposure to this synthetic compound is limited, MPTP has been instrumental in the understanding of pathways contributing to degeneration of DAergic neurons. To date, MPTP remains the best-characterized PD model and has provided the strongest support for the role of oxidative stress in disease pathogenesis. MPTP is highly lipophilic and readily crosses the blood-brain barrier (BBB). In the brain, MPTP is metabolized by monoamine oxidase B in glial cells to an unstable intermediate, followed by spontaneous oxidation yielding its toxic metabolite, 1-methyl-4-phenyl pyridinium (MPP+) (Heikkila et al., 1984; Nicklas et al., 1985). MPP+ is released to the extracellular space and gains entry into DAergic neurons via the dopamine transporter, where complex I of the mitochondrial respiratory chain is thought to be its primary target of inhibition. By disrupting the natural flow of electrons through the system, MPP+ is believed to cause an acute ATP deficiency and increased ROS production, particularly superoxide (Nicklas et al., 1985; Cleeter et al., 1992; Hasegawa et al., 1999).

Many aspects of MPTP toxicity are attenuated in mice over expressing SOD2 and exacerbated in mice with a partial deficiency in SOD2, confirming the role of mitochondrial superoxide (Andreassen et al., 2001; Klivenyi et al., 1998). In addition to mitochondrial-induced ROS generation, MPP+ produces ROS via extrinsic mechanisms. ROS from the auto-oxidation of dopamine may result from the MPP+-induced release of the neurotransmitter from storage vesicles into the cytosol (Lotharius and O'Malley, 2000). The toxic effects of superoxide are well demonstrated. Protective effects against MPTP have been observed following over expression of SOD1 in cytosol and injection into the extracellular space of the SNpc (Wu et al., 2003; Przedborski, et al., 1992). Additionally, catalytic antioxidant synthetic compounds have proven effective in attenuating the toxic actions of MPTP in a variety of *in vitro* and *in vivo* models (Liang et al., 2007; Pong et al., 2000; Kaul et al., 2003).

Rotenone: a complex I inhibitor: Rotenone is a potent member of the rotenoids, a family of natural cytotoxic compounds extracted from tropical plants. It is most commonly used as an insecticide and Piscicide (Uversky, 2004). Rotenone is highly lipophilic, easily crosses the BBB and accumulates in

subcellular organelles including the mitochondria (Talpade et al., 2000). Rotenone binds specifically to complex I, inhibiting the flow of electrons through the respiratory chain. The specific interactions of rotenone with complex I were demonstrated by replacing the endogenous subunit in human neuroblastoma cells with the single-subunit NADH dehydrogenase from *Saccharomyces cerevisiae*. The substitution of this rotenone-insensitive complex I attenuated rotenone's toxic effects (Sherer et al., 2003). In a manner similar to MPP+, rotenone-induced complex I inhibition results in acute ATP deficiency and generation of ROS. Following rotenone-induced inhibition, electron flow through complex I is slowed at upstream sites that are prone to electron leakage. Therefore, with electrons remaining in the site for a longer period of time than normal, molecular oxygen can react via one-electron reduction to produce superoxide which is released in the mitochondria. In addition to the consequences of mitochondrial superoxide generation, the toxicity of both MPTP and rotenone is linked with glial cell activation (Sherer et al., 2003).

Knockout studies in mice have further identified microglial NADPH-oxidase mediated ROS generation as a key contributor to rotenone neurotoxicity (Gao et al., 2002). Rotenone also inhibits the formation of microtubules leading to the accumulation of toxic tubulin monomers (Brinkley et al., 1974; Marshall and Himes, 1978). Rotenone exposure *in vitro* is characterized by a progressive depletion of GSH, oxidative damage to protein and DNA, and induction of apoptosis (Sherer et al., 2003; Greenamyre et al.,2001), all of which are blocked by the antioxidant α-tocopherol. An inhibitor of DA synthesis also attenuated these effects, although its efficacy was limited to DAergic cells, further suggesting a selective vulnerability of these neurons to rotenone-induced oxidative stress (Sakka et al., 2003). Consistent with its potential as a PD toxicant, rotenone is selectively toxic to dopaminergic neurons as compared to GABAergic neurons in primary mesencephalic cultures (Marey-Semper et al., 1995; Sakka et al., 2003), an effect that is greatly exacerbated in the presence of microglia. In contrast with MPP+, rotenone does not appear to possess a specific transport system. Therefore, it is hypothesized that the DAergic neurons in the nigrostriatal pathway are selectively vulnerable to complex I inhibition, an assertion that has been

supported *in vivo*. Chronic exposure to low doses of rotenone in rats resulted in uniform inhibition of complex I throughout the brain, while DAergic neuron numbers in the SN were decreased by 30% (Betarbet et al., 2000). This selective degeneration of nigrostriatal neurons was accompanied by α-synuclein-positive LB-like inclusions.

Animals models utilizing rotenone have been complicated by three factors: 1) significant reductions in non-DAergic striatal neuronal populations (Hoglinger et al., 2003), 2) wide variations in nigrostriatal effects within the same treatment paradigm, and 3) technical difficulty of rotenone use in animals (Betarbet et al., 2000). Nevertheless, the presence of LB-associated DAergic neurodegeneration in this model proved a useful model for exploring the role of protein aggregates in neuronal death. While the rotenone model has provided intriguing evidence for a role of complex I inhibition and subsequent mitochondrial dysfunction and oxidative stress in the etiology of PD, the risk of human exposure to this compound is limited. Rotenone breaks down readily in the environment, with a half-life ranging between 1 and 3 days in water and soil (Bove et al., 2005).

Paraquat: a redox cycling agent: PQ is a quick-acting, non-selective widely applied herbicide that destroys green plant tissue on contact. PQ belongs to a class of bipyridyl herbicides characterized by a structural backbone of two covalently linked pyridine rings. Epidemiological studies suggest an increased risk for developing PD following chronic exposure (Liou et al., 1997). Increased risk for PD is also associated with diquat, another member of the family of bipyridyl herbicides often applied as a mixed preparation with PQ (Di Monte et al., 2003). Despite its striking resemblance MPP+, these two compounds exert their deleterious cellular effects via different mechanisms, although induction of oxidative stress is a shared property (Richardson et al., 2005).

PQ's toxicity is related to its ability to redox cycle, accepting an electron from an appropriate donor with subsequent reduction of oxygen to produce superoxide while also regenerating the parent compound. Redox reaction of PQ produces superoxide in addition to regeneration of the parent compound. SOD overexpression and SOD mimetics are capable of inhibiting the actions of

PQ, supporting the notion that superoxide is a key mediator in the herbicide's toxicity (Day et al., 1996; Patel et al., 1996). Moreover, the redox cycling process is thought to deplete intracellular stores of NAD (P)H due to its increased oxidation. PQ·+ can also reduce iron (III) and iron (III)-chelates, in turn catalyzing the formation of hydroxyl radicals via the Fenton reaction (Clejan et al., 1989). However, the high oxygen content in the brain presumably favors the reaction of PQ·+ with molecular oxygen over iron (III). The rate-limiting step in the redox cycling process is the reduction of PQ by an appropriate electron donor.

In recent years, the involvement of mitochondria in the mechanism of ROS production by PQ has emerged, although studies examining a direct role of mitochondria in redox-cycling with PQ are extremely limited. Fukushima et al.,1993 showed that PQ may generate ROS by accepting electrons from purified complex I of the respiratory chain. Recent study (Castello et al.,2007) have demonstrated that PQ can be taken up into intact, respiring brain mitochondria which represent a major subcellular source in PQ-induced ROS production. Furthermore, ROS production by PQ in brain mitochondria at micromolar concentrations was dependent on mitochondrial membrane potential and significantly attenuated by antimycin A, an inhibitor of complex III. Rotenone showed only limited inhibition, suggesting a role for complex III in PQ-induced mitochondrial ROS production.

An indirect excitotoxic mechanism in response to PQ has also been proposed. This is predicated on data showing that PQ stimulates glutamate efflux from neurons resulting in calcium influx through non-NMDA receptor channels. This influx of calcium can activate neuronal NOS, which may contribute to the formation of peroxynitrite and also participate in the redox cycling process (Shimizu et al., 2003). Activation of the NADPH oxidase of microglia cells has also been shown as a key mediator of PQ toxicity in DAergic cells. The ability of PQ to cross the BBB is severely limited following systemic exposure. However, the process occurs via a carrier-mediated mechanism involving a neutral amino acid transporter as pretreatment with valine or phenylalanine prevents PQ-mediated neurodegeneration in mice (Shimizu et al., 2001; McCormack et al., 2003). PQ is demonstrated to reduce the number

of DAergic neurons in rat organotypic midbrain cultures in a concentration-dependent manner, which is prevented by inhibitors of NMDA, NOS, and caspases (Shimizu et al., 2003).

With the advent of stereological counting, sublethal dosing of PQ over a three-week time course has produced a decrease in TH-positive stained cells in the SNpc. In addition, DAergic neurons in the SN and striatum appear particularly sensitive to PQ, as other subpopulations of neurons were unaffected (McCormack et al., 2002). While no significant depletion of striatal DA has been observed following PQ *in vivo*, evidence for enhanced DA turnover is suggested by increases in TH activity and altered DA metabolite levels (Thiruchelvam et al., 2000).

Mechanisms of selective uptake, mitochondrial-dependent effects, or increased vulnerability of SN neurons may play critical roles in PQ-induced neurodegeneration. Additional evidence to support PQ's status as a Parkinsonian toxin comes from data demonstrating up regulation and aggregation of α-synuclein within SNpc neurons in treated mice (Manning-Bog et al., 2002). Significant increases in 4-HNE-positive neurons and nitrotyrosine immunoreactivity in nigral cells of PQ-treated mice provides further evidence for oxidative injury in the SN (McCormack et al., 2005). A direct role for oxidative stress in PQ-mediated neurodegeneration was observed in SOD1 or glutathione peroxidase (GPx) transgenic mice (Thiruchelvam et al., 2005). Moreover, synthetic SOD/catalase mimetics are capable of decreasing PQ-mediated DAergic neuronal cell death *in vivo* (Peng et al., 2005; Mollace et al., 2003).

3.0 *DROSOPHILA* AS AN ANIMAL MODEL

Drosophila melanogaster is a pomace fruit fly and sexually dimorphic in nature. The word *Drosophila* was coined by Fallen, 1823 and popularized by Morgan in 1901 - a pioneer Drosophilist who developed concepts called Transmission genetics (sex linked inheritance) and mutation (inbreeding & x-rays) (Ex. White eyed, pink eyed mutants) which revolutionized biological research and became one of the powerful model organism in science.

Kingdom–Animalia; Phylum–Arthropoda, Class–Insecta; Order– Diptera, Family– Drosophilidae; Genus – Drosophila; Species – melanogaster

Life cycle: Drosophila life cycle have complete metamorphosis type. It consists of egg stage, larval (1^{st}, 2^{nd} & 3^{rd} instar), pupal and adult stage. Life cycle of has only 10-11 days. Within 24hrs of egg laying, it will hatch into 1^{st} instar larva. Again within 24hrs after 24hrs it will develop into 2^{nd} instar larva. Subsequently, it enters into the 3^{rd} instar larva where it stays for 48hrs. Because its active crawling behavior and voracious feeder this larval model has been extensively employed to study various biological problems. After this it will get into the pupal stage where it stays for 4days and which is considered to be inactive stage. After 4 days of resting period, pupa will emerge into adult fly which is ready to lay eggs within 8hrs of its eclusion. Advantage of using DM as model organism is: Smaller size and short life span, easy to operate and less expensive and Genome sequencing and hence access to transgenic and mutant flies.

Drosophila melanogaster genome: It has four pairs of chromosomes, one sex chromosome either X or Y and three autosomes 2,3 and 4. Genome size is 165 million bases containing 14,000 genes where as human genome size 3,400 million bases containing 22,500 genes. Online data base called “Homophila” where individual genes of *Homo sapiens* were compared to *Drosophila*. According to Reiter et al., 2001 based on online Mendelian inheritance of man (OMIM) database among 929 human disease genes total found, 714 human disease genes are closely related to *Drosophila* gene (548 disease gene) that is 77% disease gene homology and 50% fly protein sequence have mammalian analogues.

Similarities of flies with humans: Generally speaking, fundamental aspects of cell biology are quite similar in man and flies, including regulation of gene expression, membrane trafficking, the cytoskeleton, neuronal connectivity, synaptogenesis, cell signaling, and cell death. Many genes and pathways that originally were studied in flies have subsequently been identified in mammals.
As an example, the *wingless* pathway in *Drosophila* was named for a mutation originally identified in a spontaneously occurring mutant that was noted to have

no wings (Sharma and Chopra., 1976). The mammalian homolog of the *Drosophila wingless* gene, Wnt, is now known to stand at the apex of the Wnt pathway also conserved in mammals that is crucial for cell polarity, differentiation, and migration, cytoskeletal regulation, synapse formation, and axon guidance during neuronal development (Bejsovec, 2005).

Difference between flies and humans: There are also important differences between flies and humans. As an example, flies have much simpler circulatory systems and cognitive processes. In some circumstances, the relative simplicity of fly as compared with human genomic organization provides benefits with regard to genetic analysis. Often, redundancy exists in humans, where duplicated versions of genes are identified that are present in only one copy in flies; this lack of redundancy can simplify analysis of biological process in the fly. Perhaps the most important aspect of invertebrate approaches is the availability of a number of genetic manipulations that are impossible or impractical to carry out in mammals. Large numbers of flies can be mutagenized and screened in a short period of time, thus permitting the identification of even rare mutations. Given the considerable success that fly genetic approaches have had in delineating processes such as cell cycle control, signal transduction, and pattern formation, it is reasonable to anticipate that similar approaches to the study of neurodegeneration will continue to yield powerful insights into disease mechanisms.

Drosophila **as a model of human disease: Advantages**

Fundamental aspects of cell biology in flies have been conserved throughout evolution in higher-order organisms such as humans. A recent report demonstrating that approximately 75% of the disease-related loci in humans have at least one *Drosophila* homologue cements this high degree of conservation present in flies (Reiter, 2001). Furthermore, studies of developmental events in the fly and subsequent similar studies in higher animals have revealed a stunning degree of functional conservation of genes, as exemplified by the *hedgehog* gene which encodes a cell-cell communication molecule (Shubin et al., 1997). (ii) In flies, size does not matter at least with respect to their brains. Indeed, the fly brain is estimated to have, strikingly

enough, in excess of 300,000 neurons and similarly to mammals is organized into areas with separated specialized functions such as learning, memory, olfaction and vision. (iii) an unparalleled advantage of invertebrates is the ability to carry out large-scale genetic screens inexpensively and rapidly for mutations affecting a relevant process. And (iv) *Drosophila* has an unrivalled battery of genetic tools including a rapidly expanding collection of mutants, transposon-based methods for gene manipulation, systems that allow controlled ectopic gene expression and balancer chromosomes. The latter are special chromosomes made up of multiple inverted segments that suppress recombination together with visible and molecular markers, which allow chromosomal features (e.g. lethal mutations and deletions) to be followed indefinitely through crosses and generations.

The above characteristics of such a minuscule system model, combined with the rapid generation time, inexpensive culture requirements, large progeny numbers produced in a single cross and a small highly annotated genome devoid of genetic redundancy, are poised to yield seminal insights into human disease.

Neurodegenerative disease models in *Drosophila*

Pioneering studies performed in the Benzer lab (Rogina et al., 1997; Kretzschmar et al., 1997) led to the identification of a number of interesting Drosophila mutants such as bubblegum, swiss cheese, and drop-dead. These mutants were isolated by first screening for flies with reduced life span and then analyzing their brain pathology. Each mutant showed distinct patterns of degeneration in specific brain regions. The mammalian homologs of some of these genes also caused neurodegeneration phenotypes when mutated, indicating that this approach can identify genes that are essential for conserved mechanisms that maintain nervous system integrity (Akassoglou et al., 2004). Generally, mutant neurodegenerative disease genes act in a dominant manner in humans and are assumed to result in gained toxic functions. Consequently, models of these diseases can be established in Drosophila by transgenic expression of the toxic proteins. In many cases, transgenic animals over expressing the wild-type versions of the disease associated genes develop

nearly identical disease phenotypes (Feany and Bender, 2000; Fernandez-Funez, 2000). These observations, together with the fact that neurodegenerative conditions are frequently associated with mutations in proteins prone to misfolding and aggregation, lead to the notion that defects in protein homeostasis and general cellular quality control systems contribute to disease pathogenesis. Other familial neurodegenerative diseases are transmitted recessively, suggesting that they are likely caused by a loss-of function mechanism. The method of choice to model these diseases is to disrupt the function of endogenous genes homologous to human disease genes via a wide array of techniques available for disrupting gene function in Drosophila. Transposon-mediated mutagenesis, transgenic RNA interference (RNAi), and homologous recombination–based gene knockout can be used for this purpose (Greene et al., 2003; Pesah et al., 2004; Yang et al., 2003; Rong and Golic, 2000).

A pharmacological approach can be used to model neurodegenerative diseases and to test candidate therapeutics in animals. This approach has been widely used in mammalian systems, and some of the toxic compounds have been successfully used in flies to recapitulate disease phenotypes (Coulom and Birman, 2004; Betarbet et al., 2000). Drugs can be mixed with food and readily delivered to the flies. One possible advantage of the fly system is the lack of a stringent blood-brain barrier- which allows compounds to easily gain access to the nervous system. Together with the smaller body size, lower cost, and shorter life cycle, this approach makes Drosophila an attractive model for the testing and screening of therapeutic compounds.

Modeling Alzheimer's disease in Drosophila: Most of the genes implicated in AD pathogenesis have clear fly homologs. There is a fly homolog of APP, flies deficient for APPL exhibit a behavioral abnormality that can be rescued by a human APP transgene, indicating functional conservation between APPL and human APP (Luo et al., 1992). Furthermore, study showed that Aβ peptide can be generated in *Drosophila* from a modified human APP transgene (Fossgreen et al., 1998; Greeve et al., 2004), a human BACE transgene was co-expressed with a human APP transgene and a fly Psn transgene, Transgenic flies expressing all three transgenes developed β-amyloid plaques and age-

dependent neurodegeneration. Genetic and pharmacological manipulation of γ-secretase activity was shown to modulate the severity of the disease phenotype in this model (Greeve et al., 2004). Transgenic flies expressing Aβ-42 developed (a) diffuse amyloid deposits, (b) age-dependent learning defects, and (c) neurodegeneration, indicated by neuronal loss and vacuole formation in various brain regions. Aβ-40-expressing flies developed only age-dependent learning defects but no amyloid formation or neurodegeneration of AD and at screening for drug targets and potential therapeutics (Finelli et al., 2004).

Modeling Tauopathy in Drosophila: The first evidence of tau-induced neurotoxicity in flies was provided by transgenic studies of a bovine tau–green fluorescent protein reporter that caused axonal degeneration in sensory neurons (Williams et al., 2000). Subsequently, fly models of tauopathy were created by expressing wild-type or FTDP-17-linked mutant forms of human tau (Wittmann et al., 2001; Jackson et al., 2002). The fly genome includes a fly homolog of tau (known as dtau). Overexpression studies showed that directed expression of wild-type vertebrate tau or dtau in adult mushroom body neurons, which control olfactory learning and memory in *Drosophila*, strongly compromised associative olfactory learning and memory in the absence of detectable neurodegeneration (Mershin et al., 2004).

Fly Polyglutamine model: Significant insights into the pathogenic pathways of poly Q diseases were obtained from the SCA-1 model. In loss-of-function and over expression screens for genes that can modify expanded ataxin-1-induced neurodegeneration, genes involved in a number of biological processes were identified (Fernandez-Funez et al., 2000). Molecular chaperones, which have previously been implicated in regulating poly Q toxicity, were recovered in the SCA-1 screen. This screen also identified modifier genes involved in RNA processing, nuclear transport, transcriptional regulation (histone deacetylation), and cellular detoxification.

Amyotrophic lateral sclerosis and prion diseases: Prion diseases have been successfully modeled in mice. Prion diseases are unique among NDD in that the causative agents are thought to be proteins with abnormal confirmation

that can alter the conformation of normal cellular proteins. A recent study (Gavin et al., 2006) suggests that *Drosophila* may be a useful model for studying prion diseases. Transgenic flies heterologously expressing diseaseassociated mouse prion protein (PrP) in cholinergic neurons exhibited severe locomotor dysfunction and premature death as larvae and as adults. Clinical abnormalities were accompanied by age-dependent accumulation of misfolded PrP molecules, intracellular PrP aggregates, and neuronal vacuoles. These transgenic flies thus exhibited several hallmark features of human Gerstmann– Str¨aussler–Scheinker syndrome (Gavin et al., 2006).

Modeling Parkinson's disease in Drosophila*:* The advantages of Drosophila as a model for PD are: (i) Rapid growth/ reproduction, and genetic tractability (ii) The fly brain has six clusters of DA neurons and robust locomotive abilities (iii) The phenotypes of PD fly models resemble human patients.

i) α-syn models: Although *Drosophila* does not have a clear α-Syn homolog, over expression of wild-type- and FPD-associated mutant α-Synin *Drosophila* has reproduced key features of PD, including Lewy body–like aggregate formation, selective degeneration of dopaminergic neurons, and locomotor behavior abnormality as detected by climbing ability in the negative geotaxis response (Feany and Bender, 2000; Auluck et al., 2002). This is also consistent with the finding that genomic triplication of α-Syn in humans can cause PD (Singleton et al., 2003). The fact that *Drosophila* does not encode an α-Syn like protein may have facilitated the modeling of α-Syn-related toxicity, as flies may not have evolved an effective defense mechanism against the toxic effects of α-Syn misfolding or aggregation.

ii) Parkin models: Two prominent features of *parkin*-mutant flies are mitochondrial pathology and apoptotic muscle degeneration (Greene et al., 2003; Pesah et al., 2005; Cha et al., 2005).. In addition, these mutant flies exhibit sterility, reduced life span, reduced cell number and size, and hypersensitivity to oxidative stress. A study using whole mount confocal analysis reported notable dopaminergic neuron loss in the protocerebral posterior lateral 1 cluster (Whitworth et al., 2005). Using a different approach,

parkin loss of- function phenotype was created by transgenic RNAi, which allows tissue- and cell type–specific gene knockdown (Yang et al., 2005).

iii) DJ-1 models: There are two DJ-1 homologs in *Drosophila*, DJ-1A and DJ-1B. Although both fly DJ-1 proteins are highly similar to human DJ-1, a putative catalytic triad characteristic of cysteine proteases is conserved in DJ-1A but not in DJ-1B, suggesting that DJ-1A is a closer homolog of human DJ-1 than DJ-1B. In one study, DJ-1A and DJ-1B double-knockout flies were generated by genomic micro deletions. These animals were viable and fertile and had a normal life span; however, they displayed a selective sensitivity to environmental toxins such as paraquat and rotenone. This sensitivity was thought to result primarily from loss of DJ-1B protein, which becomes modified upon oxidative stress (Meulener et al., 2005). In another study, DJ-1B loss-of-function mutants were found to have an extended survival of dopaminergic neurons and resistance to paraquat stress, but they also had an acute sensitivity to hydrogen peroxide treatment. Using a different approach, DJ-1A was knocked down by transgenic RNAi. Inhibition of DJ-1A function through cell type–specific RNAi resulted in accumulation of reactive oxygen species (ROS), organismal hypersensitivity to oxidative stress, and dysfunction and degeneration of dopaminergic and photoreceptor neurons (Yang et al., 2005).

iv) Pink1 models: *Drosophila* Pink1 models of PD were generated by transposon-mediated mutagenesis and RNAi approaches. Genetic removal of *Drosophila* Pink1 (dPink1) function results in male sterility, apoptotic muscle degeneration, defective mitochondrial morphology and increased sensitivity to multiple stresses including oxidative stress. Mitochondrial cristae are fragmented in *dPink1* mutants (Clark et al., 2006). In addition to these phenotypes, another group found that *dPink1* mutants exhibit dopaminergic neuronal degeneration accompanied by defects in locomotion (Park et al., 2006). Using the transgenic RNAi approach, researchers have shown that inhibition of dPink1 function results in energy depletion, shortened life span, and degeneration of both indirect flight muscles and select dopaminergic neurons (Yang et al., 2006). The muscle pathology was preceded by mitochondrial enlargement and disintegration. These phenotypes could be

rescued by the wild-type but not by the pathogenic C-terminal deleted form of human Pink1 (hPink1), indicating functional conservation between fly and human Pink1 (Yang et al., 2006). Another independent study (Wang et al., 2006) using the RNAi approach showed that, inactivation of dPink1 results in progressive loss of dopaminergic neurons and photoreceptor neurons, which can be rescued by expression of human Pink1. Further, expression of human SOD1 and treatment with the antioxidants SOD and vitamin E can significantly inhibit photoreceptor degeneration in *dPink1* RNAi flies (Wang et al., 2006).

v) Pharmacological models: A recent study showed that pharmacological treatment could be used in *Drosophila* to model sporadic PD (Coulom and Birman, 2004). Chronic exposure to rotenone in *Drosophila* resulted in PD-related neurodegenerative and behavioral effects. Rotenone-treated flies presented characteristic locomotor impairments as measured by the negative geotaxis response assay. At the cellular level, a selective loss of dopaminergic neurons was detected in all of the brain dopaminergic clusters. Feeding the flies with L-dopa (3,4-dihydroxy-Lphenylalanine), the precursor of dopamine in the biosynthetic pathway, rescued the behavioral deficits but not neuronal death. In contrast, the antioxidant melatonin (N-acetyl-5-methoxytryptamine) alleviated both symptomatic impairment and neuronal loss, suggesting that antioxidant agents such as melatonin may be beneficial for the treatment of PD.

More recently, Chaudhuri et al., 2007 successfully demonstrated that environmental toxin such as the herbicide paraquat, appear to be risk factors, and have proposed that susceptibility is influenced by genetic background. Further paraquat can replicate a broad spectrum of Parkinson's behavioral symptoms in *Drosophila* that are associated with loss of specific subsets of dopaminergic neurons. Interestingly, protection against the neurotoxicity of paraquat is conferred by mutations that elevate dopamine pathway function, whereas mutations that diminish dopamine pools increase susceptibility. Pharmacological treatment in *Drosophila* thus provides a complementary avenue for studying mechanisms of dopaminergic degeneration.

Drosophotoxicology: An emerging science by using *Drosophila*

The Molecular Libraries Program has compiled over 300,000 compounds for which it seeks high throughput assays to unveil unique biologically active reagents for the biomedical research community. Of particular importance is the ability to evaluate toxicity of the more than 2000 new chemicals introduced each year that are added to the list of 80,000 chemicals registered with the National Toxicology Program. The requirements for high throughput screening (HTS) make choosing a toxicological endpoint a balance between the complexity of the anticipated outcome and the ease of detection and quantification. Disruption of neurogenesis and neuronal and glial patterning in the embryonic nervous system is a very powerful endpoint for screening. In this case microscopy is essential for detection, and thereby introduces technical challenges for adaptation to high throughput analyses **(Figure 2)**. Yet, recent advancements in genetically encoded fluorescent reporters and automated imaging make this approach very attractive and amenable to HTS methods (Barolo et al., 2004; Yeh et al., 1995). Peripheral neurons in larvae and pupae are relatively accessible for imaging and exhibit many features common to mammalian structures, such as synaptic boutons at the neuromuscular junction (Budnik, 1996).

Larvae also display quantifiable behaviors in motility, odor response and memory that can be used to determine fitness of nervous system function (Sawin-McCormack et al., 1995). Eclusion is perhaps the simplest lethality endpoint and can be done without the aid of a microscope. A wide variety of assays for adult flies have been devised to determine morphological defects and neurological fitness. The most common assays examine changes in adult structures such as the eye or wing. These are useful proxies for effects upon underlying molecular pathways. Common adult behavioral assays assess simple locomotive behaviors viz., geotaxis, photo- or chemotaxis (Fernandez-Funez et al., 2000; Louis et al., 2008). More complex behaviors such as courtship, circadian rhythm and even associative learning and memory are now understood to have a genetic component in Drosophila and are rapidly being established as phenotypes for screening strategies (Sokolowski, 2001).

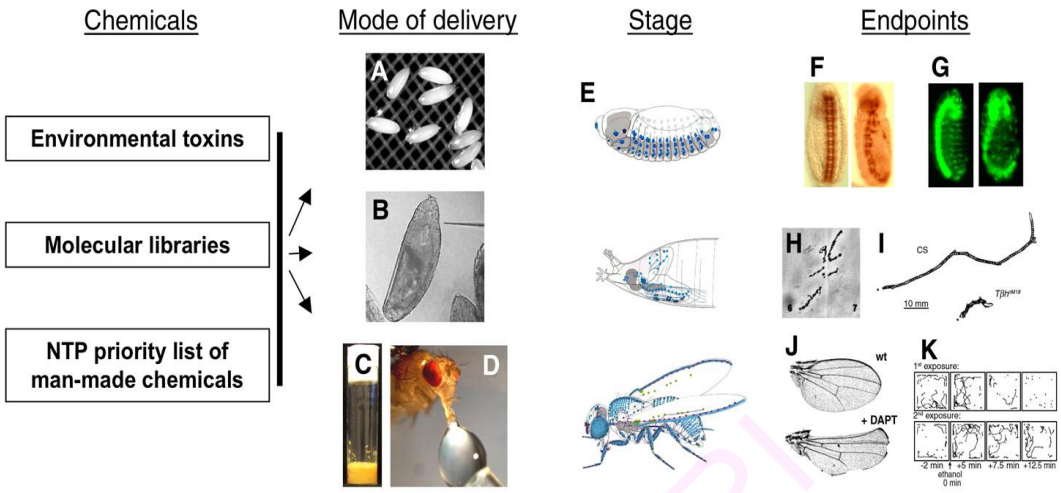


Figure 2

Drosophila as a model system to screen therapeutic compounds

Drosophila models of NDD are excellent systems for in vivo testing of therapeutic effects of various compounds. In a successful application of the chemical approach to neurodegeneration in *Drosophila*, feeding the *bubblegum* mutant with one of the components of Lorenzo's oil, glyceryl trioleate oil, prevented neurodegeneration (Min and Benzer, 1999). In two *Drosophila* models of polyQ disease,histone deaceylase (HDAC) inhibitors reduced cellular toxicity associated with polyQ repeat expansion and arrested neuronal degeneration (Steffan et al., 2001). Based on the observations that mammalian target of rapamycin (mTOR) is sequestered in polyQ aggregates and that sequestration of mTOR impairs its kinase activity and induces autophagy (Ravikumar et al. 2004) tested the effect of the specific mTOR inhibitor rapamycin on Htt accumulation and cell death in cell models of HD. Further experiments showed that rapamycin protects against neurodegeneration in a fly model of HD, and the rapamycin analog CCI-779 improved performance in behavioral tasks and decreased aggregate formation in a mouse model of HD (Berger et al., 2006). Based on the evidence that Hsp70 overexpression can prevent α-Syn-induced toxicity (Auluck et al., 2002; Auluck and Bonini 2002)

tested the effect of feeding flies with geldanamycin, a naturally occurring benzoquinone ansamycin that (*a*) specifically binds and inhibits HSP90, a negative regulator of heat shock factor and (*b*) can induce stress response in cell culture. Geldanamycin treatment induced HSP70 expression and prevented α-Syn-induced dopaminergic neuronal loss. As mentioned above, melatonin was also effective in preventing dopaminergic degeneration in a fly rotenone model of sporadic PD (Coulom and Birman, 2004). More recently our own data shown, feeding *Drosophila* with *Bacopa monnieri* extract and creatine monohydrate can rescue flies from rotenone induced oxidative stress, dopamine depletion and locomotor deficits (Hosamani and Muralidhara, 2009; Hosamani et al., 2010).

In addition to testing candidate compounds, the fly's small size, high fecundity, and low cost make *Drosophila* disease models excellent systems for performing high-throughput screening of random chemical libraries for compounds with therapeutic potential (Tables 1-4).

Table 1

Compounds / salient findings	**Reference**
Porphyrans (polysaccharides from *Porphyra naitanensis*) *Salient findings:* Extends lifespan and Improved vitality against stress in wild flies	Zhao et al., 2008
SOD mimetic: Euk-8 & 134 *Salient findings:* Extends lifespan in SOD deficit flies	Magware et al., 2006
Congo red, Cystamine and SAHA (In combination) *Salient findings:* Identified non-cytotoxic dose Failed to rescue fly from Huntington's induced neurodegeneration.	Agrawal et al., 2005
Fraxetin (1mM) *Salient findings:* Protects oxidative damage Increases GSH & Decreases peroxidative damage. Improves survival parameters.	Fernandez-Puntero et al., 2001

Table 2

Compound / salient findings	Reference
Vitamins: α & - tocopherols, Vitamin E & A,Ascorbic acid Negative effect on longevity; extends lifespan of SOD-deficit flies and lifespan of wild flies	Bahadorani et al., 2008; Zou et al., 2007
Geldanamycin Protects α-synuclein induced neurotoxicity in *Drosophila.*	Auluck et al., 2005
L-Dopa (3, 4 dihydroxy L-Phenyl alanine) Rescues rotenone-induced behavioral deficits, but not neuronal death in flies.	Coulom and Birman, 2004
Dopamine agonists : Pergolide, bromocriptine and 2,3,4,5 tetrahydro 7,8, dihydroxy 1-phenyl-1H 3- benzazepine (SK& 38393) Rescues locomotor deficits in transgenic Parkinson's model of *Drosophila*	Pendleton et al., 2002
R-deprenyl and aliphatic propargylamin Increases mean & maximal lifespan in galactose model of adult flies	Jordens et al., 1999

Table 3

Compound / salient findings	Reference
Melatonin, glutathione, serotonin, minocycline, lipoic acid and ascorbic acid. *Salient findings:* Melatonin protects more efficiently paraquat induced oxidative stress followed by glutathione and minocycline.	Bonilla et al., 2006
Melatonin (N-acetyl 5-methoxy tryptamine) *Salient findings:* Alleviates behavioral deficits & neuronal loss induced by rotenone in *Drosophila*.	Coulom and Birman 2004
Melatonin (100ug/ml daily) *Salient findings:* Extends maximum lifespan (33.2%) and median life span (13.5%) in wild flies	Bonilla et al., 2002
Melatonin (2mM) *Salient findings:* Preferentially suppresses protein oxidative damage compared to lipids in *Drosophila* model.	Coto-Montes and Hardeland, 1999

Table 4

Compounds / salient findings	Reference
Creatine monohydrate *Salient findings:* Protects oxidative stress, mitochondrial dysfunction, and dopamine depletion induced by rotenone in wild *Drosophila*	Hosamani et al., 2010
Gallic acid, Ferulic acid, caffeic acid, Coumaric acid, propyl gallate, epicatechin, epigallocatechin and epigallocatechin gallate *Salient findings:* Propyl gallate and epigallocatechin gallate protects from paraquat and iron toxicity. Maintained movement abilities in flies.	Jimenez-Del-Rio et al., 2009
Polyphenols: Green tea catechins (GTC) 10mg/ml *Salient findings:* Increases survival of flies (36%) & mean lifespan (16%). Correlated with 17% decreased LPO in Oregon-R-C but not in SOD and Cat knockout mutant flies. Up regulated SOD and Cat activity.	Li et al., 2006

4.0 NEUROPROTECTIVE APPROACHES

A better understanding of intracellular pathways and pathogenesis engaged in the neuronal cell death now offers us new targets for developing putative neuroprotective agents targeting vulnerable neurons and effective strategies for their replacement. Also pharmacological or genomic intervention aimed to modulate the expression of endogenous neuroprotective is a very promising strategy. Taking into account enormous complexity of biochemical cascades involved in neurodegenerative processes; multi potential or combined pharmacological, neuroprotective and surgical approaches seems to be more efficient in combating degenerative brain diseases.

Anti-excitotoxic approaches: Glutamate is the primary excitatory transmitter in the mammalian central nervous system and a primary driver of the excitotoxic process. Excessive NMDA receptor activation by glutamate could increase an intracellular calcium level that activates cell death pathways (Yacoubian et al., 2009). Increased glutamate release may increase Ca^{2+} influx into the cells and increase formation of nitric oxide by activating nitric oxide

synthase (NOS). This may be particularly harmful because the defect in mitochondrial complex I may make the dopamine neurons vulnerable. In support of this toxicity mechanism, neuronal NOS inhibitors and classical anti-excitotoxic pharmacological intervention in rapid cell death mechanisms are obtained by the inhibition of Ca^{2+} influx mainly through NMDA receptors the first of which, remacemide is now in clinical trials (Rodriguez et al., 1999).

Antiapoptotic and neurotrophic agents: Anti-apoptotic agents, which act mainly to inhibit caspase-3, which is an effector caspase in both intracellular and extramitochondrial cell death pathways. Apoptosis, or programmed cell death, is a mechanism that has been demonstrated to participate in neural development and to play a role in some forms of neural injury (Yacoubian et al., 2009). A wide range of factors can protect nigral dopamine neurons *in vivo* in animal models of PD, notably glial cell line-derived neurotrophic factor (GDNF), neurturin, basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), neurotrophins 3, ciliary neurotrophic factor and transforming growth factor-β. CEP-1347, an inhibitor of mixed lineage kinases that can activate the c-Jun N-terminal kinase pathway involved in cell death is another anti-apoptotic agent that showed promise in preclinical studies (Hagg, 1998).

Anti-inflammatory strategies: Considering the involvement of inflammatory processes in mechanism of neurodegeneration, activated microglia and astrocytes are a rich source of oxygen radicals, nitric oxide, neurotoxic and proinflammatory cytokines. Several epidemiological studies have indicated that the long-term use of NSAIDs most of which are cyclooxygenase (COX) inhibitors may reduce the risk of Alzheimer's disease. Pioglitazone - a peroxisome proliferator-activated receptor-gamma agonist with anti-inflammatory properties appears to have therapeutic potential for human ALS (Kiaei et al., 2005). An initial study has also shown that ibuprofen lowers the risk of PD by 45% (Chen et al., 2003).

Gene and cell-based therapies: Genetic approaches to the treatment of neurodegenerative disorders involve mainly constructing of viral vectors for targeted neurotrophic protein delivery. Also viral vector-mediated gene delivery of a dopamine-synthesizing enzyme into the striatum was reported to

restorelocal dopamine production and allowed for behavioral recovery in animal models of PD (Li et al., 2006). Another genomic strategy is the RNA interference (RNAi) method in silencing the expression of specific toxic genes but it is still only experimentally studied.

Neural transplantation: Neural grafting in PD is based on the idea that dopamine supplied from cells implanted into the striatum can substitute for the lost nigrostriatal neurons. Clinical trials have shown that mesencephalic dopamine neurons obtained from human embryo cadavers can survive and function in the brains of patients with PD (Dunnett et al., 1999).

Antioxidants and mitochondrial stabilizers: The best known neuroprotectants and free radical-neutralizing agents include polyphenols e.g. quercetin, vitamin E, lazaroids and endogenously produced melatonin and estrogens. Also, a combination of iron chelation and antioxidant therapy may be one of significant approaches to neuroprotection. Multifarious neuroprotective activities of green tea catechins have brain-permeable, radical scavenger properties that were shown in a wide array of cellular and animal models of neurological diseases (Hardeland et al., 2006). Coenzyme Q_{10} (CoQ_{10}) is a cofactor in the electron transport chain in mitochondria and has been shown to reduce dopaminergic neurodegeneration in mouse PD models (Beal et al., 1998).

Dietary antioxidants: The use of plant-derived supplements for improving health is gaining popularity because most people consider these natural products to be safer and produce lesser side effects than synthetic drug (Raskin et al., 2002). There have been several studies on the antioxidant activities of various herbs/plants with medicinal values (Dragland et al., 2003). Currently, there has been an increased interest globally to identify antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine. Natural antioxidants mainly come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols) ascorbic acid and carotenoids (Ali et al., 2008). There are clinical evidences that neurodegeneration can be ameliorated upon dietary intake or supplementary intake of natural antioxidants. These

natural antioxidants prevent oxidation of proteins, lipid peroxidations and prevent generation of ROS thus act as upstream therapeutic barrier to oxidative stress (Opazo et al., 2002).

Role of Phytochemicals in neuroprotection

Ginkgo biloba: This ancient herb has many pharmacological properties, blood flow enhancing, decreasing of free radicals production, protection of striatal dopaminergic system, until inhibition of monoamine oxidase (conserving neurotransmitters in synaptic cavity) (Youdim and Josep, 2001). It has been reported that *Ginkgo biloba* extract inhibited β-amyloid induced hippocampal cell death and H_2O_2 production.

Centella asiatica: Earlier studies showed that oral administration of an aqueous extract of Centella asiatica (CA) reduces brain malondialdehyde levels and increases the glutathione levels in whole brain of adult rats (Veerendra Kumar and Gupta, 2003). Recently, CA was demonstrated to accelerate nerve regeneration *in vivo* and increased neurite elongation in vitro (Soumyanath et al., 2005). Notable bioactive compounds of CA are the triterpenesaponins, madecassocide and asiaticoside with their respective ursane type sapogenins *viz*., madecassic and asiaticacid (Mangas et al., 2006). some of which have been shown to be potent antioxidants. Also recently, CA has the propensity to modulate both endogenous and neurotoxicant induced oxidative impairments in the brain regions of mice (Shinomol and Muralidhara, 2009). Also, recently Centella asiatica leaf powder has shown to ameliorate 3-NPA-induced oxidative stress in mitochondria both *in vivo* and *in vitro (*Shinomol and Muralidhara, 2008).

***Bacopa monnieri* as a neuromodulatory phytochemical**

BM has been used by Ayurvedic medical practitioners in India for almost 3000 years and is classified as a medhyarasayana, a drug used to improve memory and intellect (medhya). The earliest chronicled mention of BM is in several ancient Ayurvedic treatises including the Charaka Samhita (6th century A.D.), in which it is recommended in formulations for the management of a range of mental conditions including anxiety, poor cognition and lack of concentration,

and the Bravprakash Var-Prakarana (16th century A.D.). Brahmi is currently recognized as being effective in the treatment of mental illness and epilepsy.

Botanical aspects of Bacopa monnieri: (Family: Scrophulariaceae). BM is a small creeping herb with numerous branches and small fleshy, oblong leaves. Flowers and fruits appear in summer. The stem and the leaves of the plant are used (Mathew, 1984).The genus Bacopa includes over 100 species of aquatic herbs distributed throughout the warmer regions of the world.

Chemical constituents: Bacopa contains alkaloids such as Hydrocotyline, Brahmine, Herpestine (Dutta and Basu, 1963), Brahamoside, Brahminoside, Brahmic acid, Isobrahmic acid, Vallerine, pectic acid, fatty acids, tannin, volatile oil, ascorbic acid, thanakunic acid, asiatic acid. The other major chemical constituents from the alcoholic extract are dammarane type triterpenoid saponins with jujubogenin and pseudojujubogenin as the aglycones including bacosides A1-A3, bacosaponins A-G and bacopasides I-V (Hou et al., 2002; Chakravarthy et al., 2003).

Neuropharmacological activity: *Effects on learning and memory*

The plant, plant extracts and isolated bacosides have been extensively investigated in several laboratories for their neuropharmacological effects and a number of reports are available confirming their nootropic action.Preliminary studies established that the treatment with the plant (Malhotra and Das, 1959) and with the alcoholic extract of BM plant (Singh and Dhawan, 1982) enhanced learning ability in rats. Subsequent studies indicated that the cognition-facilitating effect was due to two active saponins, bacosides A and B, present in the ethanol extract (Singh and Dhawan, 1992). These active principles, apart from facilitating learning and memory in normal rats, inhibited the amnesic effects of scopolamine, electroshock and immobilization stress (Dhawan and Singh, 1996). The mechanism of these pharmacological actions remains conjectural. It has been suggested that the bacosides induce membrane dephosphorylation, with a concomitant increase in protein and RNA turnover in specific brain areas (Singh et al., 1990). Further, BM has been shown to enhance protein kinase activity in the hippocampus which could also contribute to its nootropic action (Singh and Dhawan, 1997). A study of (Bhattacharya et

al.,1999) reported that a standardized bacoside-rich extract of BM, administered for 2 weeks in rats, reversed cognitive deficits induced by intracerebroventricularly administered colchicines and by injection of ibotenic acid into the nucleus basalis magnocellularis.

Administration of BM for two weeks, also reversed the depletion of acetylcholine, the reduction in cholineacetylase activity and the decrease in muscarinic cholinergic receptor binding in the frontal cortex and hippocampus, induced by neurotoxin, colchicines (Bhattacharya et al., 1999). In a recent study, standardized extracts of BM and Ginkgo biloba (GB) were used to evaluate the antidementic and anticholinesterase activities in adult male Swiss mice (Das et al., 2002). Antidementic activity was tested against scopolamine (3 mg/kg ip)-induced deficits in passive avoidance (PA) test. Three different extracts of BM (30 mg/kg) and of GB (15, 30 and 60 mg/kg) were administered daily for 7 days and 60 min after the last dose of scopolamine, i.e. transfer latency time (TLT) amnesia and has, instead, a memory-promoting action in animals and man (Singh and Dhawan, 1992; Dhawan and Singh, 1996). These results were confirmed by Shanker and Singh (2000) who reported that BM extract possessed an anxiolytic effect.

Antidepressant and anticonvulsive activity: More recently, the standardized methanolic extract of BM was investigated for potential antidepressant activity in rodent models of depression. The effect was compared with the standard antidepressant drug imipramine (15 mg/kg ip). When given in the dose of 20 and 40 mg/kg, orally once daily for 5 days, the extract was found to have significant antidepressant activity in forced swim and learned helplessness models of depression and was comparable to that of imipramine (Sairam et al., 2002). Anticonvulsive action another important use of BM in traditional medicine is anticonvulsive action, as reported in different experimental studies. Shanmugasundaram et al. (1991) have reported that the crude water extract of BM controls epilepsy in experimental animals. Successively, BM was studied in mice and rats, at oral doses ranging between 1 and 30 g/kg, for its effect on the central nervous system.

Antioxidant activity: Data from different laboratories suggest that the cognition-promoting functions of BM may be partially attributed to the antioxidant effects of the bacosides. One study determined the effects of alcohol and hexane extract of BM on lipid peroxidation by ferrous sulphate and cumene hydroperoxide in rat liver homogenate (Tripathi et al., 1996). The alcohol fraction exhibited a greater protection against both inducers. A more recent study explored the effect of BM extract on the rat brain frontal cortical, striatal, and hippocampal superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities, following administration for 7, 14 or 21 days (Bhattacharya et al.,2000). The results were compared with the effect induced by deprenyl, a well-known neurological anti- oxidant for the same time period. BM induced a doserelated increase in SOD, CAT, and GPX activities in all brain regions investigated, after treatment for 14 or 21days. On the contrary, deprenyl induced an increase in SOD, CAT, GPX activities in the frontal cortex and striatum, but not in the hippocampus, after 12 or 21 days. (Pawar et al., 2001) demonstrated that the hydroalcoholic extract of the whole BM plant exhibited an inhibitory effect on superoxide released from polymorphonuclear cells in nitroblue tetrazolium assay. A more recent study, affected by the same research group, indicated the protective effect of BM extract on morphine-decreased brain mitochondrial enzyme activity in rats (Sumathy et al., 2002).

In a study to test the hypothesis that BM directly decreases the production of free radicals, Paoletti assay was used, which excludes the Fenton-type reaction and the xanthine–xanthine oxidase system (Russo et al., 2003). Results indicate that BM is able to directly inhibit the superoxide anion formation in a dosedependent manner. The free radical scavenging activity of this methanolic extract was also evaluated through its ability to quench the synthetic radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The free radical scavenging capacity of BM, demonstrated in the Paoletti and in DPPH tests, was confirmed by protection against plasmid DNA strand scission, induced by _OH radicals, generated from UV photolysis of H_2O_2.

Clinical studies: Supporting the preclinical investigations, several clinical studies, have been carried out. Two single-blind open clinical studies have reported memory and learning enhancing effects of chronic BM treatment in both patients with anxiety neurosis (Singh and Singh, 1980) and in children (Sharma et al., 1987). Therefore, BM has been introduced onto the market in India and other countries, alone or in association with other phytocomplexes, and utilized in the treatment of memory and attention disorders (Shukia et al., 1987). The commercial preparation has shown a remarkable nootropic activity, above all in very young subjects (Dave et al., 1993). After clinical trials on human volunteers, a standardized extract of BM has now been made available for clinical use by the Central Drug Research Institute, Lucknow, India (Dhawan and Singh, 1996).

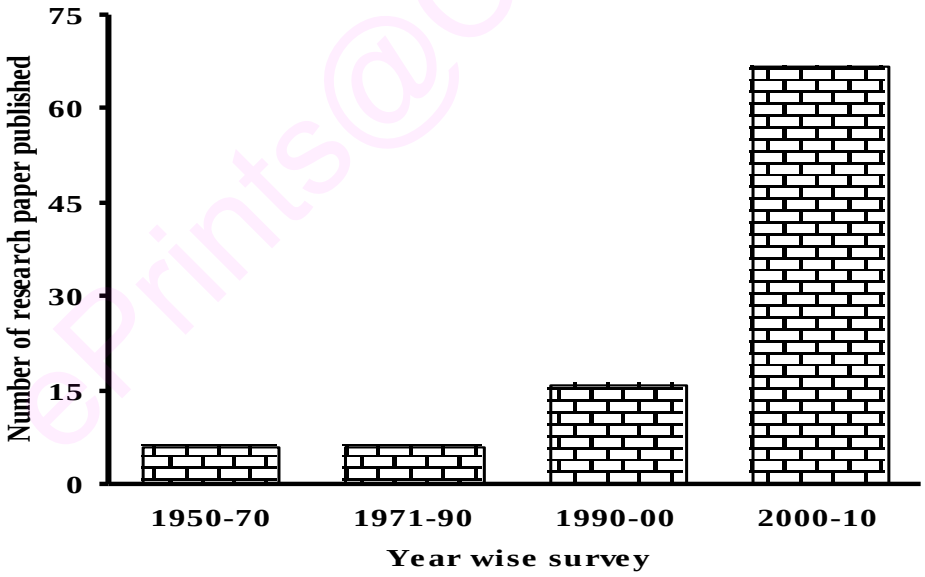


Figure 3 Graphical representation showing number of research paper published on Bacopa monnieri over the decade starting from 1950 to 2010 showing increasing importance of Bacopa monnieri research in the world.

Table 5 Beneficial effects of *Bacopa monnieri*

Salient findings	Reference
Enhances cognitive function in AD model	Uabundit et al., 2010
Ameliorative effects in epilepsy associated behavioral model	Mathew et al., 2010
Inhibitory action of lipoxygenase and cycloxygenase 2	Viji and Helen, 2010
Enhances learning and memory in rat model	Vollala et al., 2010
Protects against paraquat induced lethality and mitochondrial dysfunctions in adult *Drosophila.*	Hosamani and Muralidhara, 2010
Therapeutic role against pilocarpine-induced epilepsy in rat model	Krishnakumar et al., 2009
Protects against rotenone induced oxidative stress and neurotoxicity in *Drosophila* model	Hosamani and Muralidhara, 2009
Modulates antioxidant response in brain and kidney of diabetic rat mode	Kapoor et al., 2009
Protective role against L-NAME induced amnesia in mouse model	Saraf et al., 2009
Ameliorates β-amyloid induced cell death in primary cortical cultures	Limpeanchob et al., 2008
Neuronutrient against brain ageing	Singh et al., 2008
Modulatory effect on plasma corticosterone and brain monoamines in rat model	Sheikh et al., 2007
Prevents aluminium neurotoxicity in the cerebral cortex in rat brain	Jyoti et al., 2007
Antioxidative properties of bacoside-A	Sinha and saxena, 2006
Antistress activity, modulates HSP70 expression, superoxide dismutase and cytochrome p450 activity in rat brain	Chowdhuri et al., 2002

SCOPE AND OBJECTIVES OF THE INVESTIGATION

Currently, there is rising concern regarding the increasing prevalence of neurodegenerative diseases (NDD). NDD encompass a large group of neurological disorders. Most of these diseases have certain common features including memory loss, learning disabilities, and cognitive impairment, loss of movement or loss of control of movement and loss of sensation. Further, these conditions are connected to neurodegeneration in different anatomical areas of the brain involving multiple pathways including oxidative stress, mitochondrial damage, protein aggregation, neuroinflammation etc. A large body of evidence suggests oxidative stress mechanisms are involved in the pathophysiology of various neurodegenerative diseases. Neuronal tissue is highly susceptible to oxidative damage due to high oxygen consumption coupled with modest antioxidant defense strategies, high concentrations of Iron and rich in polyunsaturated fatty acids. Oxidative stress can induce neuronal damage, modulate intracellular signaling, ultimately leading to neuronal death by apoptosis or necrosis. Hence involvement of oxidative stress is well accepted concept among pathophysiology of various neurodegerative diseases. The molecular mechanisms of neuronal degeneration remain largely unknown and effective therapies are not currently available. However, cellular, pharmacological and genetic models have provided vital information about the affected genes and pathways and will provide the basis for evaluating potential therapeutic interventions.

Although modern approach in biomedical research has made major breakthroughs in understanding the molecular basis of CNS diseases, the knowledge about therapy is limited. Modern medicine utilizes a distinct, well-defined chemical molecule(s) for pharmacotherapy. However, the biggest challenge faced by scientists is to come up with a drug(s) that could simultaneously target multiple disease pathways without significant side-effects, be non-toxic at higher concentrations in humans and have the ability to cross the blood-brain barrier. Consequently, scientists are envisaging a paradigm shift from monotherapy involving isolated compounds to target a specific cellular pathway to multi-therapy based on various targets with a

greater probability for success. In this regard, natural substances various phytochemicals which possess highly beneficial medicinal properties are being exploited. Most of the information regarding phytochemicals has originated from long-established knowledge of natural herbs and traditional medicine that has existed for thousands of years in developing countries such as India. This has in recent years dramatically increased the interest in the use of herbal products in the western countries .Consequently; there is increasing support for combination of modern drugs with traditional medicine to evolve better therapies.

Mammalian models such as rats, gerbils and mice have been extensively employed to understand the mechanism/s underlying various neurodegenerative diseases. Since a large amount of evidence has accumulated over the years that have implicated oxidative stress as being intimately involved in the deficits seen in aging as well as age-related neurodegenerative diseases, numerous studies have examined the putative positive benefits of antioxidants in altering, reversing or forestalling the neuronal and behavioral dysfunctions. Considerable evidence suggests that mitochondrial dysfunction and oxidative damage may play a role in the pathogenesis of neurodegenerative disease and several agents have been suggested to modulate the cellular energy metabolism and could exert antioxidative effects.

Owing to the existence of rich background literature, coupled with easy culture methods and short lifespan, *Drosophila* have been extensively utilized in the recent past to understand human neurodegenerative diseases. There are now several examples of genetic manipulation of the steady state level of reactive oxygen species (ROS) or of the ability to repair oxidative damage in *Drosophila*. Increasing the levels of antioxidant defenses and repair systems is demonstrated to increase lifespan concomitant with a decrease in oxidative damage. More importantly, pharmacological interventions expected to reduce ROS levels have been shown to effectively increase the life span in *Drosophila*.

Researchers have utilized *Drosophila* mutants and transgenics to provide mechanistic insights in to mechanisms associated with neurodegenerative disease. For example Parkinson’s disease is a common

neurodegenerative condition that can result from several distinct genetic mutations and specific environmental conditions. Recent studies have investigated the effects of *α-synuclein* and *parkin* mutations and a pharmacologic agent a rotenone in the *Drosophila* model. Mitochondrial complex I inhibitors (eg. Rotenone) are capable of causing mitochondrial dysfunction that phenocopies Parkinson's disease both *in vivo* and *in vitro.* Chronic exposure to rotenone models sporadic Parkinson's disease in *Drosophila* in which both dopaminergic cell loss and locomotors impairment were recently demonstrated. In general studies in *Drosophila* have translated well to mammalian systems.

To the best of our knowledge, the *Drosophila* system has not been employed as a model to understand the neuroprotective effects of phytochemicals. Hence, it is hypothesized that *Drosophila* can be utilized as a rapid model system to test the efficacy of putative neuroprotective compounds. Further, we would like to explore whether *Drosophila* may provide a cost-effective platform for testing large matrices of phytomedicines as therapeutic drugs and to test undesirable interactions before proceeding to the mouse models or human subjects suffering from neurodegenerative disease. Accordingly, this proposal aims to investigate the utilization of *Drosophila* as an *in vivo* model to assess the efficacy of phytochemical therapies against oxidative stress -mediated neurodegeneration.

Objectives

i) To elucidate the neuroprotective properties of selected phytochemicals in modulating oxidative biomarkers in *Drosophila melanogaster*

ii) To understand the protective impact of these phytochemicals on experimentally - induced oxidative stress in *Drosophila* and to establish the attenuation of oxidative stress and mitochondrial dysfunctions.

iii) To explore the possibilities of employing them as cytoprotective agents for the treatment of the specific neurodegenerative disease such as Parkinson's disease and to obtain insights into the underlying biochemical pathways.

Materials and Methods

MATERIALS

1.0 Chemicals

1,1,3,3-tetramethoxypropane, 1-chloro-2,4-dinitrobenzene, 2,4-dinitro phenylhydrazine, 5,5-dithiobis-2-nitrobenzoic acid, adenosine triphosphate, Sulphanilamide, N-1-naphthyl-ethylene diamine, HCl, dopamine, ascorbic acid, bovine serum albumin, cetyl trimethylammonium chloride, collagenase, diphenyl-β-picrylhydrazyl (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, thiobarbituric acid (TBA), trypan blue, trypsin, ferulic acid, eugenol and reduced glutathione were procured from M/s Sigma Chemicals Co., (St.Louis, MO, USA). Ferric chloride, ferrous sulphate, Folin-ciocalteu reagent, o-pthalaldehyde, potassium ferricyanide, sodium dodecyl sulphate, trichloroacetic acid, tris, acrylamide, coomassie brilliant blue were purchased from M/s Sisco Research Laboratories (Mumbai, India). All other chemicals used were of analytical grade.

2.0 Neurotoxicants

Rotenone (≥95-98%), Paraquat dichloride x hydrate (99.9%) and Acrylamide (≥99%) were obtained from M/s. Sigma chemicals Co., (St. Louis, Mo, USA).

3.0 Phytochemicals

i) Brahmi capsule (BC)

Brahmi capsules (actives-250 mg per capsule) (a proprietary product of Himalaya Herbal Health Care, Bengaluru, India, which manufactures and sells herbal drugs for human consumption) were procured from a local pharmacy store.

ii) *Withania somnifera*

Standardized extract of *Withania somnifera* (Ashwagandha extract) was procured from M/s. Sami lab Pvt. Ltd. Bangalore (Batch no. C90472 PF).

iii) Standardized powder of *Centella asiatica*

Centella asiatica and *Bacopa monnieri* plants were collected during early summer from the state of Kerala and authenticated by Prof. C.M Joy, (Department of Botany, Sacred Heart College, Mahatma Gandhi University, Thevara, Kerala, India).

Fresh leaves of CA were shade dried, powdered and used for dietary studies. For the preparation of aqueous extract, the method of Veerendra Kumar and Gupta (2002) with minor modifications was followed. To 5g of leaf powder in a conical flask, 50 ml of double distilled water was added and kept in a boiling water bath for 2 .5 h. 100ml water was added and the conical flask was removed from the water bath after additional 2.5 h and stirred for a period of 20 min. Later the extract was cooled, filtered through a 400 µ sieve, rotary evaporated at 40^0C for 30 minutes followed by lyophilization to obtain 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).

iv) Standardized powder of *Bacopa monnieri*

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 (MGU), Kerala, India. The voucher specimen was kept at the departmental Herbarium, MGU, and Kerala. Fresh leaves of BM were shade dried and powdered in a mill without producing much heat. Whole leaf powder was employed as such for incorporation in to the commercial powdered diet.

4.0 *Drosophila* culture and husbandry

Drosophila melanogaster, wild type (Oregon K), adult male flies (8-10d old) were obtained from the Drosophila stock culture facility at Manasagangothri, University of Mysore, Karnataka, India. The flies were maintained at 25±1^0C and 70–80% relative humidity, and fed on a standard wheat flour-agar diet with yeast granules as the protein source (Ashadevi and Ramesh, 2001). One liter semisolid diet contained 100g wheat flour, 100g Jaggery (carbohydrate

source), 10g agar agar (solidifying agent) and 7.5ml propionic acid (antifungal agent). Few granules of yeast were added to ensure availability of protein source. After 24h, flies were transferred to stock bottles to avoid sticking of flies to media. Male adult flies were isolated by following brief anesthesia. *(Generally, a given number of flies are exposed to few drops of diethyl ether in a small airtight glass container for one minute. In case of over anesthetization, the wings of the flies will be found perpendicular to the body axis).*

4.1 Synchronization of adult flies and larvae

Delcour's method (Delcour, 1969) was used to generate synchronized adult flies as well as larvae.

Adult flies: Young female flies were starved for 6 h in a glass container. Small size blocks of agar were prepared by adding 3% agar-agar in 100ml boiling water, followed by addition of 3.5ml absolute alcohol and 2.5ml acetic acid. Yeast paste was spread on the blocks. These agar blocks mainly acts as platform for flies to lay eggs. Alcohol attracts the flies towards the platform and acetic acid promotes fecundity. Then synchronized eggs were collected and transferred to culture bottles. Within 24hrs, egg hatches into 1^{st} instar larvae subsequently undergo 2^{nd} and 3^{rd} instar larval stage. For the experimental purpose we have used 3^{rd} instar larvae because of active crawling behavior and voracious feeding nature. Similarly synchronized adults flies were also collected by this method.

Larvae: Synchronized eggs were collected by following Delcour's method as earlier described. Synchronized larvae were (either 2^{nd} instar or 3^{rd} instar larvae) collected from culture bottle and washed thoroughly by using phosphate buffer saline (PBS, pH 7.4). Following isolation, larvae were transferred to vial containing regular diet and allowed to feed on yeast solution containing test compound (Rotenone a neurotoxicant). After exposure to prescribed time period larvae were separated and washed with buffer and homogenized with same buffer (PBS, pH 7.4) and used for biochemical assays.

5.0 Care and maintenance of Mice

Prepubertal and adult male CFT-swiss mice (4wk and 8wk old) were used in the validation experiment to compare with Drosophila model. Animals were randomly drawn from the 'Institute animal house facility' and housed in rectangular polypropylene cages (n=3) kept on racks built of slotted angles and the cages were provided with dust free paddy husk as the bedding material. The animals were acclimatized for a week prior to the start of experiment in a controlled atmosphere with a12h light: dark cycle maintained on a commercial pellet diet (supplied from M/s Saidurga Feeds and Foods Pvt. Ltd, Bangalore, India) with free access to tap water *ad libitum* throughout the studies. All experiments, inclusive of animal handling and sacrifice were conducted strictly as per the guidelines of 'Institutional Ethics Committee' regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment of India, India.

EXPERIMENTAL PROCEDURES

1. 0 Co-exposure protocol

Known numbers of adult synchronized flies (8-9days old) were allowed to feed on a semisolid diet containing both neurotoxicant and phytochemical together for the experimental period. Fresh diet was replaced on alternative days so as to ensure availability of test compounds. At the end of the experiment, flies were mildly anesthetized and used for biochemical assays.

2. 0 Prophylaxis protocol

Known numbers of flies were pre-fed with the given phytochemical in diet for experimental period, and then exposed to an acute dose of the neurotoxicant (saturated on filter paper discs).

3. 0 Neurotoxicant exposure: filter paper disc method

Adult flies were allowed to feed on neurotoxin on filter discs (a stack of 4 filter disc, 1.5cm diameter) (paraquat or acrylamide) dissolved in 5% sucrose

solution. Flies were starved for 3-4 hrs prior to their transfer to vials containing filter discs.

4. 0 Preparation of fly homogenates

i) Whole body homogenate/cytosol was prepared by using 0.1M sodium-phosphate buffer, pH 7.4. Following homogenization, homogenate was filtered through sieve with nylon mesh (pore size, 10microns) and centrifuged at 2500Xg for 10 min at 4^0 C. Supernatant was immediately used for biochemical assay. Protein free fractions of the supernatants were obtained by treatment with 10% TCA. The resultant pellet was used for estimation of protein carbonyl levels.

ii) To prepare head and rest of the body homogenates, known numbers of heads were separated from rest of the body using a sharp blade/cutter. 20 heads/rest of the body were homogenized in 100 µl/500µl of sodium-phosphate buffer, pH 7.4, Followed by centrifugation at 2500Xg for 10 min at 4^0 C. Supernatant was used directly to estimate the dopamine level by HPLC method.

5. 0 Preparation of brain homogenates

Mice of control and treatment groups were sacrificed under mild anesthesia. Brain was isolated, rinsed in ice cold saline and processed immediately. The brain regions i.e. 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. The homogenate was centrifuged at 1000xg for 10 min. and the supernatant was used for the quantification of malondialdehyde (MDA), reactive oxygen species (ROS) and total thiols. Protein free fractions of the supernatants were obtained by treatment with 10% TCA and the resultant pellet was used for estimation of protein carbonyl levels.

6.0 Mitochondrial Fractions

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 4^0C. The homogenates were centrifuged at 1000xg for 10 min at 4^0C 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) and re-suspended in the buffer and stored at -80^0 C until use. Protein was estimated in mitochondrial suspensions according to the method of Lowry et al., (1951).

ASSAY METHODS

1.0 Non-enzymatic oxidative markers

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

Reactive oxygen species (ROS) generated in whole body homogenate and mitochondrial fraction were measured using dihydro dichlorofluorescein diacetate (DCF-DA) (a non polar compound that after conversion to its polar derivative by intracellular esterases, rapidly reacts with ROS to form a highly fluorescent dichlorofluorescein, DCF). An aliquot (equivalent to 0.1mgP) was dispensed into tubes containing Locke's buffer solution (154 mM NaCl, 5.6mM KCl, 3.6mM $NaHCO_3$, 5mM HEPES, 2mM $CaCl_2$ and 10mM glucose/lit, pH 7.4) to which was added 10ul of DCFDA (5uM) and incubated for 30min at room temperature. The fluorescence was measured with excitation and emission wavelengths at 480 and 530nm. Background fluorescence was corrected including parallel blanks. ROS generation was quantified from DCF standard curve and expressed as pmolDCF formed/min/mg protein.

Lipid peroxidation (Ohkawa et al., 1979)

The extent of Lipid peroxidation was measured by measuring the thiobarbituric acid reactive substances (TBARS) in whole fly homogenate and mitochondrial

fraction. Briefly, the reaction mixture contained 500µl fly homogenate/mitochondrial preparations, 1.5 ml acetic acid (pH 3.5, 20%v/v), 1.5 ml of TBA (0.8% w/v), 200µl sodium lauryl sulphate (SDS) (8% w/v). The mixture was heated in a boiling water bath for 45 min and adducts formed were extracted into 3 ml of 1-butanol, and the colour intensity was measured at 532 nm and quantified as malondialdehyde equivalents using 1,1,3,3-tetramethoxypropane as the standard.

Hydroperoxide levels (Wolff, 1994)

Water soluble hydroperoxide levels were determined according to the method of ferrous iron oxidation with xylenol orange (FOX 1). An aliquot of homogenate/mitochondria (equivalent to 100µg protein) were added to 1 ml of FOX 1 reagent (250µM Ferrous ammonium sulphate; 100mM sorbitol; 25mM H_2SO_4; 100µM xylenol orange), and incubated for 30 min at room temperature. The mixture was centrifuged to remove flocculent material and the absorbance was measured at 560nm and expressed as nm HP/mg protein (ε- $1.5X10^4$mol/cm).

Protein carbonyls (Levine et al., 1990)

Protein carbonyl content was quantified using dinitrophenylhydrazine (DNPH). Fly homogenate/mitochondria precipitate was obtained using 20% trichloroacetic acid (TCA) and centrifuged at 5200Xg for 10 min. Precipitate equivalent to 0.5mg protein were allowed to react with 10mM DNPH dissolved in 2N HCl and incubated for 1 h at room temperature. Following centrifugation, the pellet was washed three times with acetone to remove excess reagent and dissolved in 1ml of Tris-HCl buffer (2% SDS, 20mM tris HCl, 0.14M NaCl, pH 7.4). The absorbance was measured at 360nm and expressed as nmol carbonyl/mg protein (ε-22,000$M^{-1}cm^{-1}$).

Nitric oxide levels (Choi et al., 2002)

Nitric oxide levels were measured by using commercially available Greiss reagent. The principle of assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is based on two step diazotization

reaction in which acidified NO_2 –produces a nitrosating agent which reacts with sulphanilic acid to produce the diazonium ion which is coupled with NED, N-(1-napthylethylenediamine to form the chromophoric azo derivative which absorbs light at 540nm. Nitric oxide levels were quantified from a sodium nitrate standard curve.

2.0 Antioxidant molecules

Reduced glutathione levels (Mokrasch and Teschke, 1984)

Reduced glutathione (GSH) content was estimated based on a fluorimetric method employing *o*-phthalaldehyde (OPT). An aliquot of fly homogenate or mitochondria was precipitated with 0.1M formic acid, centrifuged at 5200Xg for 10 min. From this, an aliquot of supernatant was added to 0.1M buffer formaldehyde (1:4, Formaldehyde: 5mM EDTA) was allowed to react with OPT (1mg/ml in methanol). Following incubation for 30min at room temperature, fluorescence was measured at excitation of 345 and emission at 425nm. Glutathione levels were quantified from the standard curve and expressed as µg GSH/mg protein.

Oxidized glutathione levels (Mokrasch and Teschke, 1984)

To an aliquot of homogenate, 0.1ml buffer formaldehyde (1:4(v/v) 37% formalin: 0.1M Na_2HPO_4), and 0.1ml NaOH (0.1M) were added followed by 0.10ml OPT. After 45 min at ambient temperature, the fluorescence was measured with excitation wavelength at 345nm and emission 425nm. Concentration of GSSG was calculated from a standard curve and expressed as µg GSSG/mg protein.

Total thiols (Ellman, 1959)

An aliquot of whole fly homogenate/mitochondrial preparation (equivalent to 0.5mg protein) was added to Tris buffer (02M, pH 8.2) containing 25µl of dithiobisnitrobenzoic acid (DTNB,10mM in methanol) and 1.975ml of methanol. Following incubation for 30 min at room temperature, the tubes were centrifuged at 3,000Xg for 10 min. The clear supernatant was read at 412nm against distilled water blank and expressed as nmol DTNB oxidized/mg protein (ε -13.6/mM/cm)

Non protein thiols (Ellman, 1959)

Whole fly cytosol/mitochondrial protein (equivalent to 0.1mg protein) was added to 5% TCA and centrifuged at 10,000Xg for 30 min. An aliquot of protein-free supernatant was added to 1 ml Tri buffer (0.4M, pH 8.9) containing 25µl DTNB (10mM in methanol) and kept in dark for 15 min at room temperature. The absorbance was read at 412 nm and expressed as nmol DTNB oxidized/mg protein (ε -13.6/mM/cm).

3.0 Enzymatic oxidative markers

Superoxide dismutase (Kostyuk and Potapovich, 1989)

Superoxide dismutase (SOD) activity was determined by monitoring the inhibition of quercetin auto oxidation. Total volume of 1 ml reaction mixture contained 3-5 µg protein; 0.016M sodium phosphate buffer (pH 7.8), 8mM N,N,N,N tetramethyl ethylenediamine (TEMED) and 0.08mM Ethylenediamineteraacetic acid (EDTA). Reaction was started by adding 0.15% quercetin dissolved in dimethyl formamide. The rate of quercetin auto oxidation was monitored for 3 min at 406nm. Following addition of sample (5-10µg cytosol/mitochondrial protein), the decrease in absorbance was monitored. The amount of protein that inhibits quercetin oxidation by 50% was defined as one unit. The results were expressed as units/mg protein.

Catalase (Aebi, 1984)

Catalase (CAT) activity was determined according to a previously described method (Aebi, 1984). The rate of H_2O_2 (final concentration 8.8mM) decomposition by the enzyme was monitored by addition of an aliquot (equivalent to 10µg protein) from whole fly homogenate/mitochondria. The decrease in H_2O_2 was monitored for 3 min at 240 nm and expressed as µmol of H_2O_2 decomposed/min/mg protein (ε-H_2O_2 44.1 mM^{-1} cm^{-1}).

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

Glutathione-S-transferase (GST) activity was measured by following an increase in absorbance at 340 nm for 3 min. Reaction mixture consists of 50µl

of sample added to 0.1M sodium phosphate buffer (pH 6.5) with 1mM EDTA, 20 mM reduced glutathione, 20 mM CDNB. This indicates the rate of conjugates formation between reduced glutathione (GSH) and 1-chloro-2-4-Dinitrobenzene (CDNB). The activity was expressed as µmol conjugate formed/min/mg protein (ε -9.6/mM/cm).

4.0 Neurotoxicity markers

Acetylcholinesterase (Ellmann, 1961)

To the reaction mixture containing 1 ml phosphate buffer (0.1 M, pH 8.0), 50 µl of 5,5-dithiobis 2-nitrobenzoic acid DTNB (10mM), 50 µl test sample (whole fly cytosol) and 20µl acetylthiocholine iodide were added and the change in absorbance was monitored at 412 nm for 3 min. The enzyme activity was expressed as nmoles substrate hydrolyzed /min/mg protein**.**

Butyrylcholinesterase (Ellmann, 1961)

To the reaction mixture containing 1 ml phosphate buffer (0.1 M, pH 8.0), 50 µl of 5,5-dithiobis 2-nitrobenzoic acid DTNB, 10mM, 50 µl whole fly cytosol and 20µl butyrylthiocholine iodide (78mM) was added. Activity of butyrylcholinesterase (BChE) was measured by following change in absorbance was monitored at 412 nm for 3 min. The enzyme activity was expressed as nmoles substrate hydrolyzed /min/mg protein**.**

Dopamine levels (Dalpiaz et al., 2007)

Head and rest of the body regions isolated from adult Drosophila were homogenized in 100 and 500 µl of ice cold 0.1M phosphate buffer (pH, 7.4) containing 1mM EDTA and filtered through sieve. Following centrifugation at 2500Xg for 10 min, the supernatant was injected directly into HPLC column (Discovery C-18, 25 cm X 4.6mm, 5 µm, Supelco sigma-aldrich) equipped with a ultraviolet detector set at 280nm. Mobile phase consisted of 0.2% aqueous trifluoroacetic acid and methanol (70:30 v/v) and the flow rate was maintained at 1ml/min. Dopamine levels were calculated from the Dopamine standard graph and results were expressed as µg dopamine/mg protein.

ATPase activities (Desaiah and Ho, 1979)

ATPase activity was measured by determining the inorganic phosphate liberated from ATP according to the method of Desaiah and Ho (1979). Total ATPase was assayed in a medium containing Tris-HCl buffer (50mM, pH 7.4), NaCl (100mM), KCl (20mM), $MgCl_2$ (5mM) and 0.1mM ouabain with the omission of NaCl and KCl. The reaction was started by the addition of 50µg cytosl/mitochondrial protein and incubated for 15 min at 37^0C. The reaction was terminated by addition of 20% TCA. Following centrifugation at 1,500Xg for 10 min the inorganic phosphate in the protein free supernatant was determined according to Fiske and Subbarao (1921). Blanks without enzyme were carried throughout the entire procedure. Enzyme activity was expressed as µg inorganic phosphate liberated per mg protein for 15 min. Na^+ K^+ ATPase and $Mg2^+$ ATPase.

5.0 Iron content in flies (Peters et al., 1956)

Tissue iron levels were quantified using bathophenanthroline, a ferrous iron sensitive dye. A 5% whole fly homogenate was prepared using protein precipitating agent (TCA 0.1% v/v, 0.3ml thioglycolic acid, 0.02ml HCl). The homogenate was centrifuged at 2500Xg for 10 min and the supernatant was used for estimation. A known volume of supernatant was added to 1ml of bathophenanthroline color reagent (bathophenanthroline-thioglycollic acid: sodium acetate:water:1:20 :20) and incubated at room temperature for 10min. the absorbance was measured at 535nm and quantified using a reference standard iron graph.

6.0 Electron transport chain (ETC) enzyme activities

NADH-Cytochrome C reductase (complex I-III) (Navarro et al., 2004)

Mitochondria isolated from whole fly of adult flies (equivalent to 50 µg) were added to phosphate buffer (0.1 M, pH 7.4) containing NADH (0.2 mM) and KCN (1 mM). The reaction was initiated by addition of 0.1 mM cytochrome C and the decrease in absorbance was monitored for 3 min at 550 nm. The activity was expressed as µmol cytochrome c reduced/min/mg protein (ε -19.6 mM/cm).

Succinate: Cytochrome C reductase (Complex II-III) (Navarro et al., 2004)

An aliquot of mitochondria from whole fly (equivalent to 50 µg) were added to phosphate buffer (0.1 M, pH 7.4, 2 mM EDTA) containing succinate (20 mM) and KCN (1 mM). The reaction was initiated by addition of 0.1 mM cytochrome C and decrease in absorbance was monitored for 3 min at 550 nm. The activity was expressed as µmol cytochrome c reduced/min/mg protein (ε-19.6 mM/cm).

7.0 Assay for citric acid cycle enzymes

Succinate dehydrogenase (Penington, 1961)

Succinate dehydrogenase (SDH) activity was determined by incubating mitochondria (equivalent to 50 µg protein) with 50mM potassium phosphate (pH, 7.4) containing sodium succinate (0.01 mol/L) and p-iodonitrotetrazolium violet (2.5 µg) for 10 min. The reaction was stopped by addition of 10% TCA. The color obtained was extracted with ethyl acetate: ethanol: trichloroacetic acid (5:5:1 v:v:w) and measured at 490 nm. The activity was expressed as mmol substrate oxidized/mg protein (ε -2000/mM/cm).

Malate dehydrogenase activity (Kitto, 1969)

Malate dehydrogenase (MDH) activity was measured by the addition of whole fly mitochondrial protein (equivalent to 100 µg) to 0.1M potassium phosphate (pH, 7.5) containing NADH (14.3 mM) and oxaloacetate (20mM) and the decrease in absorbance was monitored at 340 nm for 3 min. The enzyme activity was expressed as µmol NADH oxidized/min/mg protein (ε–6.22 mM/cm)

Citrate synthase activity (Srere, 1969)

Citrate synthase activity was determined by monitoring the oxidation of DTNB at 412nm. Whole body mitochondria of adult flies (equivalent to 100 µg protein) were added to a reaction mixture containing Tris-HCl buffer (0.1M, pH 8.1 containing 0.1% Triton X-100), 0.2mM DTNB and 0.1mM acetyl Co-A. The reaction was initiated by addition of oxaloacetae (10mM). The enzyme activity was expressed as nmol thiol group oxidized/min/mg protein (ε -13.6/mM/cm).

8.0 Mitochondrial integrity assay

Membrane potential (Shimizu et al., 1999)

Whole fly mitochondria equivalent to 50 µg protein was added to 0.1M phosphate buffered saline (PBS, pH 7.4) containing Rhodamine-123 (1.5µM) and incubated at 37^0C for 30 min. Following centrifugation at 10,000Xg for 10min at 4^0C, the fluorescence intensity of the supernatant was measured at excitation and emission wavelengths of 490nm and 520nm respectively.

NADH-dependent MTT reduction (Berridge and Tans 1993)

Mitochondria isolated from whole body of adult flies were incubated in a Mannitol- Sucrose-HEPES buffer (pH 7.4) containing NADH (10mM) and 15ul MTT (5mg/ml) at 37^0C for 60 min. the formazan crystals formed were dissolved in aqueous 10% SDS-45% DMF buffer (v/v pH 7.4). The absorbance of clear solution was measured at 570nm and expressed as absorbance/mg protein.

Succinate-dependent MTT reduction (Berridge and Tans 1993)

Mitochondria isolated from whole body of adult flies were incubated in a Mannitol- Sucrose-HEPES buffer (pH 7.4) containing succinate (20mM) and 15ul MTT (5mg/ml) at 37^0C for 60 min. the formazan crystals formed were dissolved in aqueous 10% SDS-45% DMF buffer (v/v pH 7.4). The absorbance of clear solution was measured at 570nm and expressed as absorbance/mg protein.

9.0 Protein estimation (Lowry et al., 1951)

An aliquot of fly cytosol or mitochondrial protein concentrations were determined by using Lowry's reagent (2% Na_3CO_3 in 0.1N sodium hydroxide containing 1% copper sulphate and 2% sodium potassium tartarate) and incubated for 10min at room temperature. Following addition of 0.1ml of Folin-Ciocalteu reagent (1N), the reaction mixture was allowed to stand for 20min at room temperature. The absorbance was measured at 750nm and the concentration of protein was determined using bovine serum albumin as the standard.

10.0 Negative geotaxis assay (Feany and Bender, 2000)

Test flies were anesthetized and placed in a vertical glass column (length, 25cm; diameter, 1.5cm) sealed at one end. After a brief recovery period, flies were gently tapped to the bottom of the column. Following 60s observation of their climbing ability along the wall of the vertical glass column by visual method, flies that reached the top of the column and flies that remained at the bottom were counted separately. Data was expressed as percent flies escaped beyond minimum distance of 6cm in 60 seconds of interval. 20 adults per replication were used for each assay and the assay was repeated three times. The score for each replication was an average of three such trials for each group of flies including control.

11.0 Statistical analysis

Experimental data obtained were expressed as mean ± standard error (SEM) and analyzed by Student's't' test. Data obtained in modulation of oxidative damage with various phytochemicals were expressed as mean ± SEM and analyzed by one way ANOVA using SPSS professional statistical software 2009 version 11.0 followed by appropriate post hoc test. For multiple comparisons we used Tukey post hoc test, whereas comparison between control and all the treated groups we used Dunnet's test. *P* value was set at 0.05 as the minimum level of significance.

CHAPTER I

Efficacy of phytochemicals to rescue *Drosophila* against neurotoxicant-induced lethality

INTRODUCTION

In recent decades, there has been a phenomenal increase in the number of laboratories employing invertebrates to model several human neurodegenerative diseases (NDD). Drosophila system has been extensively used to understand the etiology of various neurological disorders including sporadic Parkinson's disease (PD) (Feany and Bender, 2000; Coulom and Birman, 2004; Marsh and Thompson, 2006; Whitworth et al., 2006; Bonilla et al., 2006; Chaudhuri et al., 2007; Botella,2008; Saini et al., 2010). The specific molecular mechanisms leading to neuronal death in PD are less understood. However, most insights are derived from investigations performed in experimental models of PD especially produced by neurotoxins (Bove et al., 2005). Rodent models employed to screen therapeutic interventions for modulating neurodegeneration have proved to be expensive and time–consuming. Hence, researchers are utilizing Drosophila system for basic understanding of neurodegenerative mechanism/s. More importantly since Drosophila offers the power of rapid screening analysis, this system is currently popular and widely utilized for developing newer therapeutic interventions against various NDD. In this regard, our laboratory has initiated a research programme to establish Drosophila as an alternative model to assess neuroprotective efficacy of phytochemicals and to elucidate their pharmacological properties against neurotoxicant –induced oxidative stress, mitochondrial dysfunction and neurotoxicity.

Ayurveda has classified selected plants as "medhya rasayanas" ("medhya"= intellect or cognition, and 'rasayana'= rejuvenation in Sanskrit) that are specifically beneficial in boosting memory, preventing cognitive deficits and improving brain function (Kumar, 2006; Singh et al., 2008; Gohil and Patel, 2010). Such plant products have been used in herbal and traditional medicine since they offer improved therapeutic benefits in CNS disorders with cognitive impairment and memory deficits compared to pharmaceutical drugs. One such memory booster and a natural remedy for CNS function and therapy is "Brahmi" . In most parts of India, Brahmi by and large refers to *Bacopa monniera* (BM) also termed Jalanimba (Russo and Borrelli, 2005). Early

references to Brahmi are also referred to *Centella asiatica*, (CA) the Indian penniwort more correctly known as “mandukaparni”. The entire plant is used for medicinal purposes (Satyavati et al., 1976) and specific uses include the treatment of asthma, insanity and epilepsy. The plant has been extensively utilized as a nootropic, digestive aid and to improve learning, memory and respiratory function. Our recent studies have demonstrated the neuroprotective efficacy of BM extract against neurotoxicant induced oxidative stress and mitochondrial dysfunctions (Shinomol, 2008; Shinomol and Muralidhara, 2010; Shinomol et al., unpublished).

Various neuropharmacological properties of *Centella asiatica* (CA) have been described and the major effects in experimental animals comprise of memory enhancement and cognitive function (Veerendra kumar and Gupta, 2002; Wijeweera et al., 2006), immunostimulatory activity (Wang wt al, 2006) and antiepileptic property (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 (Zainol et al., 2003; Mangas et al., 2006; Hussin et al., 2007). Our recent studies have demonstrated the neuroprotective ability of CA against 3-NPA exposure in mice models (Shinomol and Muralidhara, 2008; 2009; Shinomol et al., 2010). *Withania somnifera* commonly known as Ashwagandha has been used for centuries to treat variety of ailments as an aphrodisiac, liver tonic, anti inflammatory agent, astringent and more recently to treat bronchitis, asthama, ulcers and senile dementia. Clinical evidences support the use of Ashwagandha for anxiety, cognitive and neurological disorders, inflammation and Parkinson’s disease (Bhattacharya et al., 2001). The major biochemical constituents of Ashwagandha root are steroidal alkaloids and steroidal lactones in a class of constituents called W*ithanolides.* The main Withanoloides characterized includes withanine, somnine, withananine, pseudo-withanine, tropine etc. However, much of its pharmacological activity has been attributed to two main withanoloids, withaferin A and withanolide D (Grandhi et al., 1994).

Ferulic acid (4-hydroxy-3 methoxy cinnamic acid), a phenolic compound and a ubiquitous plant constituent arise from metabolism of phenyl alanine and tyrosine. Ferulic acid occurs as a major constituent in fruits (eg. Orange), some

vegetables (eg. Tomato, carrot) and sweet corn (Balasubhashini et al., 2004). Several pharmacological properties such as anti-inflammatory, anti-carcinogenic, antiageing, neuroprotective, radioprotective and anti-atherogenic properties have been reported (Kawabata et al., 2000; Kanaski et al., 2002; Rukkumani et al., 2004). Eugenol (4-allyl-2-methoxyphenol) occurs naturally as a component of clove oil, which is commonly used as a flavoring agent in baked products, beverages, sweets, and frozen dairy products. In addition, these compounds are widely used in cosmetics, dentistry, and traditional medicine. It has been reported that isoeugenol and eugenol show anti-oxidant activities, anti-inflammatory effects, inhibition of lipid peroxidation, and induction of Phase II enzymes (Kabuto et al., 2007).

Parkinson's disease (PD) is the most frequent neurodegenerative movement disorder and its pathological hall marks are a progressive degeneration of nigrostriatal dopaminergic neurons and the presence of neuronal cytoplasmic inclusions (the lewy bodies). The specific molecular mechanism leading to neuronal death in PD is still not understood. . Several epidemiological studies have suggested an association with environmental toxins, in particular mitochondrial complex inhibitors such as rotenone. Rotenone is a classical, well-characterized and high affinity specific inhibitor of mitochondrial NADH dehydrogenase (complex I), and 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). Inhibitors of complex I are known to increase ROS generation, which produces a state of oxidative stress leading to mitochondrial dysfunction (Wang et al., 2006). Another environmental contaminant , Paraquat (1, 1'-dimethyl-4, 4'-bipyridinium dichloride), a potent widely used herbicide is also considered a prime risk factor for PD based on both epidemiological evidences of increased incidence of PD after exposure and its chemical similarity to the Parkinsonism-inducing agent, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The mechanism of paraquat (PQ) neurotoxicty is known to be mediated *via* oxidative stress.

Superoxide anion radicals are generated by PQ through redox cycling *via* reaction with molecular oxygen and electron transfer reactions with NADH-dependent oxidoreductases. Acrylamide (AA), an axonal neurotoxin is and extensively used for the production of high molecular weight polyacrylamides which are modified to suit a wide variety of industrial applications. However, recently AA neurotoxicity is gaining lot of attention due to its presence in wide variety of processed foods such as potato chips, French fryings, biscuits and coffee. Recent studies have shown the involvement of oxidative stress in the neurotoxicity of acrylamide *in vivo.*

Accordingly, in the present study three phytochemicals along with two pure compounds were studied for their protective effects against three neurotoxins viz., Rotenone, paraquat and Acrylamide employing Drosophila as the model. The efficacy of phytochemicals to protect against neurotoxicant - induced lethality was studied and compared with a well known endogenous antioxidant, glutathione. Further, attempts were also made to understand the ability of phytochemicals to modulate the levels of endogenous markers of oxidative stress in whole body homogenate of *Drosophila.*

2.0 OBJECTIVE

The primary objective of this investigation was to assess the protective effects of selected phytochemicals and spice actives against neurotoxicant-induced lethality employing *Drosophila* as the model. Further, the effects of phytochemical-enriched diet on the endogenous level of oxidative markers were also studied.

EFFICACY OF PHYTOCHEMICALS TO RESCUE *DROSOPHILA* FROM NEUROTOXICANT-INDUCED LETHALITY

3.0 EXPERIMENTAL DESIGN

I. Lethality response of *Drosophila* to neurotoxicants

Preparation of neurotoxicant solutions

i) Rotenone was dissolved in DMSO and a stock solution was maintained at 40mM

ii) Paraquat solution was prepared in double distilled water (100mM).

iii) Acrylamide solution was prepared in double distilled water (100mM).

Lethality response of flies to rotenone (Rot) exposure in diet

To establish lethality response, synchronized adult (8-9 d) male flies (n=50, triplicates) were allowed to feed on diet (2ml) containing various concentrations of rotenone (25, 500, 1000 and 2000µM) for 7 days. Every alternative day, vials were replaced with fresh diet to ensure fresh availability of neurotoxicant. Mortality was recorded every day and terminally the cumulative percent mortality was computed.

Lethality response to Paraquat (PQ) using filter paper disc method

Stacks of filter papers (Whatman no.41) were saturated with various concentrations of PQ (10, 20 and 40mM) supplemented with 5% sucrose solution in order to avoid starvation of flies during treatment period. Synchronized adult male flies were starved for 3-4 hrs prior to their transfer to vials containing neurotoxicant-incorporated filter discs to ensure non-

interference of diet. Flies were observed regularly twice a day, for mortality and the data was expressed as percent mortality.

Lethality response to acrylamide (AA) using filter paper disc method

Stacks of filter papers (Whatman no.41) were saturated with various concentrations of acrylamide (2, 5 & 10mM) supplemented with 5% sucrose solution. Synchronized adult flies were starved for 3-4 hrs prior to their transfer to vials containing neurotoxicants to ensure non-interference of diet. Mortality was recorded at 24 and 48h. The data was expressed as percent mortality.

II. <u>Protective effects of phytochemicals</u>

Rotenone-induced lethality: Modulatory effect of phytochemicals

The phytochemicals used in this study were: *Centella asiatica* (CA)*, Withania somnifera* (WS) and *Bacopa monnieri* (BM) at 0.05 and 0.1% in diet. Synchronized adult male flies were transferred to vials containing both phytochemicals and rotenone (500µM) and allowed to feed for 7 days. Diet was changed on alternate days and mortality was recorded on regular basis. Terminally, the percent mortality was computed. Number of flies was employed for this study was 50 in each group, and such three replications were used.

Rotenone-induced lethality: Modulatory effect of ferulic acid and eugenol

In this study eugenol (a spice active principle) and ferulic acid (a flavonoid) were employed to understand their protective efficacy against rotenone-induced lethality. Here co-exposure protocol was followed, where in both eugenol (0.1, 0.2 and 0.5%)/ferulic acid (500 and 1000µg per two ml diet) and rotenone (500µM) was homogenously mixed in 2ml semisolid diet. Synchronized adult male flies (n=50, triplicates) were allowed to feed on rotenone-modulant enriched diet for 7 days. Diet was changed on alternate days and mortality was recorded and expressed as percent mortality.

Paraquat-induced lethality: Modulatory effect of phytochemicals

In this study, synchronized adult male flies were maintained on various phytochemicals at two concentrations prior to challenge them to paraquat using

filter disc method. Phytochemicals viz., *Centella asiatica (CA)*, *Withania somnifera (WS)*, *Bacopa monneiri (BM)* (0.05 and 0.1%) and pure antioxidants like glutathione (250 and 500µM)-enriched diet (2ml) was prepared. Adult (8-9 d) male flies (n=50, triplicates) were allowed to feed on these diets for 7 days. On alternate days, flies were fed with fresh diet containing test compounds. At the end of the experiment these pre-treated flies were challenged with paraquat (40mM) for 48hrs. Mortality was recorded at 24 and 48h and the protection among pretreated flies were compared with untreated group and expressed as percent mortality.

Acrylamide-induced lethality: Modulatory effect of phytochemicals

Synchronized adult male flies (n=50, triplicates) were pretreated with various phytochemicals at two concentrations (*Centella asiatica (CA)*, *Withania somnifera (WS)*, *Bacopa monneiri (BM)* (0.05 and 0.1%) for 7days. Pure antioxidant viz., glutathione (250 and 500µM) was employed for comparison. On alternate days flies were fed with fresh diet containing test compounds. At the end of the experiment these prefed flies were exposed to acrylamide (40mM) for 48hrs. Mortality was recorded at 24 and 48h and the protection among pretreated flies were compared with untreated group and expressed as percent mortality.

III. <u>Effect of phytochemicals on endogenous markers of oxidative stress</u>

Effect of phytochemicals on oxidative markers and status of thiols

For the biochemical study, phytochemicals viz., *Centella asiatica* (CA) and *Withania somnifera* (WS) were employed. Synchronized adult male flies (n=50, triplicates) were allowed to feed on CA/WS (at 0.05 & 0.1%) enriched diet for 7 days. Diet was changed on alternate days to ensure fresh availability of test compounds. Terminally whole body homogenate of flies were prepared in phosphate buffer (pH 7.4) and biochemical assays were carried out.

Extent of damage to lipids in terms of malondialdehyde (MDA), hydroperoxide and protein carbonyls were measured among flies treated with CA and WS at 0.05, 0.1%. Further, alteration in thiols status was quantified in

terms of total thiols, non-protein thiols and reduced glutathione levels among treated and untreated flies.

Effect of eugenol on oxidative markers and redox status

Synchronized adult flies (n=50, triplicates) were fed with eugenol-enriched diet at 250, 500 and 1000µg/2 ml diet for 7 days. Fresh diet containing test compounds were replaced on alternate days. Terminally, these flies were homogenized with phosphate buffer (pH, 7.4) and biochemical assays were carried out. Status of lipid peroxidation and water soluble hydroperoxides were quantified using thiobarbituric acid (TBA) and FOX reagent respectively. Further, reduced glutathione was measured among flies fed with eugenol and compared with untreated controls.

Effect of Bacopa monneiri (BM) on oxidative stress and thiols status

In this study a range of BM concentrations were screened to understand its effect on endogenous markers of oxidative stress. BM at various concentrations viz., 0.01, 0.05, 0.1 and 0.5% were incorporated into the diet (2 ml) and synchronized male adult flies (n=50, triplicates) were allowed to feed on BM-enriched diet for 7 days. At the end of experimental period flies were sacrificed, homogenate was prepared and used for biochemical assays viz., oxidative markers (ROS, MDA, protein carbonyls) and thiols status (reduced glutathione and total thiols).

4.0 RESULTS

EFFICACY OF PHYTOCHEMICALS TO RESCUE *DROSOPHILA* AGAINST NEUROTOXICANT-INDUCED LETHALITY

I. Lethality response of *Drosophila* to neurotoxicants

Lethality response of flies to rotenone (Rot) exposure in diet

Exposure of adult flies to rotenone resulted in a significant and concentration-dependent lethality during a 7day experimental period **(Figure 1.1)**. In general, significant mortality occurred between 4- 7 days among rotenone exposed flies. Terminally, the cumulative percent mortality at the tested concentrations was as follows: 250µM- 14%; 500µM -48%; 1000µM -78% and 2000µM -96%.

Lethality response to Paraquat (PQ) using filter disc method

Data on the incidence of lethality among flies exposed to PQ over 5 days is presented in **(Figure 1.2A)**. No mortality was observed at any of the concentrations up to 24 h. However at 48 h, mortality ensued only at higher concentrations (30 mM, 18%; 40 mM, 46%). Among the tested concentrations, we obtained 50% mortality (LC_{50}) at 40 mM at 48 h and this concentration was employed for further studies.

Lethality response to acrylamide (AA) using filter disc method

Among the tested concentrations of AA only 10mM induced significant mortality (33% mortality) at 24h. However at 48h significant mortality was recorded only in flies exposed to at 5 and 10mM AA. Mortality at 48h was 34% at 5mM and 71% at 10mM **(Figure 1.2B)**.

II. Protective effects of phytochemicals

Rotenone-induced lethality: Modulatory effect of phytochemicals

Adult flies exposed to Rot *per se* (500µM) in diet for 7 days resulted in 46% mortality. However, WS (0.05 & 0.1%) did not offer any protection, while CA only at higher concentration (0.1%) provided marginal protection (26%) against

Rot-induced mortality. Flies supplemented with CA/WS in diet did not have any effect on fly lethality and behavior. Interestingly BM co–exposure (0.05 and 0.1%) with rotenone markedly reduced the incidence of mortality **(Figure 1.3A, B & C)**.

Rotenone-induced lethality: Modulatory effect of ferulic acid and eugenol

Flies treated with rotenone alone showed 46% mortality at the end of 7 days. Interestingly, co-treatment of FA and rotenone resulted in significant reduction in mortality among adult flies. The percent protection was seen: 61, 17 & 44% at 0.1, 0.2 % 0.5% FA in diet **(Figure 1.4A)**. Similarly, eugenol supplementation also offered significant protection against mortality at both the tested concentration (51 & 55% at 500 & 1000µg respectively). However, FA and Eugenol *per se* did not have ant effect on fly lethality at the end of 7 days exposure period **(Figure 1.4B)**.

Paraquat-induced lethality: Modulatory effect of phytochemicals

Flies pre treated with CA and WS at 0.05 and 0.1% for 7 days were challenged with PQ at 40mM at 48h. PQ *per se* induced significant mortality (50%) at 48hrs. CA at lower concentration (0.05%) protected against mortality only marginally (by 22%). However, WS failed to protect flies from PQ-induced mortality. Similarly, glutathione at higher concentration (500µM) offered significant protection against PQ induced mortality.

Interestingly, the incidence of mortality among flies pre-fed with BM (0.1% for 7 days) and exposed to PQ was significantly reduced compared to flies exposed to PQ alone. While PQ *per se* resulted in nearly 50% mortality at the end of 48 h, BM pre-fed flies showed 20% mortality, suggesting a 40% protection due to BM prophylaxis **(Figure 1.5)**.

Acrylamide-induced lethality: Modulatory effect of phytochemicals

Adult flies fed with AA (10mM) *per se* showed significant mortality (74%). Interestingly, CA prefed flies showed lower mortality. Percent protection offered by CA was 47% and 40% at 0.05 and 0.1% respectively. Similarly, WS offered marginal protection (20%) only at higher concentration (0.1%). CA and WS *per*

se did not cause any mortality at any of the tested concentrations. Further, flies prefed with GSH at higher concentration (500µM) showed in significant protection (33%). However, at lower concentration percent protection was less (13%) **(Figure 1.6)**.

III. <u>Effect of phytochemicals on endogenous markers of oxidative stress</u>

Effects of CA and WS on oxidative markers and thiols status

Malondialdehyde levels measured in whole body homogenates of adult flies fed with CA and WS supplemented diet remained unchanged at lower concentration (0.05%), while higher concentration (0.1%), the MDA levels were significantly high (by 26% and 24%) with the CA and WS **(Figure 1.7A)**. However, hydroperoxide levels were consistently high among lower concentrations (29% and 24% at 0.05% of CA and WS respectively). Interestingly, at the higher concentration (0.1%) significant decrease was evident (percent decrease was 38% and 24%) **(Figure 1.7B)**. Interestingly, protein carbonyls levels were significantly decreased (by 20%) only at highest concentration of CA. However consistent increase (by 20-37%) in remaining treated groups **(Figure 1.7C)**.

Significant increase in reduced glutathione (by 23-33%) was observed among flies supplemented with CA at the concentration viz., 0.05 and 0.1% respectively. Similar response was observed in WS fed flies also (26-33%) **(Figure 1.8A)**. Marginal increase in total thiols among lower concentration (0.05%) of CA was evident whereas higher concentration remained unchanged. Further, WS supplemented flies showed marginal decrease in total thiols by 16-24% at both the concentration respectively **(Figure 1.8B)**. Moderate decrease in non-protein thiols (23%) was demonstrated among flies fed with CA at both concentrations. However, lower concentration of WS resulted in significant increase (39%) compared to untreated flies **(Figure 1.8C)**.

Effect of eugenol on oxidative markers and redox status

Adult flies supplemented with the eugenol for 7 days showed significant and dose dependent decrease in oxidative markers in whole body homogenate fraction. Decrease in MDA by 29, 52 & 56% and hydroperoxide level average

by 50% was evident. However, reduced glutathione (17, 37 & 46%) levels were consistently high among flies supplemented with 250, 500 and 1000µg diet respectively **(Figure 1.9 A, B & C)**.

Effect of Bacopa monneiri (BM) on oxidative stress and thiols status

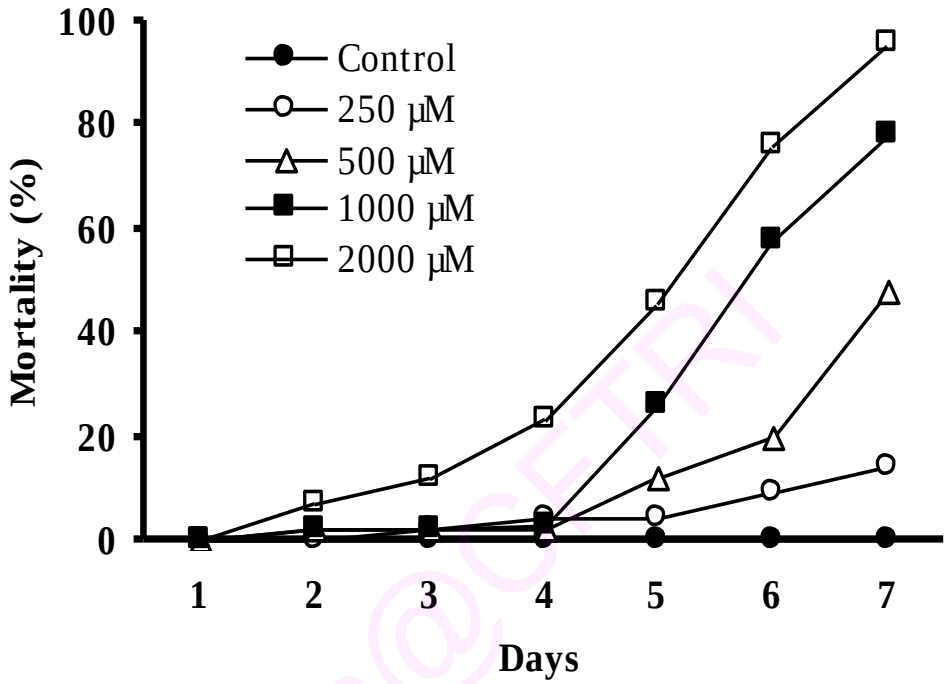
Adult flies were fed with BM for 7 days at various concentrations (0.01, 0.05, 0.1 &0.5%) in diet. Among the tested concentrations, 0.05 and 0.1% BM significantly decreased endogenous reactive oxygen species (ROS) (22 & 25%). Interestingly, MDA level was also significantly diminished at all the concentration with more robust rate (33-36%) at 0.05-0.1% respectively **(Figure 1.10 A & B)**.

Protein carbonyl content assayed among flies supplemented with BM showed significant decrease (21-26%) at 0.05 and 0.1% only. However, at lowest (0.01%) and highest (0.5%) concentration it remained unaltered. Further, hydroperoxide levels were diminished by 17, 30 and 23% at 0.01, 0.05 and 0.1% BM treated groups respectively **(Figure 1.10 C & D)**.

Reduced glutathione level was consistently high among flies supplemented with BM. The percent increase was 51, 63 and 48% at 0.05, 0.1 and 0.5% respectively. Interestingly, thiol status measured in terms of total thiols showed increased levels (25, 19% at 0.05, and 0.1%) among BM exposed flies compare to untreated flies. Similar response was observed for non-protein thiols status (20, 23, 19% at 0.05, 0.1 & 0.5%) was considered also **(Figure 1.10 E & F)**.

Figure 1.1

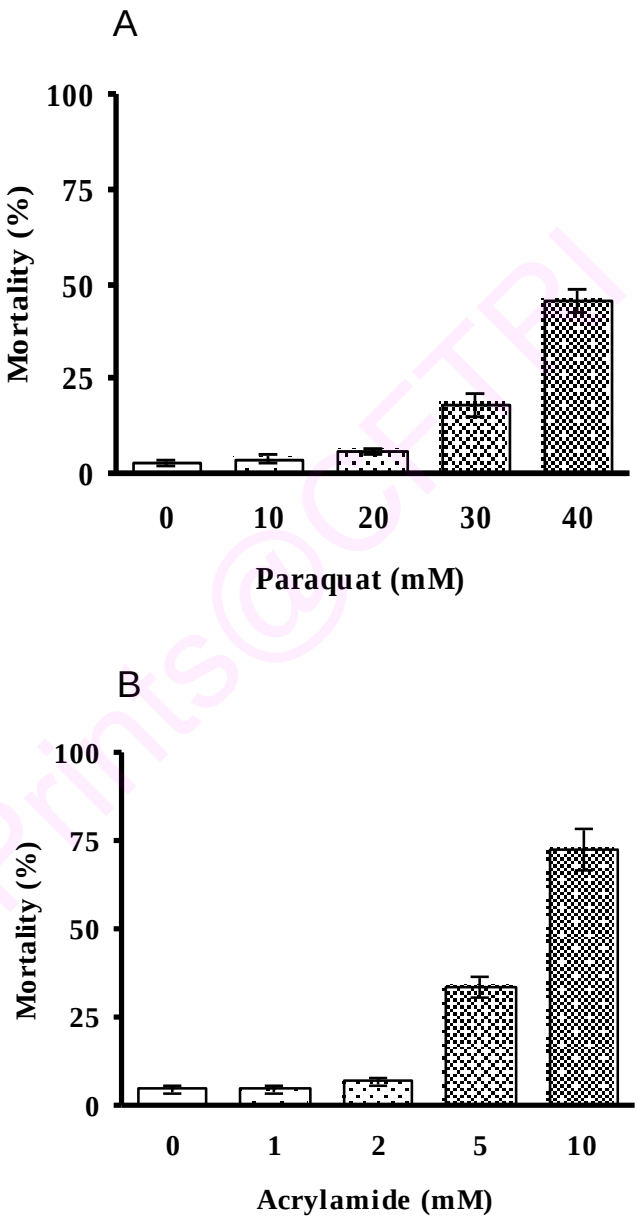
Lethality response among adult male *Drosophila melanogaster* exposed to rotenone for 7 days in diet



Rot: Rotenone in diet
n=50 flies per replicate, three such replication used for assay.

Figure 1.2

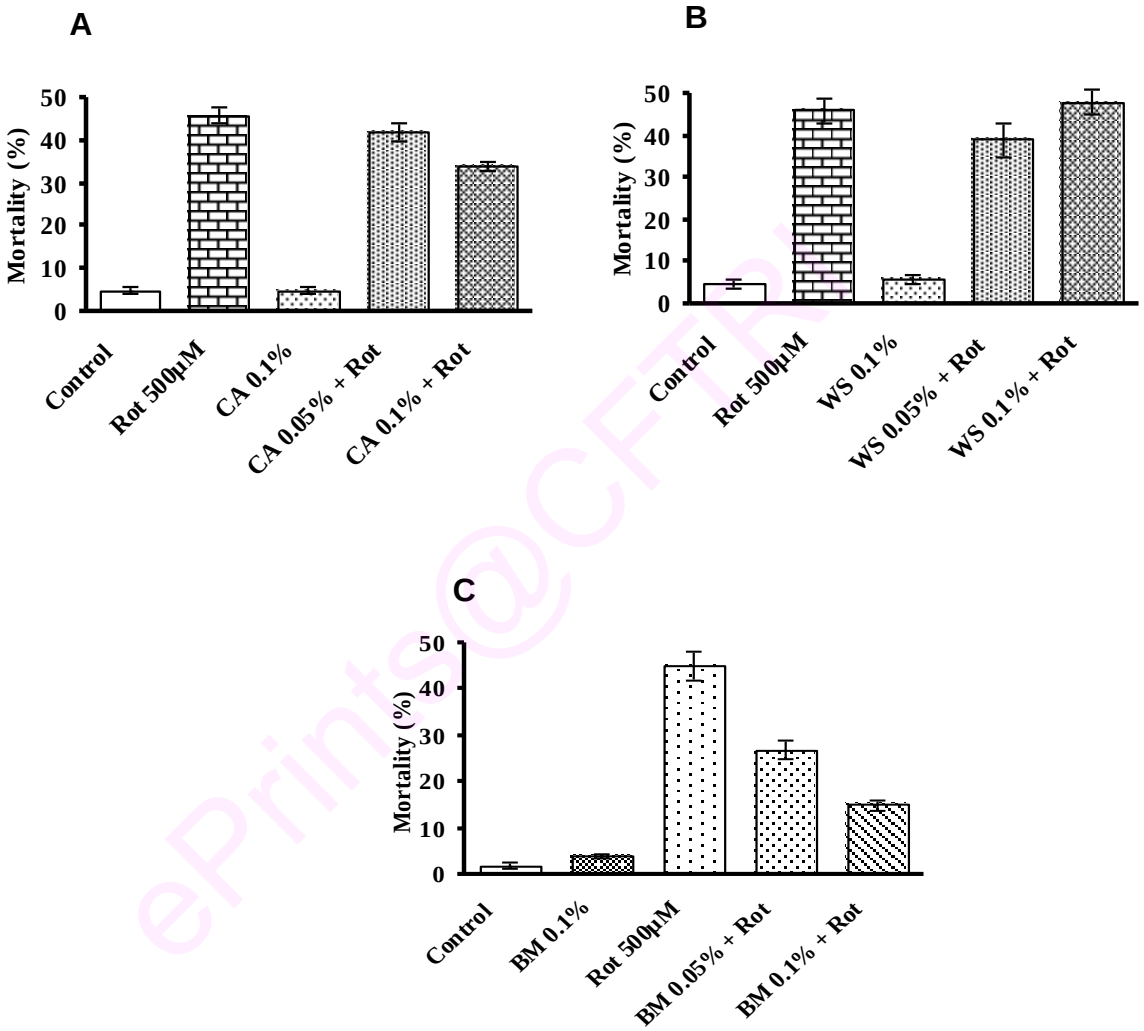
Lethality response among adult male *Drosophila melanogaster* exposed for 48h to various concentrations of Paraquat (A) and Acrylamide (B) (filter disc method)



n=50 flies per replicate, three such replication used for assay.

Figure 1.3

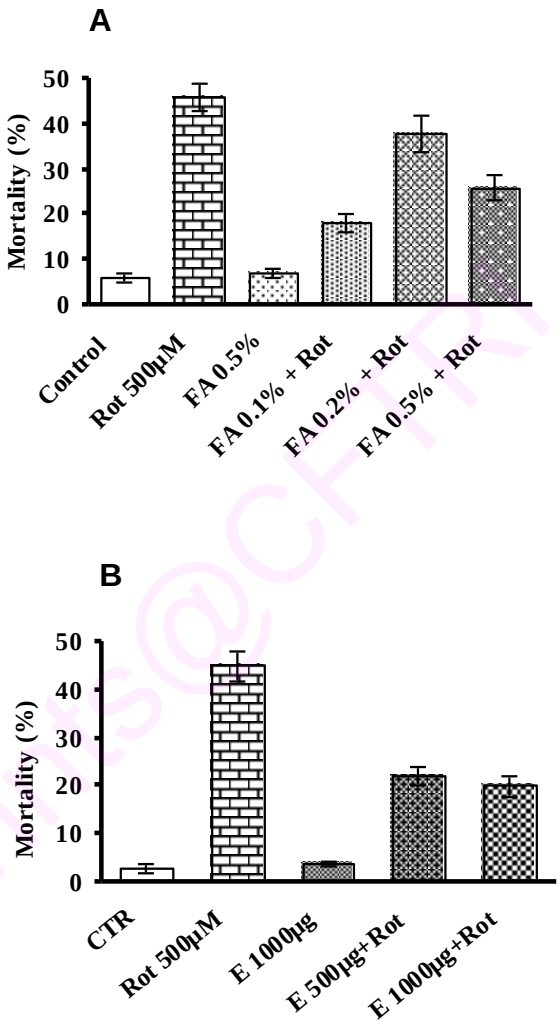
Protective efficacy of *Centella asiatica* (CA) (A), *Withania somnifera* (WS) (B) and *Bacopa monnieri* (BM) (C) against rotenone-induced lethality in adult *Drosophila*



Rot: Rotenone;
Note: n=50 flies per replicate, three such replication used for assay

Figure 1.4

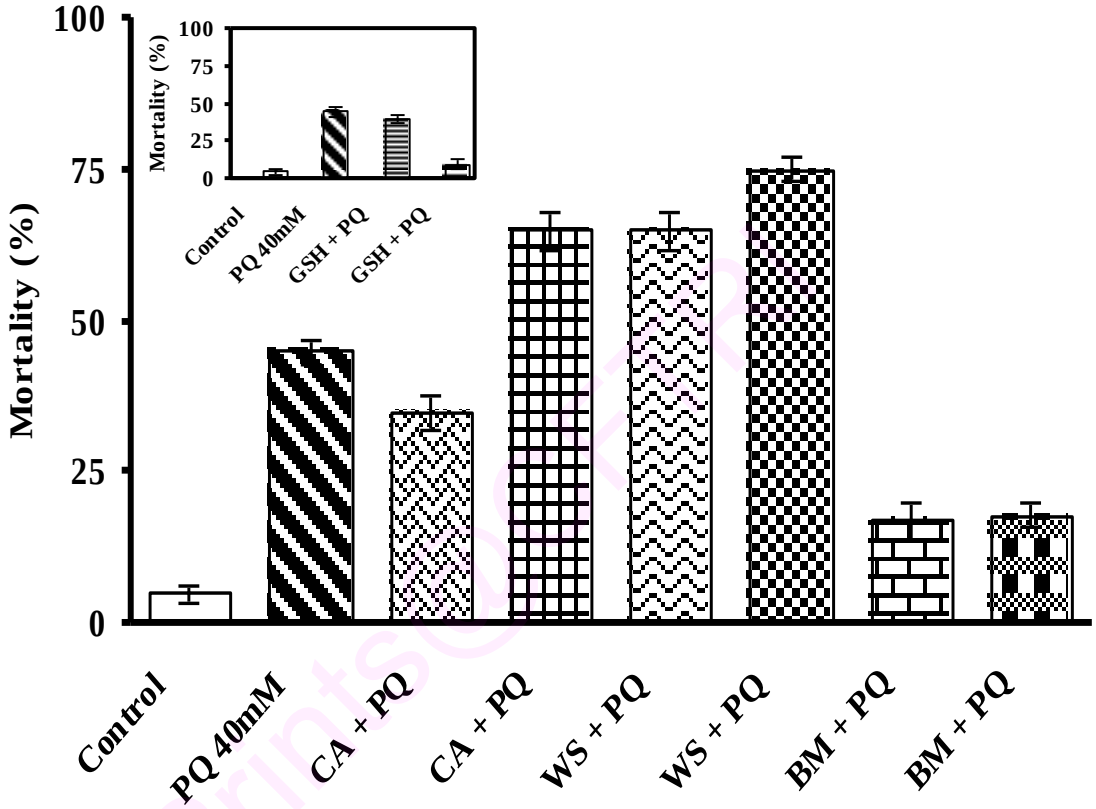
Protective efficacy of Ferulic acid (FA) (A) and Eugenol (E) (B) against rotenone induced lethality in adult *Drosophila*



n=50 flies per replicate, three such replication used for assay.

Figure 1.5

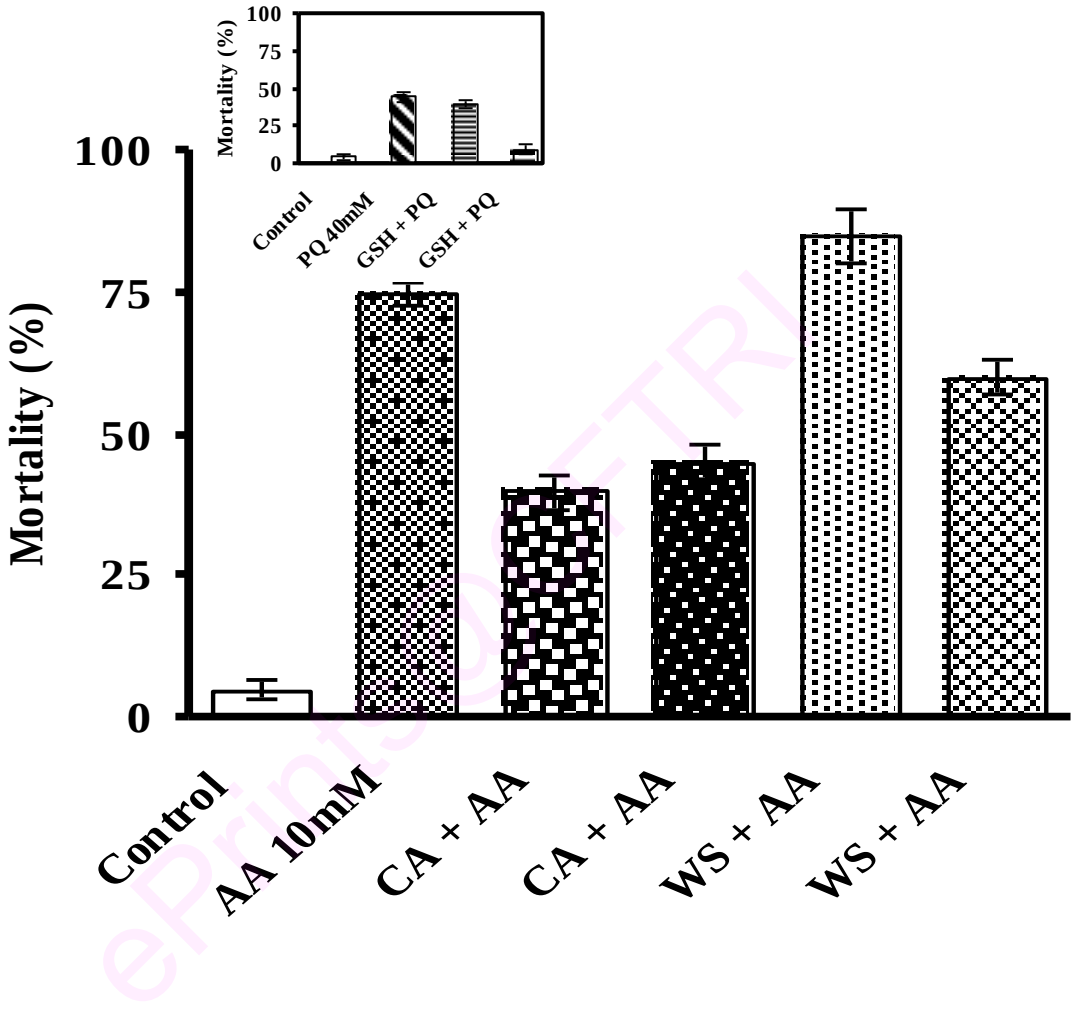
Protective efficacy of *Centella asiatica*, *Withania somnifera* and *Bacopa monnieri* against paraquat-induced mortality among adult *Drosophila*



PQ: Paraquat
BM: *Bacopa monnieri*; CA: *Centella asiatica*; WS: *Withania somnifera* (0.05 and 0.1% in diet)
n=50 flies per replicate, three such replication used for assay.
In set: protective effect of GSH (250 & 500µM)

Figure 1.6

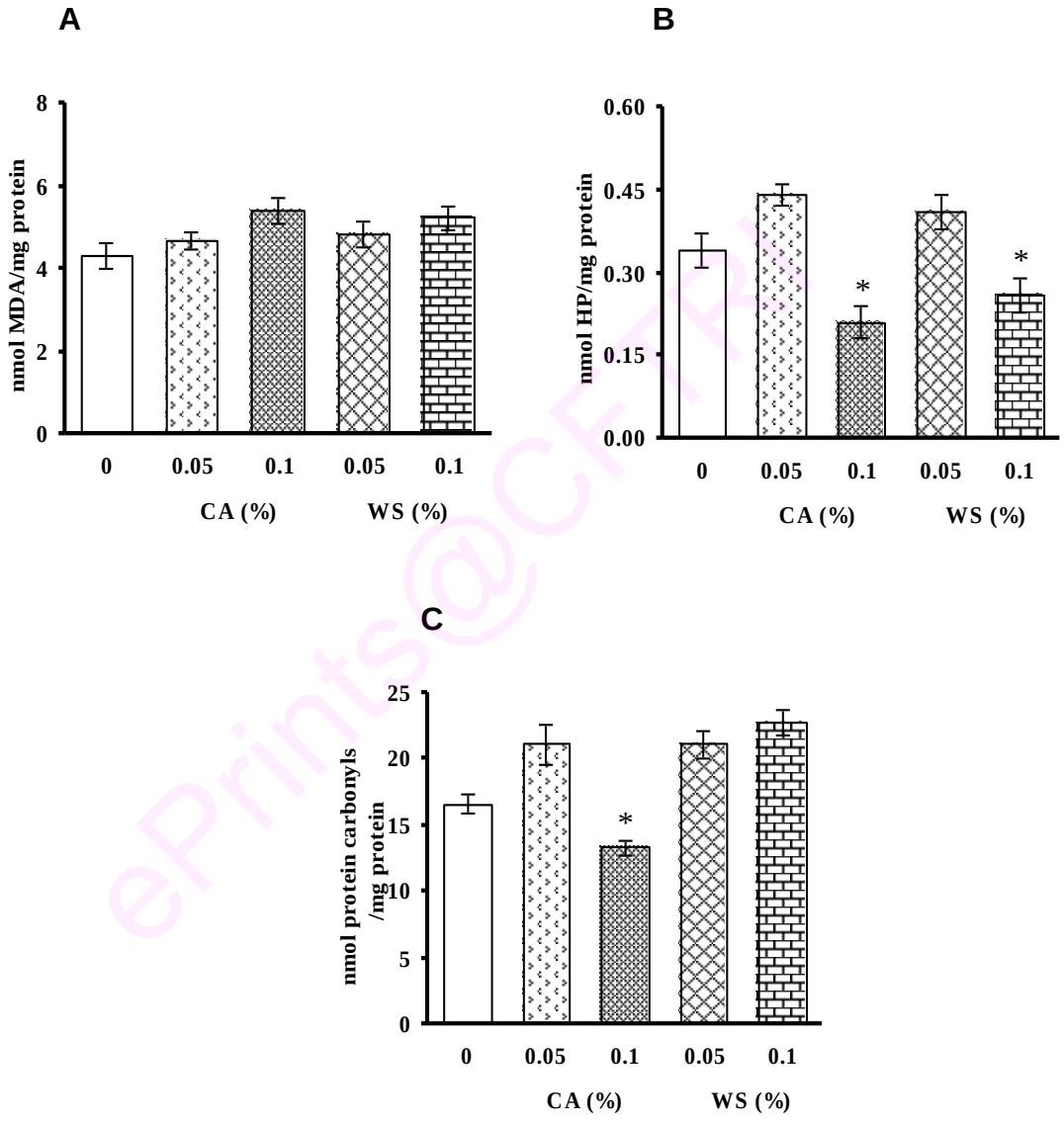
Protective efficacy of *Centella asiatica* (CA) and *Withania somnifera (WS)* against acrylamide (AA) induced mortality among adult male *Drosophila*



AA: Acrylamide in filter disc (10mM)
CA: *Centella asiatica*; WS: *Withania somnifera* (0.05 and 0.1% *in* diet)
n=50 flies per replicate, three such replication used for assay.
In set: protective effect of GSH (250 & 500µM)

Figure 1.7

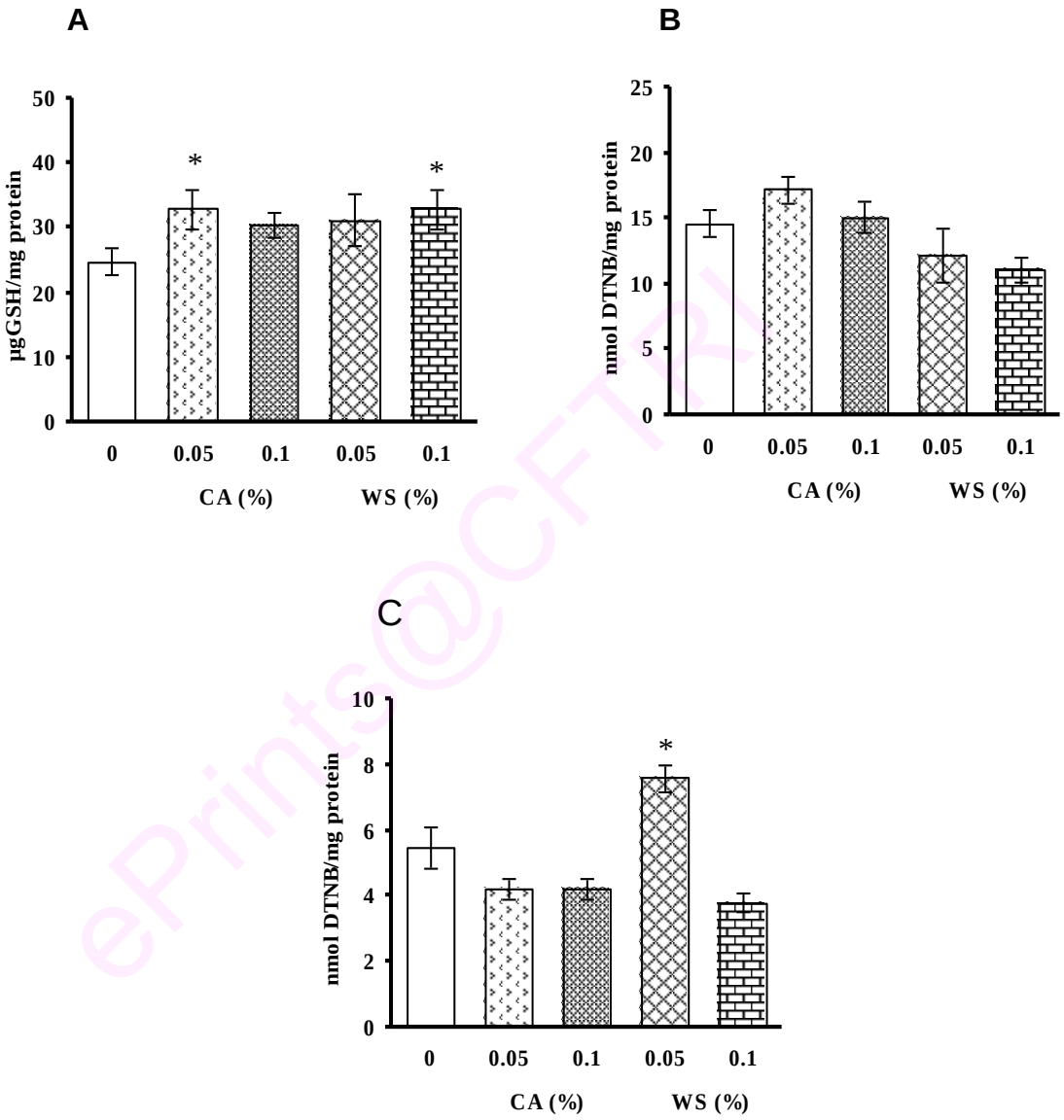
Malondialdehyde (A), hydroperoxide levels (B) and protein carbonyls (C) in adult male *Drosophila* supplemented with *Centella asiatica* (CA) and *Withania somnifera* (WS) for 7days in diet



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

Figure 1.8

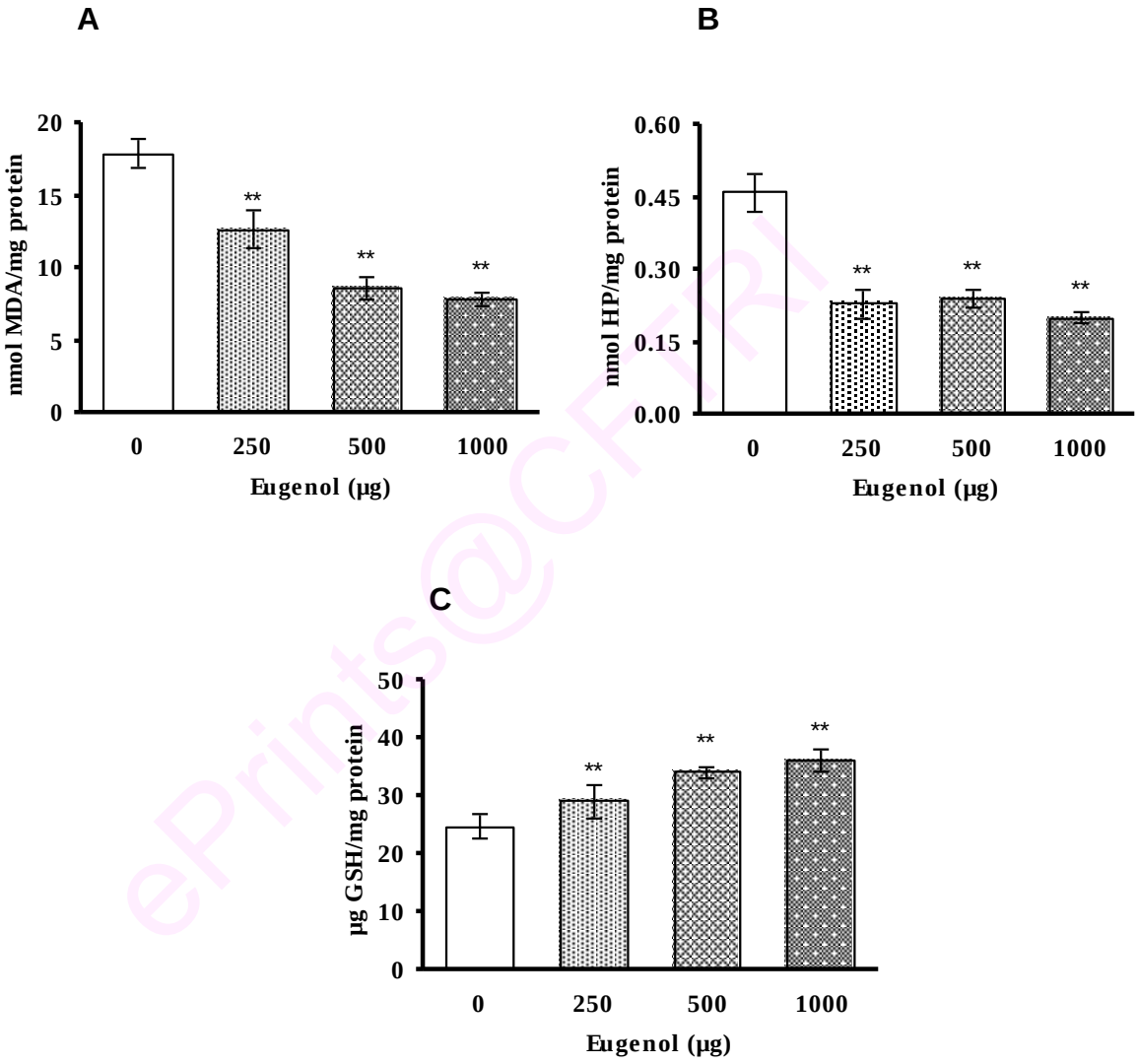
Levels of Reduced glutathione (A), total thiols (B) and non-protein thiols level (C) among adult male *Drosophila* supplemented with *Centella asiatica* (CA) and *Withania somnifera* (WS) for 7days in diet



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

Figure 1.9

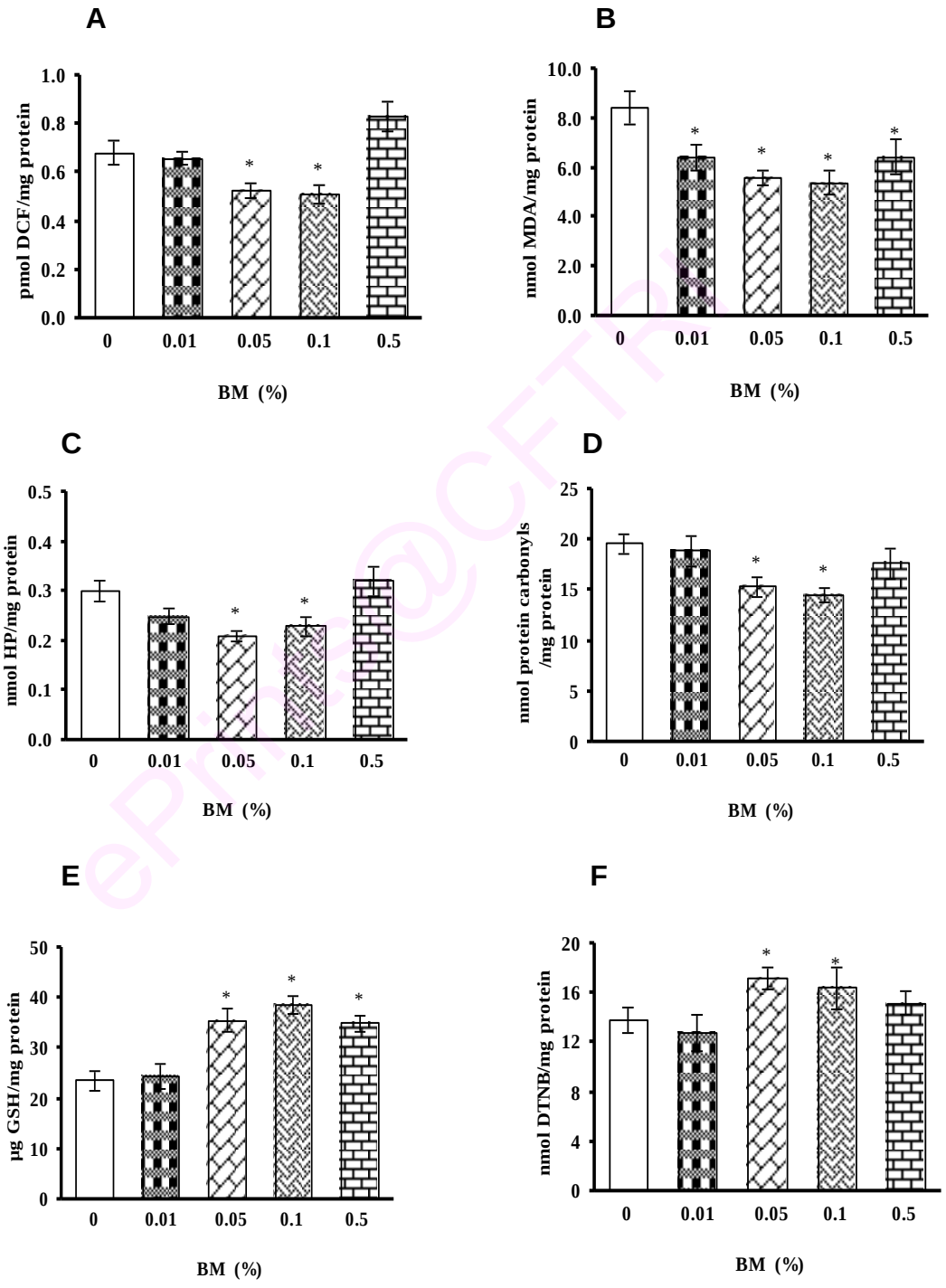
Effect of eugenol supplemented diet on malondialdehyde (A) hydroperoxide (B) and reduced glutathione (C) levels in adult male *Drosophila*



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

Figure 1.10

Effect of feeding *Bacopa monnieri* (BM) supplemented diet on ROS generation (A), Malondialdehyde (B), hydroperoxide levels (C), protein carbonyl content (D), reduced GSH (E) and total thiols (F) in *Drosophila*



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

5.0 DISCUSSION

The primary objective of this study was to assess the efficacy of phytochemicals to offer protection against neurotoxicant-induced lethality in adult *Drosophila*. Two treatment regimens viz., co-exposure and prophylaxis were employed to assess the modulation of lethality response. While co-exposure approach was employed against the rotenone models, prophylaxis was used against both Paraquat and Acrylamide model. The selection of neurotoxicants was based on the prime consideration that both rotenone and paraquat are widely employed to induce PD in flies and rodent models. The neurotoxin acrylamide is commonly formed in high amounts in everyday food stuff, mainly in fried and baked starch-enriched food and is also an industrial toxicant (Tareke et al., 2002).

Use of *Drosophila* as a PD model is considered to be more advantageous over other models for the following vital reasons i) In *Drosophila* except for cells in the gonads and some cells in the gut, there is no cell mitosis in the adult fly. Thus the adult fly might be considered as an organism of synchronously ageing cells. ii) *Drosophila* represents an unprecedented model organism not only for understanding fundamental neuropharmacological processes, but also for comparative experimental research. Indeed, the similarity between the dopaminergic network, mode of drug action, behavior and gene response in D. melanogaster and mammalian systems, has made the fly a very attractive model for antiparkinsonism drug discovery (Nichols, 2006; Marsh and Thompson, 2006; Manev et al., 2003) and iii) *Drosophila* offers the power of rapid drug screening analysis, which is not yet possible in mammalian models. Iv) Given the high degree of evolutionary conservation of the human and fly genes involved in movement disorders, *Drosophila* is an ideal system for evaluating molecules with the potential of ameliorating motor coordination (Jimenez-Del-Rio et al., 2010).

Currently herbal remedies are back into prominence because the efficacy of conventional medicines, which once had near universal effectiveness against range of disease, is on the wane. In Ayurveda, about 2000 plant species are considered to have medicinal value. Ayurveda based drug discovery uses ‘reverse pharmacology’ in which drug candidates are first

identified based on the large scale use in the population, then validated in clinical trials. The study of antioxidants is becoming one of the most important subjects in PD research. It is in this contest, it was envisaged to screen a few phytochemicals for their protective efficacy against selected neurotoxicants. Since our laboratory is interested in both *Centella asiatica* and *Bacopa monnieri,* both the phytomedicines were selected along with *Withania somnifera*, Eugenol a spice active principle and ferulic acid. The criteria employed to assay their efficacy was (i) their potency to inhibit toxicant-induced lethality among adult male flies (ii) and their ability to lower endogenous levels of oxidative stress markers.

Bulk of literature demonstrates the involvement of environmental toxins particularly pesticide/herbicide such as rotenone and paraquat in the occurrence and progression of PD. Rotenone is a classical, high affinity inhibitor of complex I impairs oxidative phosphorylation by inhibiting complex I of the electron-transport chain (Uversky, 2004). Similarly paraquat is also another environmental risk factor, PQ a non-selective herbicide, is currently used to model PD (Bove et al., 2005) because of its similarity to the chemical structure of the active metabolite of the Parkinsonism-induced agent MPTP, the 1-methyl-4-phenylpyridium ion (MPP+). It is known that mechanism of toxicity of these neurotoxins in dopaminergic neurons is most likely mediated *via* oxidative stress by impairing mitochondrial function. Therefore any molecule or compound which prevents oxidative stress has the potential antioxidant capacity and rescue the subsequent damage to dopaminergic neurons.

Initially, we established the lethality profile of rotenone in adult flies using a dietary regimen, while the toxicity of both paraquat and acrylamide were assessed using a filter disc procedure. The toxicity profile of rotenone obtained in flies in our studies is consistent with previously published toxicity values (Cooks-weins and Grotewiel, 2002; Bonilla et al., 2006). It has been hypothesized that, the toxicity of rotenone and paraquat could be due to induction of oxidative stress among flies, since both toxicants are known to induce severe free radical generation *in vivo.* In the rotenone model, co-exposure of flies to BM-enriched

diet offered robust protection compared to CA- and WS-fed flies clearly suggesting the superior protective efficacy of BM. Several workers have demonstrated the protective role of BM against experimentally induced oxidative stress *via* antioxidant mechanism/s (Russo and Borrelli, 2005). Our own findings in prepubertal mice have revealed that, dietary BM significantly reduces the endogenous levels of oxidative markers and effectively offsets rotenone induced oxidative stress in brain (Shinomol and Muralidhara, 2010; Shinomol, 2008). The neuroprotective efficacies of BM have been dealt in more detail in a separate series of investigations *(Please see chapter 2)*. Further evidences in the paraquat model suggested that BM prophylaxis is also protective. Interestingly, BM prophylaxis for 7 days markedly reduced PQ induced mortality clearly suggesting that, BM effects may be multifactorial. Since PQ toxicity is known to be mediated through oxidative stress mechanism/s, it is likely that BM prophylaxis up-regulates several antioxidant defense mechanisms which collectively may be responsible for lowering the toxicity of PQ *(Please see Chapter 3)*.

In the present study, *Centella asiatica* powder also offered significant protection against all the three neurotoxicants. However, the degree of protection offered by CA was relatively lower than BM. Earlier studies have shown several neuropharmacological effects of CA such as memory enhancement, cognitive function, increased neurite elongation *in vitro*, and acceleration of nerve regeneration *in vivo* (Veerendrkumar and Gupta, 2003; Soumyanath et al., 2005; Wijeweera et al., 2006). Further, CA is known to contain triterpine saponins, caffeic acid derivatives and flavonols some of which were shown to have potent antioxidant properties. Earlier, an aqueous extract of CA was shown to reduce MDA levels and enhance glutathione levels in brain of adult rat (Shukla et al., 1999). Our recent investigations have clearly established the neuroprotective propensity of CA against neurotoxicants such as 3-NPA both *in vitro* and *in vivo* (Shinomol and Muralidhara, 2008, 2009; Shinomol et al., 2010). Hence, we speculate that, the protective effects of CA against neurotoxicants in the present study may be largely due to its ability to enhance GSH levels, thiols and antioxidant defenses in fly. However, further investigations are necessary in the fly model.

The protective effects of *Withania somnifera* (WS), (Ashwagandha) which is widely used in ayurvedic formulations to promote physical and mental health was also studied. Earlier studies have demonstrated its pharmacological properties including antioxidant, antistressor and aphrodisiac properties in various models (Mishra et al., 2000; Subbaraju et al., 2006). However, in the present study WS treatment did not offer much protection against any of the neurotoxicants at the tested concentrations. The lack of protective effects of WS in the present study may be attributed to the concentrations employed and duration of feeding.

Further, we also assessed the protective efficacy of two unrelated compounds viz., eugenol and ferulic acid along with the well known endogenous antioxidant, glutathione. Both eugenol and ferulic acid at the tested concentrations significantly rescued flies from rotenone and paraquat-induced lethality. Interestingly, protective efficacy of BM was highly comparable to that obtained with both eugenol and ferulic acid. Eugenol, (a methoxyphenol with a short hydrocarbon chain) a naturally occurring phenol extracted from cloves is known to be an antioxidant, a MAO inhibitor and is known to possess good neuroprotective activity (Reddy and Lokesh, 1992; Ito et al., 2005; Tao et al., 2005). In a recent study, eugenol treatment was found to protect mice from 6-OHDA toxicity (Kabuto et al., 2007). Accordingly, it is likely that, the protective effects of eugenol against neurotoxicant exposure in the present study may be largely related to its antioxidant and anti-inflammatory properties.

In the present series of experiments, ferulic acid-enriched diet offered significant protection against all the three neurotoxicants (Rot>PQ>AA). Ferulic acid, one of the most ubiquitous phenolic compound found in wheat, corn, rice, tomato, spinach, Cabbage etc is demonstrated to possess excellent antioxidant properties both *in vitro/in vivo* (Scot and King, 2004; Thygaraju and Muralidhara, 2008). Recent epidemiological studies have provided evidence that a higher consumption of whole grain products rich in FA reduces the risk of chronic diseases, diabetes and Alzheimer's disease (Srinivasan et al., 2006, 2007; Zhao et al., 2008). Accordingly, the protective action of FA in the fly model can be ascribed to its antioxidant properties and also may be related to

its ability to enhance antioxidant defenses particularly the levels of glutathione and the activity of GST, a phase 2 enzyme.

The protective effects of glutathione, a endogenous antioxidant was evident in both PQ and AA models. Glutathione is required for many critical cell processes including cell differentiation, proliferation and apoptosis. It plays an important role in regulation and maintenance of thiol-redox status. Disturbance in GSH homeostasis is implicated in the etiology and/or progression of number of human disease including neurodegerative disease (Ballatori et al., 2009). It has been shown that, the GSH/GSSG ratio decreased significantly with increasing age of the flies, due to an increase in GSSG content. It appears that the proxidizing shift in the glutathione redox state, the decrease in methionine content and increase in protein disulphide are associated with the life expectancy of flies (Rebrin et al., 2004). The protective effect of GSH in PQ and AA models is consistent with previous report in which glutathione enriched diet (0.22mM) significantly increased survival rate among flies exposed to PQ (20mM) (Bonilla et al., 2006).

In a satellite study, the potential of phytochemicals to modulate endogenous levels of oxidative markers among male flies was studied. Both BM and eugenol significantly reduced the endogenous levels of oxidative markers coupled with enhanced glutathione levels in whole body homogenates of *Drosophila*. It has been demonstrated that the antioxidant properties of BM include chelation of divalent iron which can decrease the formation of ROS, direct scavenging of ROS and inhibition of lipoxygenase activity.

Based on these data, we conclude that BM significantly rescued flies from the neurotoxicant induced lethality and the degree of protection offered by BM was highly comparable with known antioxidant viz., GSH. Further, BM significantly diminished endogenous markers of oxidative stress in flies. Both from lethality response data and biochemical evidences, we speculate that BM could be acting *via* antioxidative mechanism/s. Further studies on neuroprotective efficacy of BM against rotenone and paraquat have been dealt comprehensively in chapters 2 and 3 respectively.

6.0 SUMMARY

1. Exposure of adult male *Drosophila* to rotenone *per se* (250-2000µM in the diet) resulted significant and concentration dependent lethality. A concentration of 500µM was considered as the LC_{50} during 7 days exposure period.
2. Adult flies exposed (filter disc method) to varying concentration neurotoxicants, (paraquat, 10-40mM; acrylamide, 1-10mM) for 48h showed significant concentration related mortality. The 48h LC50 computed were: paraquat (PQ)- 40mM; acrylamide (AA)-10 mm.
3. Feeding of phytochemical-enriched diet or spice active-enriched diet did not have any effect on fly behavior (motor dysfunction) and caused no lethality.
4. BM-enriched diet offered robust protection at both dietary concentrations (40-66%) against rotenone induced lethality, while CA gave only marginal protection, and WS treatment did not provide any protection.
5. Among the phytochemicals tested, the incidence of PQ induced lethality was significantly lowered among flies fed on BM-enriched diet at both concentrations, while CA offered significant protection at lower dose. A similar protective effect was also evident among flies exposed to acrylamide.
6. Determination of endogenous markers of oxidative stress among flies fed phytochemicals per se revealed significant reduction in the levels only with higher dose of CA. However, both CA and WS feeding caused marginal increase in glutathione levels.
7. Eugenol-enriched diet resulted in significant and concentration related reduction in oxidative markers coupled with enhanced glutathione levels among adult flies.
8. BM fed flies showed significant diminution in endogenous markers of oxidative stress as evidenced by decrease in ROS, MDA, hydroperoxide and protein carbonyl levels coupled with redox status at both concentration.
9. Among the tested phytochemicals, BM offered significant protection against all the tested neurotoxicants in adult flies. The order of protection was BM>CA>WS, and hence was selected for further studies.

CHAPTER II

Neuroprotective efficacy of *Bacopa monnieri* against rotenone-induced oxidative stress and neurotoxicity in adult *Drosophila melanogaster*

1.0 INTRODUCTION

*Bacopa monnieri (*commonly known as Brahmi/Jalabrahmi) belonging to the family Scrophulariaceae is widely employed in Indian ayurvedic system (Kishore and Singh, 2005) due its various neuropharmacological properties. Since ancient days, it has been extensively used as memory enhancer, antianxiety, antiepilepsy, antisedative agent and also used against cognitive dysfunctions (Russo and Borelli, 2005; Ernst, 2006; Shinomol et al., 2010). Apart from the neuroprotective/neurobeneficial properties, recent evidences ascribe both antioxidant and antistress properties to *Bacopa* (Tripati et al., 1996; Bhattacharya et al., 2000; Chowdhary et al, 2002). Antioxidant property of *Bacopa monnieri* extracts have been attributed to the presence of saponins viz., bacosides and bacosaponins (Singh, 1988), which are shown to possess properties such as chelation of metal ions, free radicals scavenging and up regulation of antioxidant defense enzymes (Russo et al., 2003). Since *Bacopa* has a long history of being a neuroprotective plant, recent researchers have made attempts to exploit this medicinal plant to attenuate oxidative stress mediated neuronal dysfunctions. (Shinomol, 2008; Gohil and Patel, 2010).

Several studies have emphasized the advantages of employing *Drosophila* as a model for several NDD (Bonini and Fortini, 2003) including a genetic model of PD based on expression of human α-synuclein in *Drosophila* brain (Feany and Bender, 2000: Auluck et al., 2002). More recently, chronic exposure to rotenone model of sporadic PD has been demonstrated. Several advantage of rotenone has been explained over the expression of human α-synuclein in Drosophila model. Widespread and consistent DA neuron loss, its correlation with locomotor deficits, absence of lewy bodies and retinal degeneration suggests specificity of rotenone to induce PD like symptoms (Coulom and Birman, 2004). The selectivity of rotenone action is likely attributable to a specific sensitivity of *Drosophila* dopaminergic neurons to reactive oxygen species and oxidative damages.

Rotenone, a commonly used natural pesticide is a complex ketone derived from the roots of *Lonchocarpus* species. It is a classical, high affinity specific inhibitor of mitochondrial NADH dehydrogenase (complex I). Being

extremely lipophilic, it crosses the blood brain barrier rapidly and accumulates in sub cellular organelles like mitochondria where it impairs oxidative phoshorylation (Uversky, 2004). *In vitro*, rotenone has been shown to induce apoptosis and aggregation of α- synuclein and ubiquitin, oxidative damage and endoplasmic reticulum stress (Ryu et al., 2000; Sherer et al., 2002). Chronic systemic exposure to rotenone has been used to model PD in the rat, since it induces dopaminergic neurodegeneration, Parkinson's like behavior and occurrence of cytoplasmic inclusions similar to the lewy bodies (Betarbet et al., 2000). Significant differences have been observed among various rat strains, making it difficult to test new pharmacological treatments for PD. Previously, many workers have attempted to attenuate rotenone induced oxidative stress and neurotoxic effects using various therapeutic agents both *in vitro* and *in vivo* mammalian model (Panov et al., 2005).

Since the fly model offers a large panel of genetic approaches and allows the rapid screening of potential therapeutic drugs, this model is gaining importance. Flies chronically exposed to rotenone developed selective loss of dopaminergic neurons in the brain and severe locomotor dysfunctions (Coulum and Birman, 2004). Interestingly, they showed the efficacy of melatonin in rescuing both rotenone- induced neuronal loss and behavioral defects, while L-DOPA rescued only the later. Currently, we have initiated a programme to establish *Drosophila* as an alternative model to screen the potential of phytochemicals/pharmacological agents against specific NDD (Hosamani et al., 2009).

Using *Drosophila,* several workers have demonstrated the efficacy of various pharmacological compounds to treat accelerated ageing and neurological disorders. Dopamine and its agonists (Jordens et al., 1999), geldanamycin (Auluck et al., 2005) and melatonin (Bonilla et al., 2002) were used to protect dopaminergic neurons against both transgenic and sporadic fly models. Further, vitamins (Zou et al., 2007, Bahadorani et al., 2008), SOD mimitics (Magware et al., 2006) and various polyphenols (Bonilla et al., 2006) viz., Green tea catechins, Gallic acid, Ferulic acid, caffeic acid, Coumaric acid, propyl gallate, epicatechin, epigallocatechin and epigallocatechin gallat (GTC) have been screened for their protective efficacy against oxidative stress

mediated ageing (Jimenez-Del-Rio et al., 2010). The neuroprotective effects of BM extract have been tested against few toxicants including glutamate, aluminium, beta amyloid and nitric oxide etc in rodent models. The antioxidant and antistress activities of BM are speculated to significantly contribute to its neuroprotective action. However, there has been no data on the ameliorative effects of BM against known neurotoxicant chemicals which have been widely employed as chemical models (eg., Rotenone, paraquat, MPTP) to induce neurodegeneration either in mammalian or in *Drosophila* models.

Accordingly, in the present study, we specifically addressed questions related to the possible neuropharmacological properties of *Bacopa monnieri* leaf powder against rotenone induced oxidative stress and neurotoxicity employing wild strains of Drosophila. Initially, following short-term dietary exposure of adult flies to BM, its ability to modulate the endogenous levels of oxidative markers was determined. Further, its neuroprotective propensity was investigated in terms of its potency to attenuate rotenone induced lethality, oxidative stress and neurotoxicity. Oxidative stress response was measured by quantification of malondialdehyde/hydroperoxide levels, activities of antioxidant enzymes and protein carbonyls in whole body homogenates of flies. Neurotoxicity was assessed by a negative geotaxis assay and quantification dopamine levels in both head and body regions. Further, the neuroprotective efficacy of BM was compared with that of a commercial preparation *viz*., Brahmi capsule (BC) powder (a proprietary supplement for human consumption), a widely marketed herbal preparation in India. Furthermore the potential of BM prophylaxis to render resistance against paraquat induced lethality was also investigated.

2.0 OBJECTIVE

The primary focus of these investigations was to obtain biochemical insights into the neuroprotective efficacy of *Bacopa monneiri* (BM) extract against rotenone-induced oxidative stress and neurotoxicity by employing *Drosophila melanogaster* as a model system.

NEUROPROTECTIVE EFFECTS OF *BACOPA MONNIERI* (BM) AGAINST ROTENONE IN ADULT *DROSOPHILA*

3.0 EXPERIMENTAL DESIGN

I. Analysis of *Bacopa monnieri* powder

The standardized powder prepared from Bacopa monnieri leaves (as described earlier) was subjected to physical and chemical analysis employing standard procedures and the salient results are presented in **Table 2.1**. The total triterpenoid saponins were quantified in the leaf powder by HPLC method (Houghton and Raman, 1998).

E*xtraction of Bacopa leaf powder*

A known (500mg) amount of *Bacopa monnieri* extract was dissolved in methanol (50ml), sonicated for 3min, boiled on steam water bath for 5 min, cooled, made upto 100ml with methanol and filtered through 0.45micron membrane filter paper.

Estimation of Bacosides by HPLC analysis

The separation was performed using a Shimadzu High Performance Liquid Chromatographic system equipped with LC10A pump with SPD-M 10A*vp* Photo diode Array Detector or UV detector in combination with Class-VP software or LC 2010 A and LC 2010HT integrated system equipped with Quaternary gradient, auto injector in combination with Lab solution software . The mobile phase was consisted of 0.2 % Orthophosphoric acid and acetonitrile. The total run time was 45 min. All peaks were integrated at the wavelength of 205 nm. The flow rate was 1.5ml/min. The coulmn used was Pinnacle DB C18 column,

250mm, 4.6mm / 5 micron obtained from Restek, Part No. 9414575. Caibration curves of five saponin glycosides , bacoside A3 , bacopasiside II, bacopasaponin C isomer, bacopasaponin C, bacopaside I were prepared based on peak areas of reference standards

II. <u>Standardization of rotenone model: Effect of rotenone exposure</u>

Preparation of Rotenone (Rot) solution:

Rotenone was dissolved in DMSO, DMSO volume kept as low as possible to avoid interference with rotenone toxicity. Stock concentration was maintained at 40mM through out the investigations.

Rotenone concentration: Lethality response

In a preliminary study, synchronized adult male flies were exposed to rotenone at concentrations of 250, 500, 1000 and 2000 µM for 7 days. Mortality was monitored for every 24hrs through out the observation period of 7 days and the data was expressed as percent mortality. However, to assess the neuroprotective effects of BM and BC, only one concentration of rotenone (500µM) was employed.

Induction of locomotor deficits by rotenone: Negative geotaxis assay

At the end of 7 days exposure period, rotenone treated flies (250, 500 and 1000µM) were subjected to negative geotaxis assay in order to determine the induction of locomotor dysfunctions. Following 60 second observation of their climbing ability along the wall of the vertical glass column by visual method, flies that reached the top of the column and flies that remained at the bottom were counted separately. Data was expressed as percent flies escaped beyond minimum distance (it is average distance, where the adult untreated flies can climb the vertical column within 60 seconds) of 6cm in 60 seconds of interval. 20 adults per replication were used for each assay and the assay was repeated three times. The score for each replication was an average of three such trials for each group of flies including control.

III. Effect of BM and BC on endogenous markers of oxidative stress

Two concentrations of BM (viz., 0.05 and 0.1%) were employed to understand its effect on endogenous markers of oxidative stress. The test concentrations of BM were incorporated into the semisolid diet (2 ml). After 4-5 hrs, synchronized adult (8-10days old) male flies were transferred to vials containing test compounds by using brief anesthetic procedure and were maintained on BM-enriched diet for 7 days. Fresh diet was provided to flies on every alternate day. Terminally control and BM treated flies were briefly anaestized, whole flies homogenates were prepared. The following biochemical measurements were made in cytosolic fraction.

In order to compare the activity of BM standardized powder, we also employed Brahmi capsule (BC) actives were also tested at two concentrations (0.05 and 0.1%). Effect of both BM and BC were determined on endogenous oxidative markers interms of generation of reactive oxygen species (ROS), MDA protein carbonyls and hydroperoxide levels among whole body homogenate of adult flies. Further, redox status was measured in terms of reduced glutathione (GSH) level.

Effect on cholinergic function

The activities of acetylcholinesterase and butyrylcholinesterase were assayed among BM and BC fed flies as a marker of cholinergic function.

IV Evidence of neuroprotective effects of BM

Co-exposure procedure

For these studies, we followed co-exposure protocol. Adult flies were provided with either BM or BC (0.05 and 0.1 %) or rotenone (500 µM) or in combination of rotenone and BM/BC in the diet for 7 days and determined the modulatory effect of BM on rotenone -induced lethality, locomotor dysfunctions, oxidative impairments and dopamine levels. For these studies 40 flies were employed such three replication were used for biochemical studies.

Modulatory effect on rotenone-induced lethality response

For mortality studies, a minimum of 50 adult flies per replicate such three replicates were exposed to either BM or BC or rotenone or a combination of BM + rotenone, BC + rotenone for 7 days. Lethality of the flies were monitored for every 24hrs till the end of the experiment and data was expressed in terms of percent mortality.

Ameliorative effects on rotenone-induced oxidative stress

The status of oxidative markers viz., lipid peroxidation quantified in terms of malondialdehyde levels and hydroperoxide levels were quantified by using FOX reagent in whole body homogenates of adult *Drosophila*.

The levels of reduced GSH was measured in whole body homogenates of flies and expressed as µg GSH/mg protein.

Protective effect of BM against rotenone-induced protein oxidation was measured in terms of protein carbonyls level among adult flies co-exposed with rotenone and compared with untreated flies and rotenone *per se*.

The ameliorative effect of BM on the antioxidant enzymes status was determined by measuring the activities of selected enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferase (GST) in whole body homogenates of adult flies.

V. <u>Attenuation of rotenone-induced neurotoxicity by BM</u>

BM rescues flies from rotenone-induced locomotor deficits

Locomotor dysfunctions of adult flies among untreated, rotenone *per se,* BM *per se* and co-treated with BM (0.05 & 0.1%) and rotenone (500 µM) for 7 days were quantified by employing negative geotaxis assay. Number of flies remained at the bottom of vial were compared with the number of flies could escape due to treatment effect were determined. The data was expressed as percent flies escaped/min. Minimum of 50 flies were employed, and three trials were conducted to minimize the error.

Modulation of dopamine levels in head and body regions

Since dopamine depletion was specific marker of neurotoxicity, in the present study the effect of BM was assessed in terms of its ability to ameliorate rotenone-induced dopamine depletion among adult flies by using high performance liquid chromatography (HPLC) in both head and rest of the body region.

Modulatory effect on cholinergic function

Cholinergic functions were measured in terms of activities of acetylcholinesterase and butyrylcholinesterase in whole body homogenates of adult flies.

VI. BM prophylaxis renders flies resistant against PQ toxicity

Paraquat induced lethality response

Synchronized adult flies were starved for 4hrs, then transferred to PQ vials containing filter disc (4 stacked to maintain moisture for longer time) containing PQ saturated with 5% sucrose. Three concentrations of PQ viz., 10, 20, and 40mM of were used to determine the toxicity profile mortality was recorded at 24h and 48 h.

BM prophylaxis modulates PQ toxicity

For modulatory study only one concentration of PQ (40mM) and two concentrations of BM (0.05 and 0.1%) were used. Adult flies maintained on BM-enriched diet (0.05 and 0.1% for 7 days) were challenged with PQ (40mM in 5% sucrose solution) for 48h using filter disc procedure. Mortality was recorded among both control (BM untreated flies) and BM pretreated flies at 24 and 48h.

4.0 RESULTS

NEUROPROTECTIVE EFFECTS OF *BACOPA MONNIERI* (BM) AGAINST ROTENONE IN ADULT *DROSOPHILA*

I. HPLC analysis of *Bacopa monnieri*

The HPLC -finger print analysis of the sample was carried out as per the conditions and procedures described previously (Deepak et al., 2005). A typical HPLC chromatogram is presented in. The quantities of major saponins expressed as % w/w are: luteolin (***1***-0.29%) ; Apigenin(***2***-0.12%); Bacopaside -I (***3***-1.43%) ; Bacoside -A3 (***4***-1.60%); Bacopaside -II (***5***-2.74%) ; Jujobogenin isomer of Bacopasaponin C (***6***-1.96%); Bacopasaponin –C (***7***-1.70%) ; Bacoside -A (***8***-7.96%) and Bacosine (***9***-0.61%) **(Figure 2.1)**.

II. Standardization of rotenone model: Effects of rotenone exposure

Rotenone induced lethality response:

Exposure of adult flies to rotenone resulted in a concentration dependent lethality during a 7day experimental period **(Figure 2.2A)**. In general, mortality occurred between 4- 7 days among rotenone exposed flies. Terminally, the cumulative percent mortality at the tested concentrations was as follows: 250µM- 14%; 500µM -48%; 1000µM -78% and 2000µM -96%.

Induction of locomotor deficits (Negative geotaxis assay)

Data obtained in a negative geotaxis assay among rotenone exposed (7 days) flies to revealed concentration dependent locomotor deficits **(Figure 2.2B)**. Large number of flies showed a tendency to stay at the bottom of vertical glass column at higher concentrations of rotenone. Among untreated controls, more than 94% flies were able to reach at the top of the column within a minute, while rotenone exposed flies exhibited significant decrease in climbing ability (250µM- 44%; 500µM- 19% and 1000µM- 15%) clearly suggesting the induction of locomotor deficits.

III. Effect of BM on endogenous markers of oxidative stress

Whole body homogenates of adult flies fed with BM supplemented diet for 7 days showed significant diminution in MDA and HP levels. With BM treatment, the levels of MDA were reduced by 19 and 27% respectively at the lower and higher concentration, while the HP levels were reduced by 17 and 25% compared to the untreated control flies. Interestingly, the endogenous levels of oxidative markers among BC fed flies were also significantly reduced at both concentrations ($p<0.05$). Further, marginal increase in ROS generation was evident **(Table 2.2)**.

The levels of GSH were significantly enhanced in whole body homogenates among flies fed BM as well as BC. With BM treatment, the GSH levels were elevated by 22 and 36% at low and high concentration respectively, while BC treatment enhanced the levels by 20 and 29%. Further, significant reduction in protein carbonyl levels was also evident with both the treatments, although the percent reduction was robust with BC treatment **(Table 2.2)**.

However, the activities of antioxidant enzymes *viz*., Catalase, Superoxide dismutase and glutathione transferase assayed in whole body homogenates of flies fed BM and BC showed no significant alterations (data not shown).

Effect on cholinergic function

The activity levels of acetylcholinesterase enzyme among flies treated with BM and BC was marginally increased ($p<0.05$). However, the activity of butyrylcholinesterase enzyme was marginally elevated only with BM treatment at the higher concentration **(Table 2.3)**.

IV. Evidence of neuroprotective effects of BM

Modulatory effect on rotenone-induced lethality response

For this study, only a single concentration of rotenone (500µM) was employed. While rotenone exposure induced significant mortality (48%) among flies, co–exposure of flies to either BM or BC (0.05 and 0.1%) with rotenone markedly reduced the incidence of mortality **(Figure 2.3)**. The extent of protection offered

by BM and BC against rotenone induced mortality was concentration related (BM: 40 and 66%; BC: 49 and 60%). A significantly lowered incidence of rotenone induced lethality clearly suggested significant protective effect of both BM and BC.

Ameliorative effects on rotenone-induced oxidative stress

Feeding of BM (0.1%) to adult flies significantly ($p<0.05$) diminished the basal levels of both MDA (by 35%) and HP (by 16%). Whole body homogenates of rotenone exposed flies revealed a marked increase in MDA (by 30%) and HP (by 38%) compared to control flies. Interestingly, BM treatment completely abrogated the rotenone induced oxidative impairments as revealed by the normal levels of MDA and HP among such flies. Likewise, BC treatment also resulted in complete restoration of MDA levels, while the HP levels were not completely restored **(Figure 2.4 A&B)**.

Moderate increase in GSH level was evident among flies treated with BM (22%) and BC (16%) *per se*. Although rotenone exposure for 7days caused significant decrease (23%) in GSH level, on co-exposure with BM, flies were able to restore the depleted GSH levels **(Figure 2.5A)** clearly suggesting the ability of BM to up regulate levels of GSH. On the other hand, the levels of oxidized glutathione (GSSG) were not affected due to BM treatment. Further, rotenone exposure also caused no significant alterations in GSSG levels in any of the treatment groups (Data not shown).

The effect of BM on rotenone induced protein damage was measured as protein carbonyl content in whole body homogenates. Rotenone exposed flies showed significant enhancement (45%) in carbonyl levels, while flies co-exposed with BM or BC showed normal levels of protein carbonyls suggesting complete rescue against protein damage. However, BM and BC *per se* had no effect on the protein carbonyl levels **(Figure 2.5B)**.

Modulatory effect on activity of antioxidant enzymes

BM *per se* treatment resulted in only a marginal decrease in the activity levels of Catalase and SOD, while the activity of GST was enhanced. Rotenone (500µM) exposure induced a significant elevation in the activity of all

antioxidant enzymes measured (CAT, 17%; SOD, 32%; GST, 62%). However, among BM + rotenone exposed flies, the activity levels of catalase and SOD, were restored to normalcy, while the activity levels of GST were restored to near basal levels , although remained higher than untreated control flies (Table 2). Further among BC + rotenone, the activity levels of SOD were not restored to normalcy **(Figure 2.6A,B&C)**.

Modulatory effect on protein profile of adult fly brain

Significant modulatory effect of BM supplementation was evident on total protein profile in the adult fly brain **(Plate 1).**

V. <u>Attenuation of rotenone-induced neurotoxicity by BM</u>

Effect on rotenone-induced locomotor deficits

In a parallel experiment, we measured the locomotor deficits among flies of various treatment groups employing a similar paradigm. Rotenone treated flies exhibited severe locomotor impairments as evident by the large(95%) number of flies staying at the bottom of the glass column, while co-treatment with BM and BC significantly improved the performances of flies **(Figure 2.7)**. With BM treatment, more number of flies (45-65%) showed negative geotaxis behavior clearly indicating its neuroprotective effect. A robust protection was also evident with BC treatment (55-70%). In general, flies co-exposed to either BM or BC appeared to be more active than rotenone only treated flies. However, BM and BC exposure *per se* had no significant effect on locomotor function among flies.

Effect on dopamine levels in head and body regions

Flies treated with BM *per se* showed only a marginal increase in DA level in both regions (head, 13%; rest of the body, 15%). With rotenone treatment, significant DA depletion was evident in both the regions (head, 50%; body region, 45%) compared to untreated control flies. Interestingly, rotenone exposure among flies treated with BM caused less robust DA depletion. There was a significant degree of protection offered by BM against DA depletion and the percent protection was 33% in the head region and 44% in rest of the body **(Figure 2.8A)**. BC treatment *per se* resulted in only a marginal decrease (15%)

in DA levels in the rest of the body, while head region remained unaltered. On the other hand, rotenone exposure among flies treated with BC offered a higher degree of protection among both regions (head, 43% and rest of the body, 90%) **(Figure 2.8B)**.

Effect on cholinergic function

BM *per se* treated flies showed a marginal increase in the activity of cholinergic functions measured in terms of acetylcholinesterase and butyrylcholinesterase activity. Rotenone (500µM) exposure also showed a significant increase in the enzymes activities. Further, among BM + rotenone exposed flies, the activity remained unaltered. However among BC + rotenone, the activity levels of AChE and BChE activity were restored to normalcy **(Figure 2.9A & B)**.

VI. Attenuation of PQ toxicity by BM prophylaxis

Paraquat induced lethality response

In order to determine the LC_{50} value, we exposed flies to three concentrations (10, 20 and 40mM) of paraquat (5% sucrose solution) and determined the incidence of mortality. At the lower conc. (10 and 20mM) PQ did not result in any significant mortality during the first 72 h, while it induced significant (30-50%) mortality on day 4 (96h), which further progressed (87-100%) on day 5 (120h). At the highest concentration (40mM), PQ induced significant mortality (21%) by 48h which progressed to 50% at 72 h and attained maximum at 96h **(Figure 2.10)**.

BM prophylaxis modulates PQ toxicity

In this study, we used only one concentration of PQ (40mM for 72h) to assess the extent of resistance offered by BM against PQ toxicity. Adult flies pretreated with BM or BC (0.05, 0.1% for 7 days) showed better resistance against PQ toxicity compared to controls **(Figure 2.11)**. With BM pretreatment, the degree of protection was 54 and 43% at 0.05 and 0.1 % levels, while BC provided higher degree of pro5ection of 63 and 54% respectively.

Table 2.1

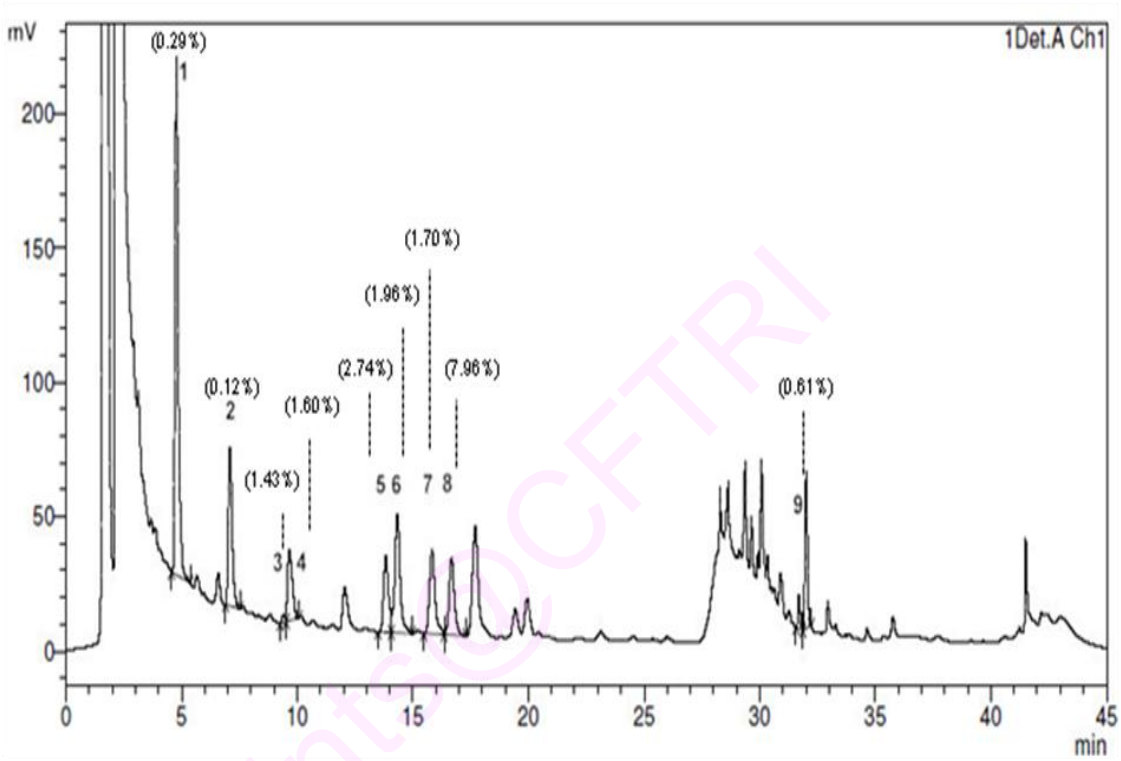
Quality control specifications of the *Bacopa monnieri* leaf powder used for the investigations

Analysis		Result
Physiochemical	Loss on drying (moisture)	NMT 5% w/w
	Ash content	NMT 10% w/w
	Heavy metals	NMT 20 ppm
Microbiological	Total viable aerobic count	<100 cfu/g
	Total fungal count (yeast and moulds)	<100 cfu/g
	E. coli	Absent
	Salmonella typhimuirum	Absent
	Salmonella aureus	Absent
Phytochemical	Bacosides	40% w/w

NMT: Not more than; cfu: colony forming units

Figure 2.1

HPLC-chromatogram of *Bacopa monnieri* leaf powder



Key to peak identities : luteolin (1); Apigenin(2); Bacopaside -I (3); Bacoside -A3 (4); Bacopaside -II (5); Jujobogenin isomer of Bacopasaponin C (6); Bacopasaponin –C (7) ; Bacoside -A (8) and Bacosine (9)

Table 2.2

Effect of feeding *Bacopa monnieri*-enriched diet for 7 days on the endogenous markers of oxidative stress in adult *Drosophila*

Parameters	*Bacopa monnieri (%)*			*Brahmi actives (%)*	
	0	**0.05**	**0.1**	**0.05**	**0.1**
Malondialdehyde[1]	3.31± 0.22	2.69± 0.18	2.41± 0.20*	2.84± 0.17	2.23± 0.20*
Hydroperoxide[2]	0.24± 0.07	0.20± 0.008	0.18± 0.006*	0.18± 0.01*	0.19± 0.007*
ROS[3]	0.21± 0.012	0.23± 0.021	0.18± 0.011	0.19± 0.022	0.17± 0.015
Protein carbonyls[4]	21.6± 1.15	17.10± 2.96*	17.33± 0.93*	16.13± 1.21*	15.77± 0.88*
Reduced Glutathione[5]	31.8± 1.71	38.86± 1.72*	43.37± 0.25*	38.12± 1.38*	40.96± 1.82*

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Tukey' test (* $p<0.05$)

[1] nmol malondialdehyde /mg protein
[2] nmol hydroperoxide /mg protein
[3] picomol DCF formed /mg protein
[4] nmol protein carbonyls /mg protein
[5] µg GSH/mg protein

Table 2.3

Effect of feeding *Bacopa monnieri*-enriched diet for 7 days on the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes in adult *Drosophila*

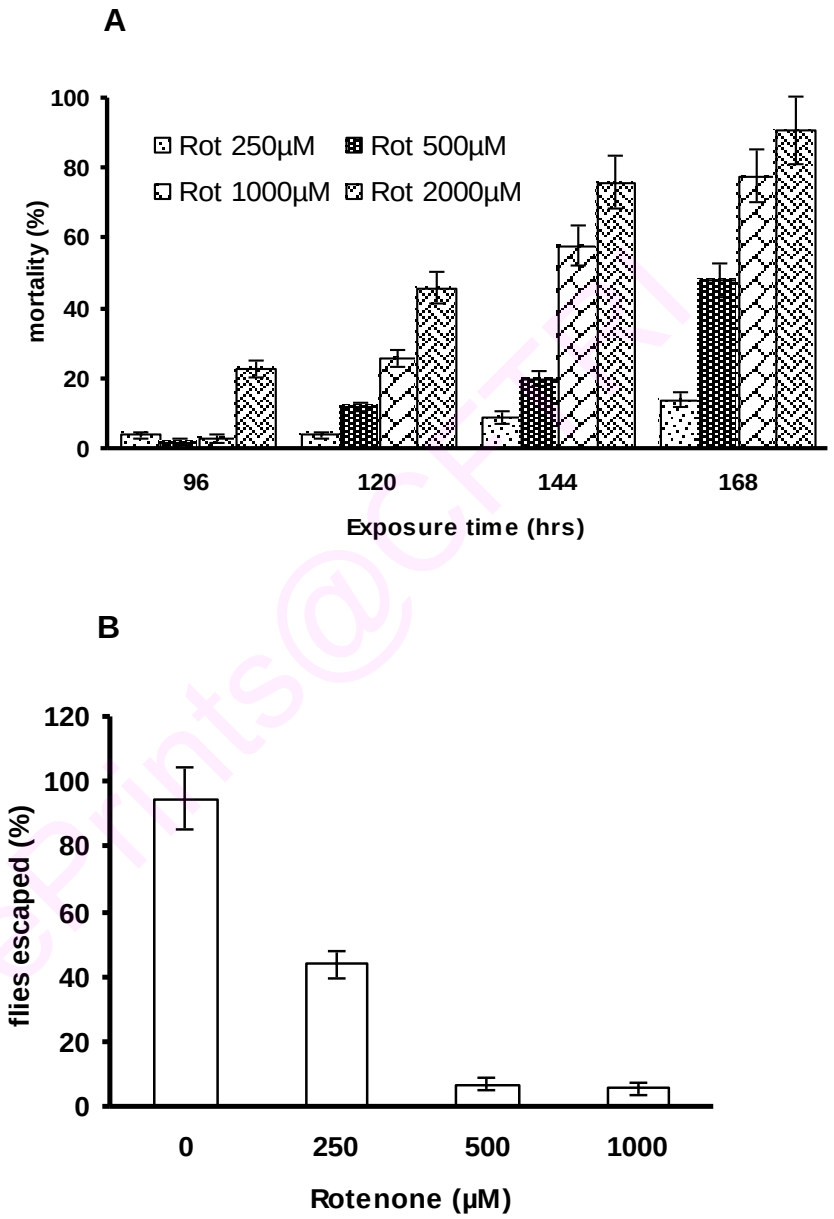
Parameter	*Bacopa monnieri (%)*			*Brahmi actives (%)*	
	0	**0.05**	**0.1**	**0.05**	**0.1**
AChE[1]	0.06± 0.01	0.07± 0.006	0.077± 0.003	0.070± 0.004	0.071± 0.003
BChE[2]	0.030± 0.004	0.034± 0.001	0.036± 0.001	0.032± 0.001	0.033± 0.002

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Tukey' test (* $p<0.05$)

[1, 2] ρmol DTNB/min/mg protein

Figure 2.2

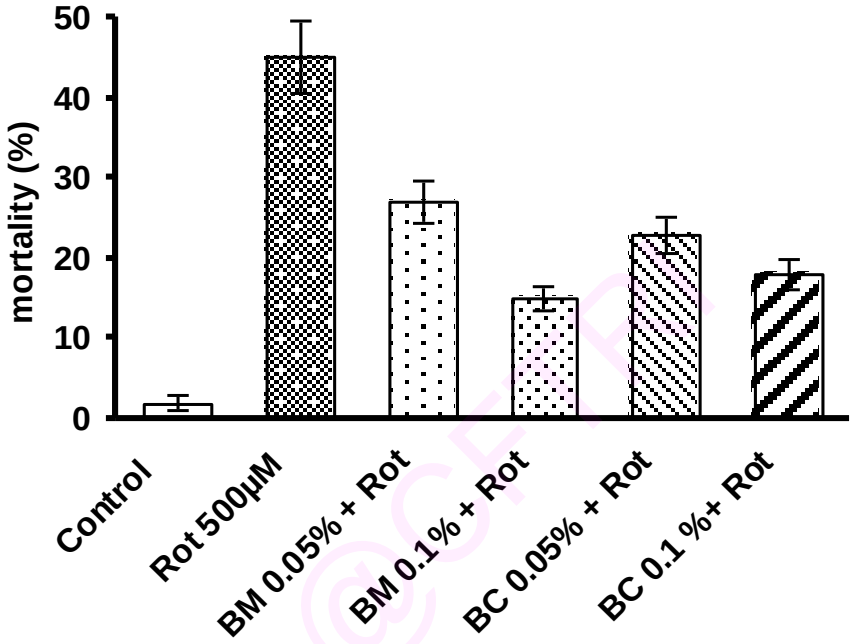
Lethality response (expressed as percent mortality) (A). Incidence of locomotor deficits (B) (expressed as percent flies escaped) determined in a negative geotaxis assay among adult male *Drosophila* exposed to rotenone in the diet



Rot: Rotenone
n=50 flies per replicate, three such replication used for assay

Figure 2.3

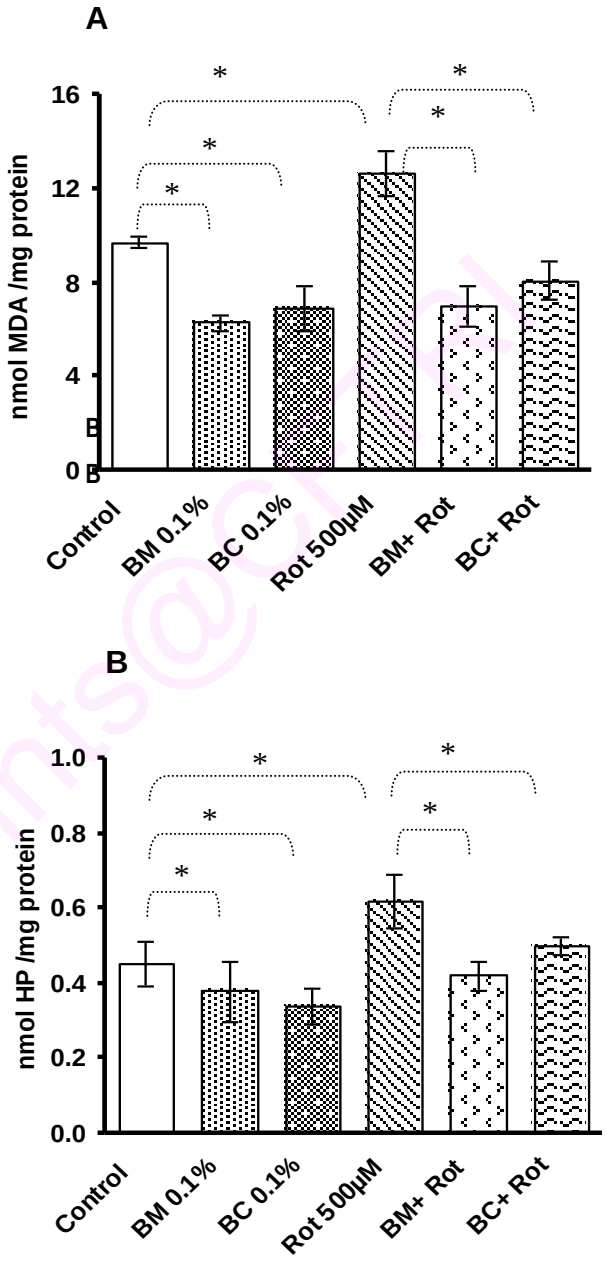
Incidence of mortality among adult male *Drosophila* exposed to rotenone, BM, BC *per se* or a combination rot + BM or rot + BC



Rot: Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
n=50 flies per replicate, three such replication used for assay.

Figure 2.4

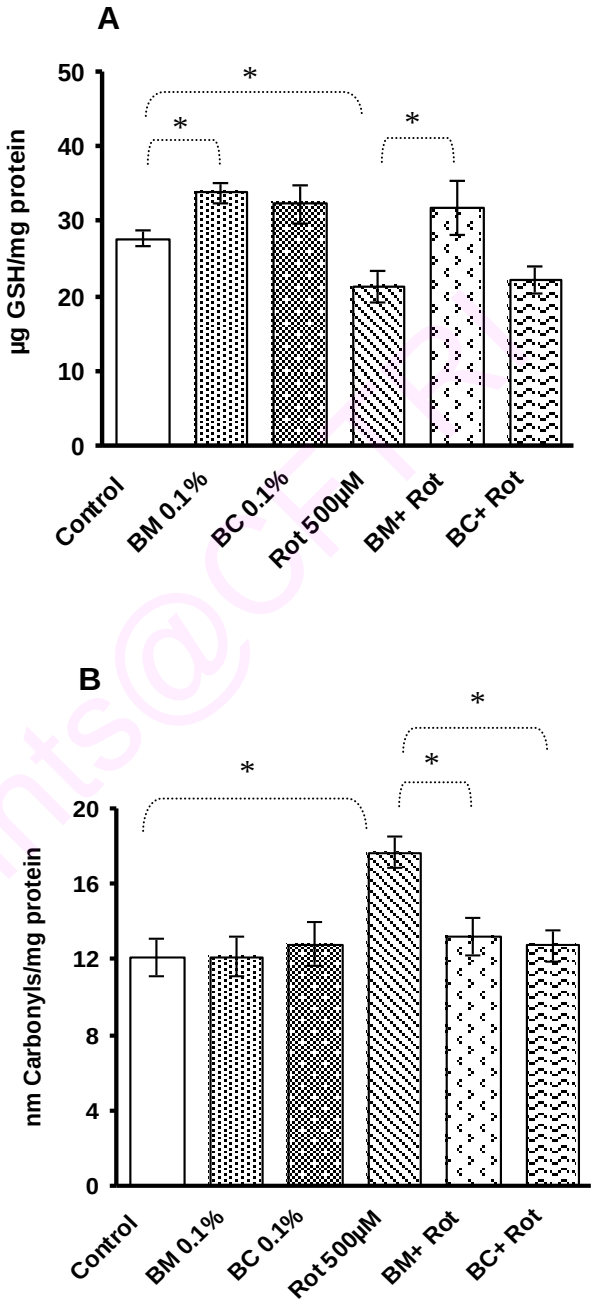
Modulatory effect of *Bacopa monnieri* and Brahmi capsule powder treatment on rotenone-induced oxidative stress measured as malondialdehyde (A) and hydroperoxide (B) levels in whole body homogenates of adult *Drosophila*



Rot: Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control vs BM, BC, rotenone; rotenone vs rot + BM, rot + BC.

Figure 2.5

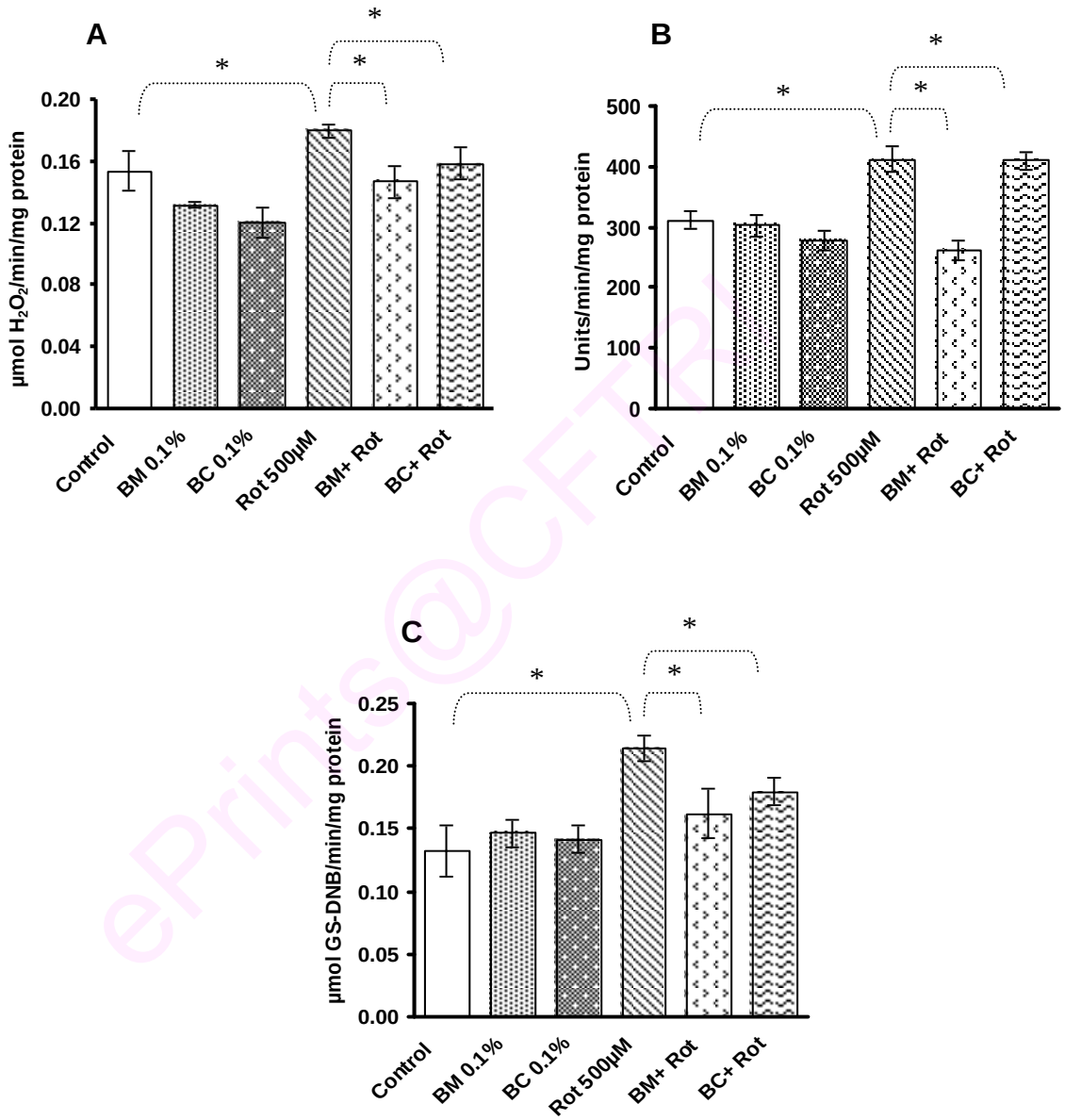
Modulatory effect of *Bacopa monnieri* and Brahmi capsule powder treatment on Rotenone induced alterations in reduced glutathione levels (A) and protein carbonyl content (B) in adult *Drosophila*



Rot: Rotenone; BM:*Bacopa monnieri*; BC: Brahmi capsule
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control vs BM, BC, rotenone; rotenone vs rot + BM, rot + BC. (n=50 flies per replicate, three such replication used for assay).

Figure 2.6

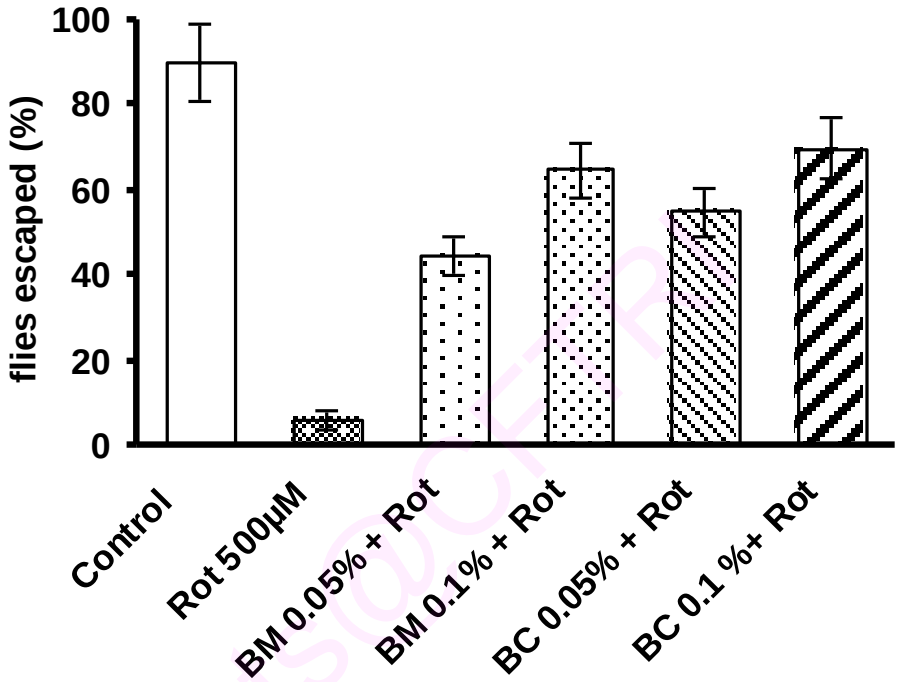
Modulatory effect of *Bacopa monnieri* extract and Brahmi capsule on the activities of antioxidant enzymes Catalase (A), Superoxide dismutase (B) and Glutathione-S-transferase (C) in whole body homogenates of flies



Rot: Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control vs BM, BC, rotenone; rotenone vs rot + BM, rot + BC. (n=50 flies per replicate, three such replication used for assay).

Figure 2.7

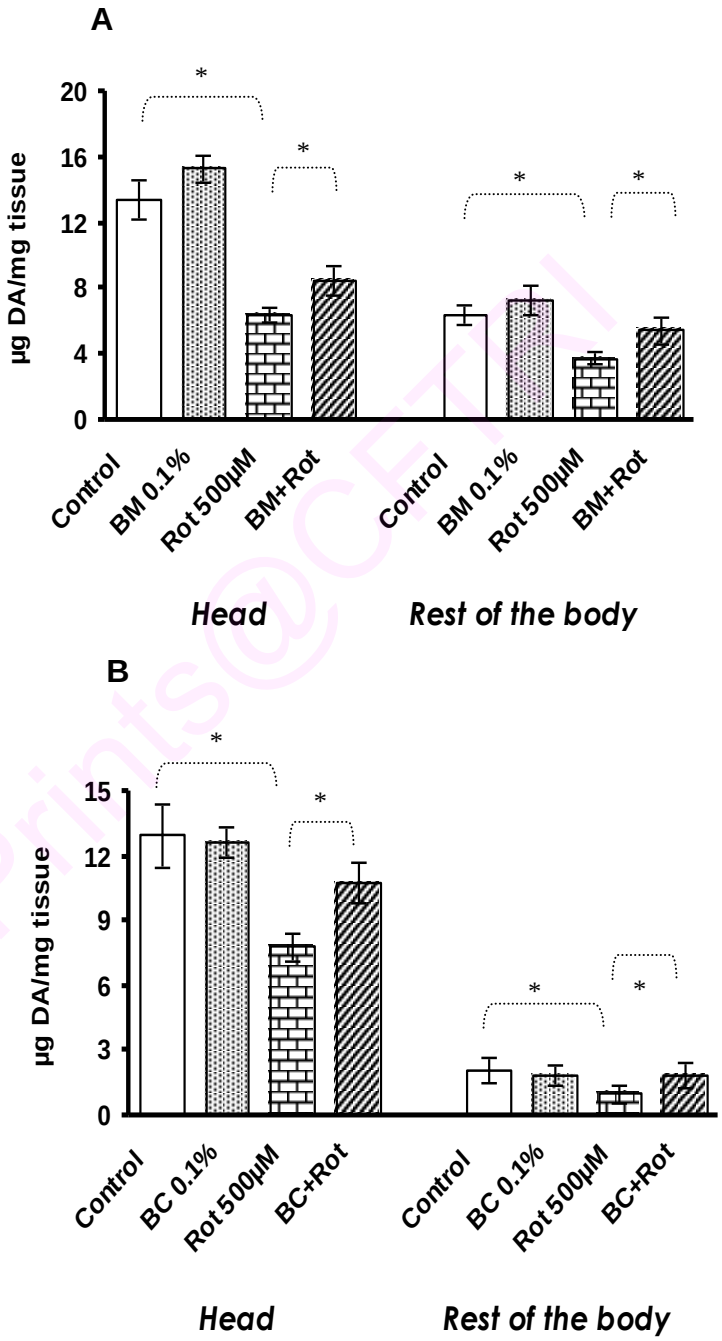
Modulation of rotenone-induced locomotor (expressed as percent flies escaped) deficits among adult male *Drosophila* by *Bacopa monnieri* and Brahmi capsule powder treatments



Rot: Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
n=50 flies per replicate, three such replication used for assay

Figure 2.8

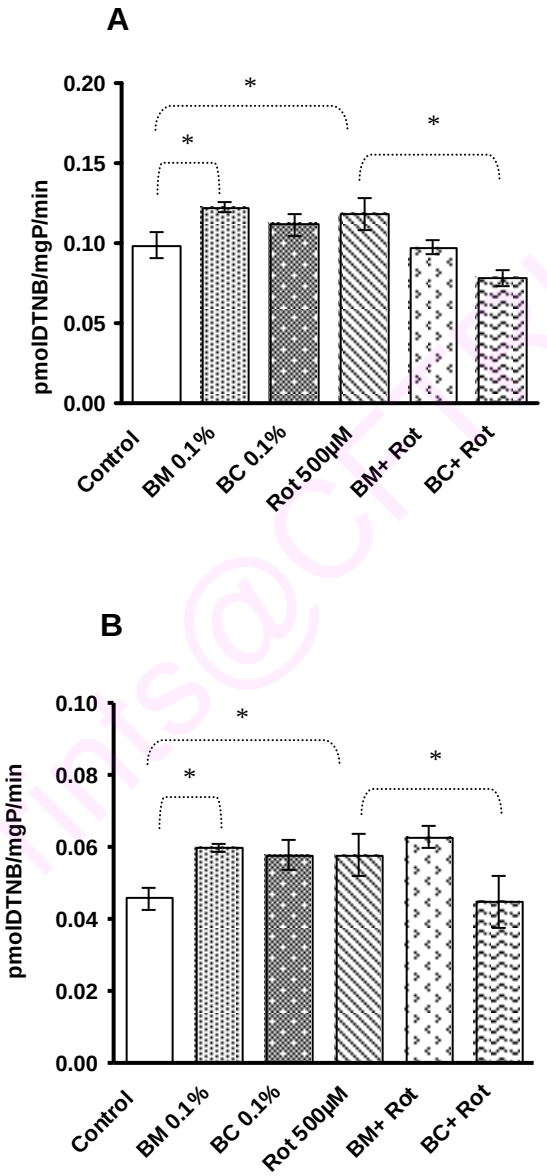
Modulatory effect of *Bacopa monnieri* (A) and Brahmi capsule powder (B) treatment on Rotenone-induced dopamine depletion in adult male *Drosophila* measured in head homogenates and rest of the body homogenates



Rot:Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control vs BM, BC, rotenone; rotenone vs rot + BM, rot + BC. (n=50 flies per replicate, three such replication used for assay).

Figure 2.9

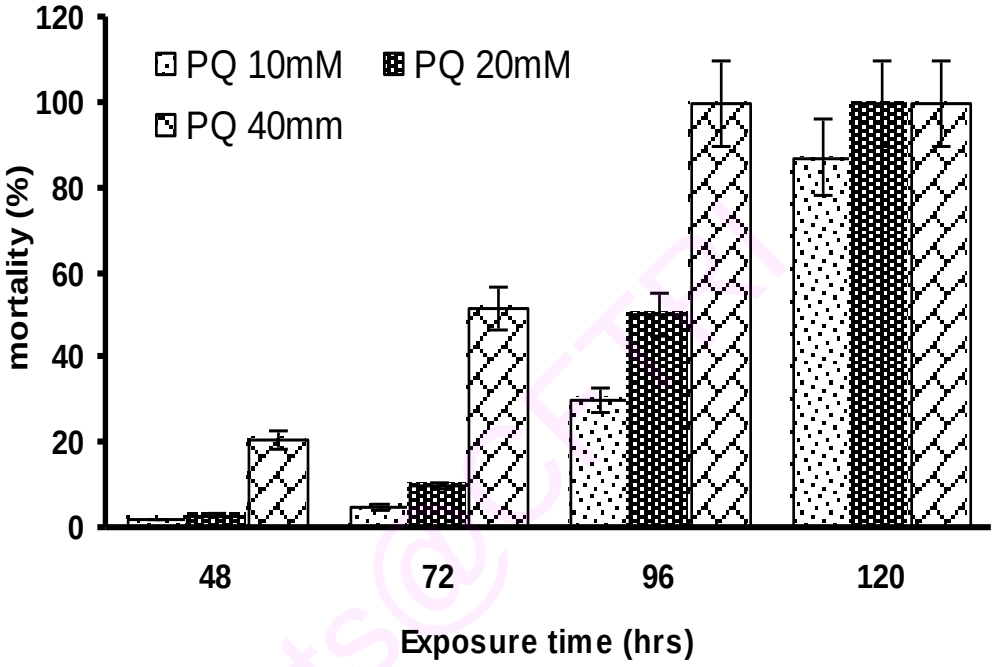
Modulatory effect of *Bacopa monnieri* and Brahmi capsule powder treatment on Rotenone induced alterations in activities of acetylcholinesterase (A) and butyrylcholinesterase (B) in adult *Drosophila*



Rot: Rotenone; BM: *Bacopa monnieri*; BC: Brahmi capsule
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control vs BM, BC, rotenone; rotenone vs rot + BM, rot + BC. (n=50 flies per replicate, three such replication used for assay).

Figure 2.10

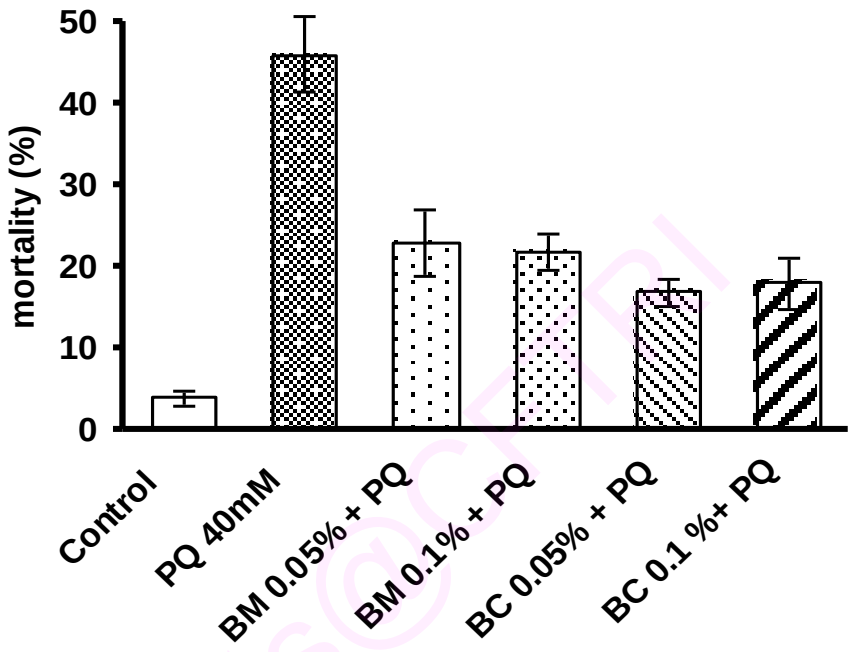
Time-course lethality response expressed as percent mortality among adult male *Drosophila* exposed to various concentration of paraquat (PQ)



n=50 flies per replicate, three such replication used for assay

Figure 2.11

Modulation of paraquat induced mortality response among flies subjected to *Bacopa monnieri* and Brahmi capsule prophylaxis.



PQ: Paraquat; BM: *Bacopa monnieri*; BC: Brahmi capsule
n=50 flies per replicate, three such replication used for assay

5.0 DISCUSSION

Human consumption of *Bacopa monnieri* is on the increase owing to its multiple beneficial effects (Shinomol et al., 2010; Gohil and Patel, 2010), which has been attributed mainly to the presence of characteristic saponins (Kishore and Singh, 2005). Currently, our laboratory is engaged in understanding the possible mechanism/s by which *Bacopa monnieri* (BM) offers protection against known neurotoxicants in Drosophila and prepubertal mice models (Shinomol and Muralidhara, 2006; Shinomol, 2008). In the present study, our primary focus was to examine if one can reproduce the neuroprotective effects of BM in an insect system such as *Drosophila,* a widely employed system for modeling neurodegenerative disorders (Coulom and Birman, 2004; Nichols, 2006). Further, *Drosophila* system offers several advantages, the primary being it allows rapid screening of potential therapeutic agents /phytochemicals against several NDD.

Initially, we studied the effects of BM on endogenous levels of oxidative markers in whole body homogenates of flies. BM exposure at both dietary concentrations caused significant reduction in the levels of oxidative markers such as MDA, HP levels and protein carbonyls in adult flies. These results are consistent with our previous findings in prepubertal mice wherein dietary feeding of BM enhanced the antioxidant defense mechanisms in both mitochondria and cytosol of different anatomical areas of prepubertal mice brain. There were also evidences of decreased MDA formation, ROS and hydroperoxide generation and reduced formation of protein carbonyls. (Shinomol and Muralidhara, 2010). The diminished levels of oxidative markers coupled with enhanced GSH levels in whole body of homogenates of *Drosophila* are suggestive of the antioxidative action of BM *in vivo*. Interestingly , the *in vivo* antioxidative activity of BM corroborates well with the free radical scavenging activity of BM actives observed in a number of *in vitro* systems reported earlier (Shinomol, 2008). Earlier findings from our laboratory have shown BM to be highly effective against free radicals like nitric oxide, superoxide and hydroxyl radical. Further, BM also effectively chelated iron,

protected against deoxyribose oxidation and exhibited significant reducing capacity.

Previously, the neuroprotective effects of BM extract have been tested against various toxicants including glutamate, aluminium, beta amyloid, and nitric oxide. The antioxidant and anti-stress activities of BM contribute significantly to its neuroprotective function. It was shown that the BM extract inhibits multiple components of the beta-amyloid induced oxidative stress pathway that can contribute to Alzheimer's pathology and reduced beta-amyloid levels in the brain of an Alzheimer's disease (AD) transgenic mouse model (PSAPP mice) (Dhanasekaran et al., 2007). The mechanism of action of BM-mediated protection against the beta-amyloid toxicity in primary cortical cultured neurons is attributable to its antioxidant potential which suppresses the neuronal oxidative stress. There are evidences showing that amyloid peptides could increase acetylcholinesterase (AChE) in cultured cells (Sberna et al., 1997), which could be part of its neurotoxic properties. Since BM inhibits AChE activity, this property might also play a role in its neuroprotection against amyloid toxicity (Limpeanchob et al., 2008). Recent studies revealed the neuroprotective effects of BM against Aluminium-induced neurotoxicity (jyoti et al., 2007), protect the brain from morphine induced inhibition of antioxidant enzyme systems (Sumathy et al., 2002) and protected rat brain from oxidative stress caused by chronic exposure to cigarette smoke (Anbarasi et al., 2005). BM was also shown to be protective in the animal model of ischemia-induced brain oxidative stress (Saraf et al., 2010).

Accumulation of free radicals and consequent neurodegeneration in specific brain regions has been proposed as causal factors in various NDD such as Alzheimer's and Parkinson's disease (Halliwell, 2006). This accumulation of oxidative free radicals is due to decreased activities of the free radical defense enzymes. Hence, a potential therapy which augments antioxidative defense system may prove beneficial under various NDD conditions. Since rotenone is a widely employed model for inducing PD in mammalian model, we chose the fly model to test the hypothesis that dietary feeding of BM can mitigate rotenone induced oxidative stress and neurotoxicity. Another compelling reason was a recent report of Coulom and Birman (2004) in

which they convincingly demonstrated that chronic exposure of *Drosophila* to sub lethal doses of rotenone, recapitulates the main symptomatic features of PD viz., a selective loss of dopaminergic neurons including locomotor defects. Further rotenone, employed as an insecticide/herbicide is also hypothesized to be a environmental factor contributing significantly towards the occurrence of sporadic PD in humans along with other chemicals such as paraquat. Based on these, we chose to investigate the neuroprotective efficacy of BM on rotenone induced lethality, locomotor deficits, oxidative stress and dopamine depletion.

Stress elicits a defensive response in organisms such as induction of stress gene expression, enhanced antioxidant protection and enhanced toxin clearance. BM facilitates each of these adaptive resources by modulating Hsp 70 expression (Anbarasi et al., 2006), and enhancement of activity of both superoxide dismutase and cytochrome P450 enzymes in stressor exposed rat brain (Chowdhuri et al., 2002). Thus, BM may facilitate the capacity of the brain to withstand stress, and help it to function under adverse conditions. These findings support the medhya rasayana classification of Bacopa in ancient Ayurveda (Mills and Bone, 2000; Blumenthal, 2003). Recently, the adaptogenic effect of BM under different stressful conditions in acute and chronic unpredictable stress was verified in the brain. The results indicated that the adaptogenic activity of BM might be due to the normalization of stress induced alteration in plasma corticosterone and levels of monoamines like noradrenaline, 5-Hydroxy Tryptamine and Dopamine in cortex and hippocampus regions of the brain, which are more vulnerable to stressful conditions (Sheikh et al., 2007).

In the present study, rotenone exposure to flies induced concentration related mortality and locomotor deficits which are in agreement with previous reports (Coulom and Birman, 2004). However, the precise mechanism of rotenone action which leads to neurodegeneration is not well understood. Evidences suggesting the involvement of oxidative stress mechanism/s stem from both *in vitro* and *in vivo* models relevant to PD (Sherer et al., 2003). Likewise in the insect model, rotenone induced neurotoxicity is attributed to the specific sensitivity of dopaminergic neurons to reactive oxygen species and oxidative damage. Interestingly, similar signs of oxidative damage have been

detected frequently in dopaminergic neurons from PD patients, suggesting an implication of oxidative stress in this disease (Beal, 2003; Jenner, 2003). Earlier studies have shown that feeding L-DOPA to flies did not reduce cell loss, suggesting that the drug acts simply, as a dopamine precursor to rescue endogenous dopamine deficits (Coulom and Birman, 2004). In contrast, prevention of both locomotor deficits and DA neuronal loss in *Drosophila* by the antioxidant melatonin implicates that rotenone induced cell death probably results from oxidative stress. Several report on the antioxidative nature of melatonin, and its ability to up regulate the activities of antioxidant enzymes such as SOD and catalase in mammalian models supports the antioxidant hypothesis (Coto-Montes et al., 1999; Reiter et al., 2001; Tan et al., 2002).

In the present study, we found significant induction of oxidative stress among flies exposed to rotenone (500µM) for 7 days as evidenced by the marked elevation in MDA and hydroperoxide level coupled with significant increase in activities of antioxidant enzymes such as Catalase, SOD and GST which suggested an increased generation of ROS and toxic aldehydes. This is consistent with previous observations in experimental rats exposed to rotenone (Saravanan et al., 2007). Reduced GSH is a tripeptide and most abundant soluble antioxidant molecule which plays a crucial role in detoxifying ROS either by reacting directly with radicals non-enzymatically or acting as a substrate in GST catalyzed reactions. Severe depletion in cellular GSH levels upon rotenone exposure in *drosophila* adds further evidence that a state of oxidative stress exists *in vivo* which may lead to mitochondrial damage, increase in free radical generation and peroxidation of membrane lipids (Sen, 1997). Further, rotenone exposure also induced significant protein damage among flies as evident by enhanced protein carbonyl levels indicating high rate of protein oxidation.

Interestingly, co-exposure of rotenone with BM resulted in complete reduction in oxidative markers clearly indicating the antioxidant ability of BM extract. Further, BM restored depleted GSH pool and normalized the protein carbonyl levels. This protective ability of BM may be attributed to the presence of bioactives which may be responsible for quenching of free radicals or alternatively it may be due to the up regulation of antioxidative defenses. It is

documented that some of the phytochemicals are known to exert their protective effects against oxidative stress by enhancing GSH levels. Though the exact molecular mechanism of action is not clear, it is speculated that, these phytochemicals may act at gene level by increasing the expression of GSH synthesizing enzymes (Sen, 1997).

We have carried out studies to elucidate the neuroprotective effects of BM leaf extract against 3-nitropropionic acid (3-NPA) and rotenone induced neurotoxicity in mitochondrial and cytosolic fractions of prepubertal mouse brain regions. Significant protection was evident from the amelioration of increased lipid peroxides, ROS, Hydroperoxides and protein carbonyls. Further, the alterations induced by 3-NPA and rotenone on mitochondrial function and antioxidant enzymes, antioxidant molecules like glutathione and other thiol antioxidants were also restored by BM exposure. Significant protection was also evident against the alterations in mitochondrial complex enzymes, altered membrane potential, Na^+-K^+ ATPase activity. BM also protected against dopamine depletion and motor dysfunctions associated with rotenone toxicity. Studies on dopaminergic cell lines gave further substantiated the neuroprotective role of BM extract (Shinomol et al, unpublished).

In the current study, rotenone caused high mortality (50-60%) among flies during a 7 day exposure period. But the low incidence of mortality among BM co-exposed flies clearly indicates the presence of protective bioactive compounds in BM. Further, rotenone induced neurotoxicity could be evidenced by high rate of locomotor deficits as measured in the negative geotaxis assay. Flies with locomotor deficits have tendency to stay at the bottom of glass column and do not appear to coordinate their legs in a normal fashion. This phenotypic expression has been explained earlier due to high energy requirement of ambulatory and flight muscles which are rich in mitochondria. Although speculative, it is likely that uncoupled mitochondrial machinery may be responsible for the same under conditions of severe complex I inhibition (Shinomol and Muralidhara, Unpublished). Another possible reason might be due to the differential and significant depletion of dopamine pool as observed in both head and body regions. Surprisingly, both BM and BC were able to rescue the flies significantly from deteriorating locomotor dysfunctions

indicating their potential to protect at the mitochondrial level with possible restoration of dopamine pool. This is consistent with earlier findings such as the significant correlation between locomotor dysfunction and dopamine deficiency (Celotto and Palladino, 2005). However, further biochemical investigations on rotenone induced mitochondrial dysfunctions in flies would be necessary in this regard. Our results obtained in 'paraquat resistance assay' also suggest that BM prophylaxis has the propensity to protect against neurotoxicant exposure largely due to its antioxidative potential. Paraquat is a well established free radical generator and also known to have role in selective neurodegeneration. Prophylactic feeding of flies with either BM or BC significantly protected the flies against both the rate and the total incidence of mortality. It appeared to effectively protect against paraquat induced radical generation as evidenced by the increased longevity of the flies clearly demonstrating antioxidative potential of BM.

Based on our biochemical evidences, we propose that dietary feeding of BM powder to *Drosophila* for a short duration has the propensity to attenuate rotenone induced oxidative stress owing to its antioxidative nature and its ability to modulate the activities of antioxidant defenses such as reduced GSH and antioxidant defenses. Additional evidences viz., lower incidence of rotenone induced mortality and higher resistance to paraquat among flies pretreated with BM clearly support such a mechanism. Further, its neuroprotective property was clearly evident by its ability to significantly abrogate rotenone induced oxidative stress, improve locomotor performance and restoration of dopamine levels. In this model, the neuroprotective activity of BM powder was highly comparable to Brahmi capsule powder, a commercial preparation advocated for human consumption. More importantly our data further confirm the utility value of *Drosophila* system as a primary model to rapidly screen potential compounds for their neuropharmacological properties prior to their testing in mammalian models and final therapeutic use in humans.

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6.0 SUMMARY

1. Exposure of adult flies to rotenone (250-1000 µM in diet) induced concentration dependent mortality and robust locomotor impairments.
2. BM-enriched diet (0.05 and 0.1% for 7 d) caused significant diminution in the levels of endogenous oxidative markers viz., MDA, ROS and HP levels, and was accompanied with reduced protein carbonyls and enhanced glutathione clearly suggesting its antioxidative activity *in vivo.*
3. Marginal elevation in the activities of acetylcholinesterase and butyrylcholinesterase enzymes among flies fed on BM-enriched diet showed its ability to significantly modulate cholinergic function.
4. The incidence of rotenone (500 µM) induced mortality was significantly reduced among flies fed with BM-enriched diet suggesting its ameliorative effect under co-exposure regimen. Brahmi capsule actives at similar concentrations also yielded comparable protection.
5. Co-exposure with BM completely offset rotenone induced oxidative stress as evidenced by the normalized MDA/hydroperoxide/ protein carbonyl levels which was accompanied by restoration of GSH in whole body homogenates.
6. Rotenone induced alterations in the activities of antioxidant enzymes (catalase, SOD and GST) among flies fed on BM-enriched diet adult flies were significantly ameliorated.
7. Rotenone induced neurotoxicity was clearly evident among flies as measured by severe locomotor impairments. Interestingly, flies co-exposed with BM/BC showed marked improvement as their performance was better in the negative geotaxis assay (65-70% protection).
8. Rotenone exposure caused significant depletion in dopamine level in both head and body region as measured by HPLC analysis. The neuroprotective effect of BM was clearly discernible as evident by the significant restoration of dopamine levels.
9. Flies given BM prophylaxis (0.05 and 0.1% for 7 d) showed marked resistance to acute paraquat (filter disc method, 40mM, 48h) challenge as evident by the lower incidence of mortality.

CHAPTER III

Prophylactic neuroprotective efficacy of *Bacopa monnieri* against paraquat-induced oxidative stress and mitochondrial dysfunctions in *Drosophila*

1.0 INTRODUCTION

Exposure to agricultural chemicals such as rotenone and paraquat has been widely postulated as a potential environmental risk factor for PD (Gorell et al., 1998: Petrovitch et al., 2002; Abbott et al., 2003; Baldi et al., 2003). Paraquat (PQ), (1, 1'-dimethyl-4, 4'-bipyridinium dichloride) is a potent, non-selective herbicide which is currently used to model PD in both rodents and *Drosophila* (Thiruchelvum et al., 2000; Ossowska et al., 2005; Bove, 2005). Based on both epidemiological evidence of increased incidence of PD and its chemical similarity to the Parkinsonism –inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP), PQ is considered a prime risk factor.

Although the precise mechanism of action of PQ toxicity is not well understood, its toxic effects are well attributed to damage in mitochondria and subsequent oxidative damage (Fukushima et al., 2003; Thiruchelvum et al., 2005; Castello et al., 2007). While the direct involvement of mitochondria in PQ toxicity is debatable, large pool of literature supports uncoupling of electron transport chain to be responsible for the production of free radicals viz., superoxide anions, singlet oxygen as well as hydroxyl and peroxyl radicals (Cocheme and Murphy, 2008). Numerous other evidences favour such a possibility. For example, mitochondrial expression of human peroxiredoxin -5 protects yeast more effectively against PQ toxicity than its expression in the cytosol (Tien Nguyen-nhu and Knoops, 2003). Drosophila over expressing catalase in mitochondria are resistant to PQ, whereas enhancement of cytosolic catalase was not protective (Mocket et al., 2003). RNA interference silencing of Mn-SOD in Drosophila caused hypersensitivity to PQ (Kirby et al., 2002), and mice heterozygous for Mn-SOD showed greater sensitivity to PQ than wild-type (Van Remmen et al., 2004). Further mitochondrial swelling is one of the earliest ultrastructural changes upon PQ exposure *in vivo*. Paraquat exposure is known to induce movement disorder in some mammalian models (Brooks et al., 1999; Chanyachukul et al., 2004). However, in other mammalian studies in which PQ induces dopaminergic neurodegeneration, neuronal loss has not been sufficiently extensive to emulate PD movement characteristics (McCormack et al., 2002; Di Monte, 2003).

Since PQ is a strong inducer of free radicals, numerous researchers have employed this toxicant to induce oxidative stress in *Drosophila* strains (wild and transgenic) following acute exposure to paraquat (Kirby et al., 2002; Mockett et al., 2003; Coulom and Birman 2004; Magwere et al., 2006; Chaudhuri et al., 2007). This assay popularly known as “paraquat resistance test” is often employed to assess the development of resistance among *Drosophila* fed various pharmaceutical drugs/antioxidants. Recent studies have demonstrated survival benefits among *Drosophila* fed known antioxidants *viz.,* Ascorbic acid, Vitamin E, Melatonin, Glutathione etc., employing PQ oxidative stress bioassay (Suntres, 2002). Further, the neuroprotective efficacy of polyphenols in PQ and iron-induced PD model has been conclusively illustrated (Jimenez-Del-Rio et al., 2010).

Our previous findings in *Drosophila* clearly showed the propensity of dietary BM to markedly attenuate rotenone induced oxidative stress in a ‘co-exposure regimen’ (Chapter 2). Evidences such as reduced incidence of rotenone induced mortality and higher resistance to PQ among flies pre treated with BM clearly suggested its antioxidative potential *in vivo*. Based on our observations in the rotenone model, the prophylactic neuroprotective ability of BM was investigated in a paraquat model of neurotoxicity. Further, repeated exposure of *Drosophila* to sublethal doses of PQ has been demonstrated to recapitulate the main symptomatic features of PD (Chaudhuri et al., 2007). However, no comprehensive data exists on the biochemical perturbations in *Drosophila* following exposure to sublethal concentrations of PQ.

Accordingly, in this study the following issues have been addressed: i) Early oxidative stress induction among flies exposed to acute PQ ii) the impact of PQ exposure on mitochondrial oxidative stress and associated mitochondrial dysfunctions and iii) The prophylactic efficacy of dietary *Bacopa monnieri* to ameliorate PQ induced lethality, oxidative stress and mitochondrial dysfunctions among adult *Drosophila*.

2.0 OBJECTIVE

The focus of these investigations was primarily to establish paraquat induced early oxidative stress and mitochondrial dysfunctions in Drosophila under acute exposure conditions. Further, using this experimental regimen, the prophylactic neuroprotective efficacy of *Bacopa monnieri* was studied.

PROPHYLACTIC NEUROPROTECTIVE EFFICACY OF *BACOPA MONNIERI* AGAINST PARAQUAT IN *DROSOPHILA*

3.0 EXPERIMENTAL DESIGN

I. Determination of toxicity profile of paraquat (PQ)

PQ induced lethality response: Time-course (96 h exposure)

Lethality response of synchronized adult flies (8-9 d old) to paraquat exposure was determined using filter paper disc method. Four circular filter paper discs (Whatman 41 diameter 1.5 cm, stacked one above the other) were saturated with various concentrations of PQ (10- 40mM in 5% sucrose solution) which were placed in vials and flies were allowed to feed for 96h. The incidence of mortality was recorded at 24h intervals and expressed as percent mortality.

In a separate study, synchronized flies were exposed to varying concentration of PQ (10-50mM) by filter disc method procedure. And the mortality data for 48h presented by graphical method to obtain $LC_{50.}$

II. PQ-induced early oxidative stress induction (48h exposure)

Synchronized adult male flies were exposed to three concentrations of PQ (10, 20 and 40mM) for a period of 24h. Following exposure, both control and PQ exposed flies were briefly anesthetized and whole boy homogenates were prepared. In order to assess the extent of oxidative stress induction, following biochemical determinations were made.

Oxidative stress markers

ROS generation, status of lipid peroxidation and hydroperoxide levels were quantified as described earlier. The extent of protein oxidation was assessed by measuring protein carbonyl content.

Redox status and activities of antioxidant enzymes

The redox status in cytosol was measured by determining the levels of reduced glutathione and total thiols in both control and PQ treated flies. The activity levels of antioxidant enzymes viz., catalase, SOD and GST were measured in cytosol.

Measurement of cholinergic function

The effect of PQ exposure on the activities of acetylcholinesterase and butyrylcholinesterase were assayed to assess cholinergic function.

III. PQ-induced mitochondrial oxidative stress and dysfunctions

Employing a similar exposure regimen as described above, the effect of PQ exposure on mitochondrial oxidative stress, and associated biochemical dysfunctions were determined as follows.

Mitochondrial oxidative stress markers, redox status and free iron level

Induction of mitochondrial oxidative stress was assessed by measuring the lipid peroxidation status and generation ROS level as described above. Further the redox status was assessed by determination of reduced glutathione and total thiol level. In cytosolic fraction, free iron levels were also quantified to assess the possible participation of iron in PQ induced oxidative stress.

Superoxide generation, Mn-SOD and GST activity

Since PQ is known to generate superoxide in other systems, the effect of PQ on the activity of Mn-SOD and its ability to generate superoxide were measured in mitochondrial fractions. Further, the effect of PQ on phase II detoxification enzyme, GST was also determined.

Effect on Electron transport chain (ETC) enzymes

Since PQ is a known inducer of superoxides, its possible effect on the activity of mitochondrial complex enzymes viz., complex I-III & complex II-III were assayed in mitochondria.

Associated mitochondrial dysfunctions

The activity of succinate and NADH dependent MTT reduction was assayed in both control and treated flies. Further, the activity levels of TCA cycle enzymes viz., succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and citrate synthase (CS) activities were also determined. The impact of PQ exposure on membrane function was determined by measuring the membrane potential and the activity of Mg^{+2} ATPase.

IV. Prophylactic neuroprotective effects of BM against PQ-induced oxidative stress and mitochondrial dysfunctions

Bacopa monnieri prophylaxis

Synchronized adult male flies fed on BM-enriched diet at two levels (0.05 and 0.1%) for 7d were used for all studies.

Modulation of PQ induced lethality response

Both control flies and flies given BM prophylaxis were challenged with paraquat (40mM for 24h) using filter disc method. Mortality was recorded as and when it occurred. A minimum of 50 flies and three such replicates were used to assess the mortality pattern.

Attenuation of PQ induced oxidative stress and mitochondrial dysfunctions

Synchronized adult male flies pre-fed on BM-enriched diet for 7 days were challenged with an acute dose of PQ (40mM, for 24h). The neuroprotective efficacy of BM prophylaxis was determined by measuring the attenuation of PQ induced oxidative stress in cytosol/mitochondria, redox status, activities of

mitochondrial antioxidant enzymes and mitochondrial functional status. The following biochemical determinations were made in various groups of flies.

Ameliorative effect of BM on cytosolic oxidative stress markers

Protective efficacy of BM against PQ induced oxidative damage was assessed by determination of selected oxidative markers viz., ROS generation, status of lipid peroxidation and hydroperoxide levels in cytosol.

Ameliorative effect of BM on redox status

The modulatory effect of BM on PQ induced redox status was ascertained by measuring and reduced glutathione level.

Modulatory effect on mitochondrial antioxidant enzymes

Activities of selected antioxidant enzymes viz., SOD and GST were assayed in mitochondrial fraction.

Ameliorative effect of BM on ETC enzymes and TCA enzymes

Since PQ is known to exert its toxic effects *via* mitochondrial dysfunctions, present investigation, we chose to study its effect on the activities of selected ETC enzymes viz., complex I-III and complex II-III in whole body mitochondrial fractions.

Effects on TCA cycle enzymes viz., succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and citrate synthase (CS) activities were determined among adult flies of control and treated groups.

4.0 RESULTS

PROPHYLACTIC NEUROPROTECTIVE EFFICACY OF *BACOPA MONNIERI* AGAINST PARAQUAT IN *DROSOPHILA*

I. Determination of toxicity profile of paraquat (PQ)

PQ-induced lethality response: Time course (96 h exposure)

Data on the incidence of lethality among flies exposed to PQ over 5 days is presented in **(Figure 3.1)**. No mortality was observed at any of the concentrations up to 24 h. However at 48 h, mortality ensued only at higher concentrations (30 mM, 18%; 40 mM, 46%). Further, a concentration dependent mortality was observed at 72 h (5, 15, 33, 72% for 10, 20, 30, 40 mM respectively). On day 4, 50% mortality was evident at 20 mM, whereas the mortality incidence reached a peak (91% and 100%) at 30 and 40 mM. On day 5, 100 % mortality was found at all the concentrations. Among the tested concentrations, we obtained 50% mortality (LC_{50}) at the concentration of 40 mM at 48 h and this concentration was employed for our modulation experiments **(Table 3.1)**.

II. Pattern of PQ-induced early oxidative stress induction (48h exposure)

Cytosolic oxidative stress markers

In general oxidative markers viz., malondialdehyde levels in whole body cytosolic fraction of adult flies treated with acute paraquat showed no significant change in any of the tested concentrations. However, hydroperoxide levels were significantly high (32-39%) at both the concentrations **(Table 3.2)**.

Redox status and activities of antioxidant enzymes

Short-term exposure of adult flies to paraquat showed significant and dose-dependent increase in reduced glutathione (30-46%) levels at both the concentrations. Similarly, total thiols levels were also enhanced significantly (23-60%) indicating up regulation of antioxidant defense to combat against paraquat induced early oxidative stress *in vivo* **(Table 3.2)**.

The activities of antioxidant enzymes viz., catalase, superoxide dismutase and glutathione-S-transferase in whole body homogenate of adult flies fed with paraquat were determined at both the concentration. Significant increase in catalase (21-24%) **(Figure 3.2 A)** and Superoxide dismutase (25%) **(Figure 3.2 B)** activity was evident at both 20 and 40mM paraquat treated flies. However, glutathione S transferase activity **(Figure 3.2 C)** was highest (24%) at lower concentration only where as it remained unchanged at higher concentration.

Measurement of cholinergic function

Cholinergic function measured interms of acetylcholinesterase (21%) **(Figure 3.3A)** and butyrylcholinesterase (18%) activity showed significant inhibition among PQ treated flies compared to control at higher concentration only; at lower concentration the activities remained unaffected **(Figure 3.3B)**.

III. PQ-induced mitochondrial oxidative stress and dysfunctions

Mitochondrial oxidative stress markers redox status and free iron level

Malondialdehyde levels were significantly higher (43%) in whole body mitochondria of adult flies treated with acute paraquat at higher concentration only. while, at lower concentration, MDA levels remained unchanged. Reactive oxygen species generation remained unchanged **(Table 3.3)**.

On exposure to acute dose of paraquat, adult flies showed significant increase in reduced glutathione levels (by 33-37%) at both the doses. Interestingly in cytosolic fraction, paraquat treated flies showed higher free iron levels (18-25%) at both the concentrations compared to control **(Table 3.3)**.

Super oxide generation, Mn-SOD and GST activities

As anticipated, flies exposed to PQ showed significant and concentration dependent increase in superoxide generation (20-38%) **(Figure 3.4A)**. Further, antioxidant enzymes viz., Mn SOD (30-50%) and glutathione-S-transferase (38-41%) activity were significantly enhanced among paraquat treated flies at both the concentrations compared to untreated flies clearly reaffirming ongoing oxidative stress *in vivo* **(Figure 3.4 B&C)**.

Effect on the activities of electron transport chain (ETC) enzymes

Adult flies exposed to acute dose of paraquat *per se* showed in significant reduction (PQ 20mM, 42%; 40mM, 25%) of complex I-III activity at both the concentrations **(Figure 3.5 A)** . However, the activity of complex II-III was found significantly decreased (30%) only at higher concentration **(Figure 3.5 B)**.

Associated mitochondrial dysfunctions

Dehydrogenase activities were measured in terms of NADH and succinate dependent MTT reduction. Marginal increase in NADH (19-29%; 10-21%) **(Figure 3.6 A & B)** and succinate dependent MTT reduction was observed among adult flies exposed to paraquat at both the concentrations.

Effect on Mg^{+2} ATPase and membrane potential

Concentration-dependent reduction of Mg^{+2} ATPase (22-32%) was evident among paraquat exposed flies **(Figure 3.7 A)**. Further as anticipated, elevated level of membrane potential (17-20%) was evident among paraquat exposed adult flies at both the concentrations **(Figure 3.7 B)**.

Effect on succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and citrate synthase (CS) activities

Citric acid cycle enzymes were measured in terms of SDH, MDH and CS in adult flies. Significant and dose dependent inhibition of succinate dehydrogenase activity (32-78%) was observed among paraquat treated flies at both the tested concentrations **(Figure 3.8 A)**. In contrary, malate dehydrogenase activity (20-38%) was significantly elevated at both the doses **(Figure 3.8 B)**. However, citrate synthase activity was enhanced marginally (22%) at lower concentration coupled with significant decrease (30%) was evident at higher concentration **(Figure 3.8 C)**.

IV. Prophylactic neuroprotective effects of BM against PQ-induced lethality, oxidative stress and neurotoxicity

Modulation of PQ-induced lethality response

In order to examine whether BM prophylaxis renders resistance to PQ treatment, adult flies were exposed to PQ (LC_{50} concentration, 40 mM for 48 h). Interestingly, the incidence of mortality among flies pre-fed with BM (0.1% for 7 days) and PQ-exposed flies was significantly diminished compared to PQ only exposed flies. While PQ *per se* resulted in nearly 50% mortality at the end of 48 h, BM pre-fed flies showed 20% mortality, suggesting a 40% protection due to BM prophylaxis **(Figure 3.9)**.

Ameliorative effect of BM on cytosolic oxidative stress markers

BM pre treatment *per se* diminished endogenous MDA level by 22%, while ROS generation remained unaltered. Further, significant induction of ROS (44%) and MDA (32%) was observed among PQ exposed flies. Interestingly, in combination group (BM+PQ) PQ-induced oxidative markers were brought to normalcy **(Figure 3.10 A & B)**.

Ameliorative effects of BM on Hydroperoxide levels and redox status

Among adult flies pre-fed with BM *per se* showed significant decrease in hydroperoxide level (56%) and enhanced glutathione level (30%). As anticipated, PQ *per se* exposed flies resulted in marked elevation in hydroperoxide (57%) and glutathione (44%) status. Surprisingly, prophylactic flies challenged with PQ showed complete protection from hydroperoxide generation and further enhanced glutathione level **(Figure 3.11 A & B)**.

Modulatory effect on mitochondrial antioxidant enzymes

Alterations in the activities of antioxidants enzymes viz., SOD and GST were determined among whole body mitochondrial fractions. No significant change in the activities of SOD and GST were observed among BM *per se* supplemented flies. PQ exposed flies showed significant increase in SOD (19%) and GST (41%) activities. However, in BM and PQ combination group SOD activity failed

to restore to normalcy and GST activity was enhanced further (59%) **(Figure 3.12 A & B)**

Modulatory effect on BM ETC and TCA cycle enzymes

In another study, prophylactic efficacy of BM on PQ-induced inhibition of electron transport chain (ETC) enzymes was determined. BM treatment *per se* caused a significant elevation in the activities of complex I-III (by 21%) and complex II-III (by 65%) in mitochondria obtained from whole body homogenates. However, PQ exposure (24 h) resulted in significant decline in ETC enzyme activities (complex I-III, 26% and complex II-III, 30%), indicating clearly its ability to exert toxicity *via* uncoupling of ETC enzymes. Interestingly, the activities of both enzymes were restored to normalcy among flies given BM prophylaxis (0.1% for 7 days) suggesting significant protection **(Figure 3.13 A & B)**.

BM treatment *per se* caused a significant elevation in the activities of citric acid cycle enzymes (SDH, 76% and MDH, 32%). However, PQ exposure (24 h) resulted in significant decline in the activity of SDH (29%), while the activity of MDH was elevated (19%). Interestingly, the activity of SDH was restored to near normalcy among flies given BM prophylaxis, while the activity of MDH was elevated further by 50% **(Figure 3.14 A & B)**.

Figure 3.1

Concentration and time-dependent mortality response among adult male *Drosophila melanogaster* exposed to PQ (filter disc method)

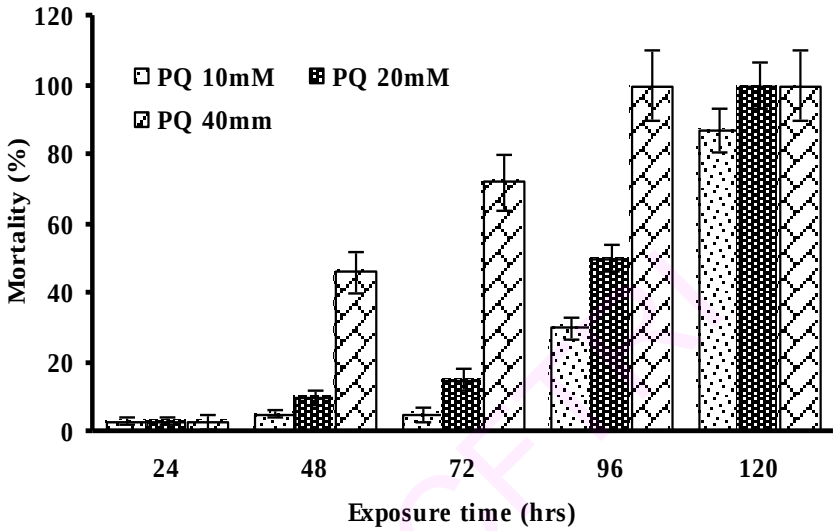


Table 3.1

Incidence of mortality among *Drosophila* exposed to paraquat for 48h (Filter disc method)

Paraquat (PQ) (mM)	Mortality (%) At 48h
0	0
10	6
20	14
40	50
50	60

n=50 flies per replicate, three such replication used for assay

Table 3.2

Oxidative perturbations in whole body homogenate of adult male *Drosophila* exposed to Paraquat (PQ) for 24 h

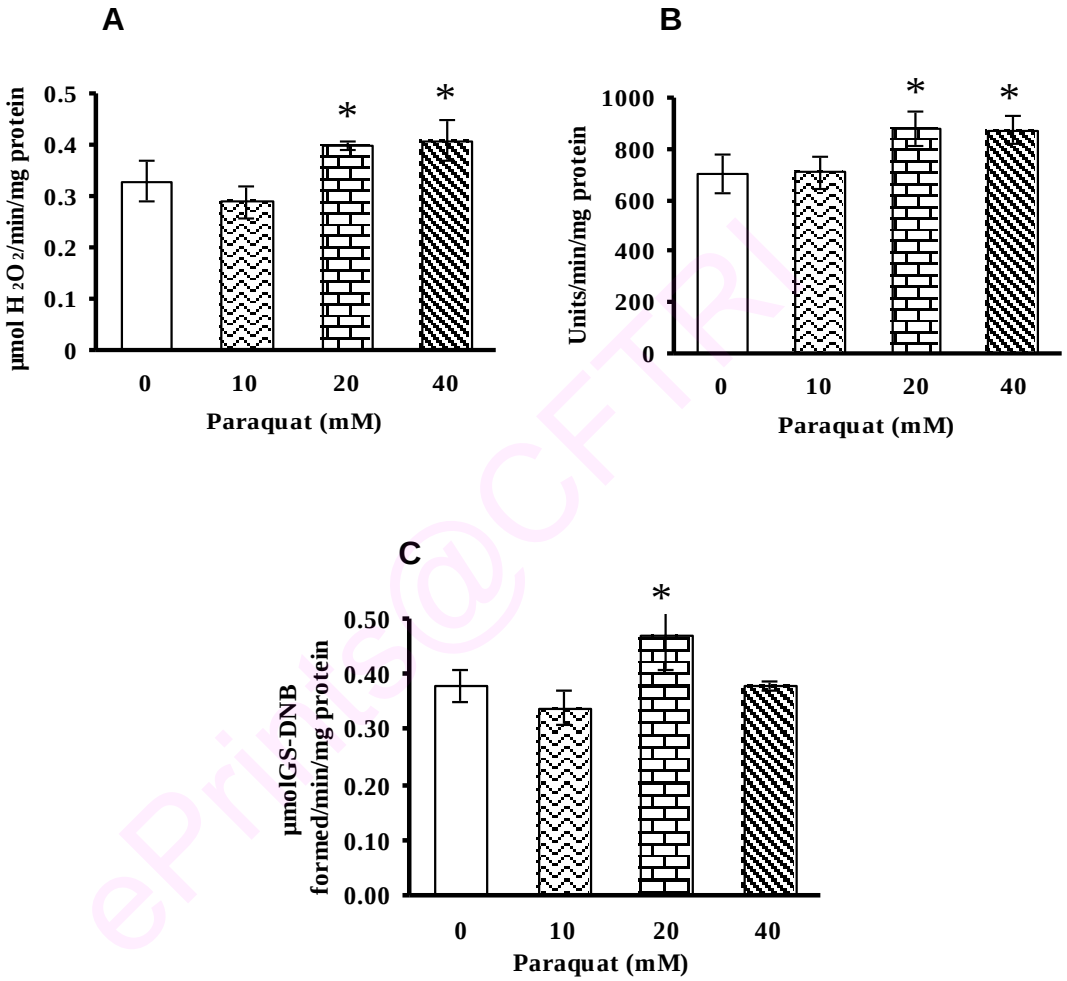
Parameter	Paraquat (mM)			
	0	10	20	40
MDA[1]	3.89±0.25	3.79±0.21	3.46±0.46	3.57±0.31*
HP[2]	0.28±0.0050	0.26±0.007	0.39±0.003	0.37± 0.004
GSH[3]	26.4±2.44	28.11±2.11	34.21±1.7*	38.52±2.81*
Total thiols[4]	0.049±0.01	0.052±0.01	0.78±0.01*	0.060±0.01*

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$).

[1]nmol MDA/mg protein
[2]nmol HP/mg protein
[3]µg GSH/mg protein
[4]mmol DTNB/mg protein

Figure 3.2

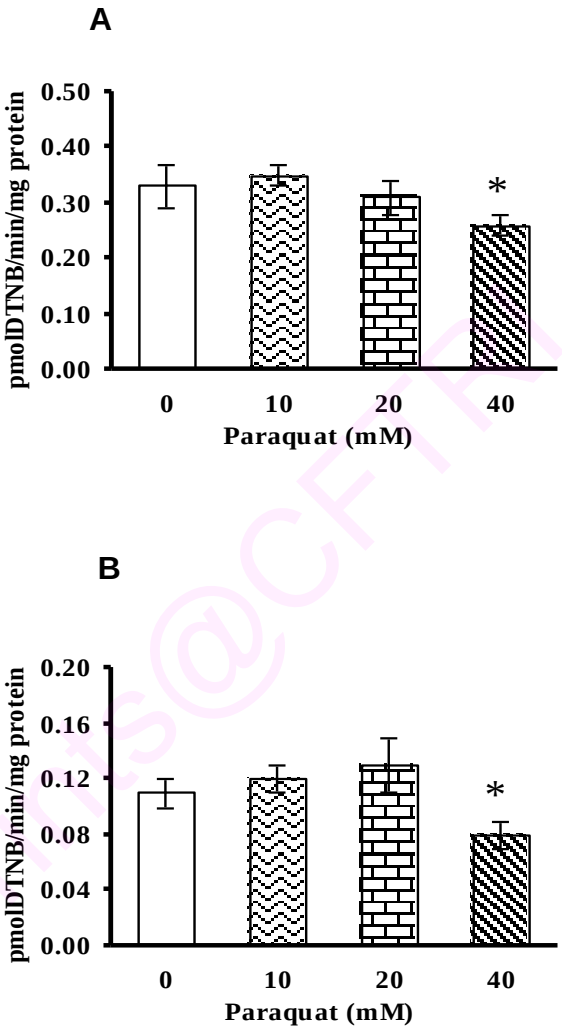
Effect of paraquat exposure (24h) on the activities of catalase (A), superoxide dismutase (B) and glutathione S transferase (C) enzymes in whole body homogenate of adult *Drosophila*



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

Figure 3.3

Effect of paraquat exposure (for 24h) on the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme in whole body homogenate of adult *Drosophila*



Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$).

Table 3.3

Acute paraquat exposure (for 24hrs) induced oxidative markers measured in terms of MDA level, ROS generation and GSH level in whole body mitochondrial fraction and free iron level in cytosol of adult *Drosophila*

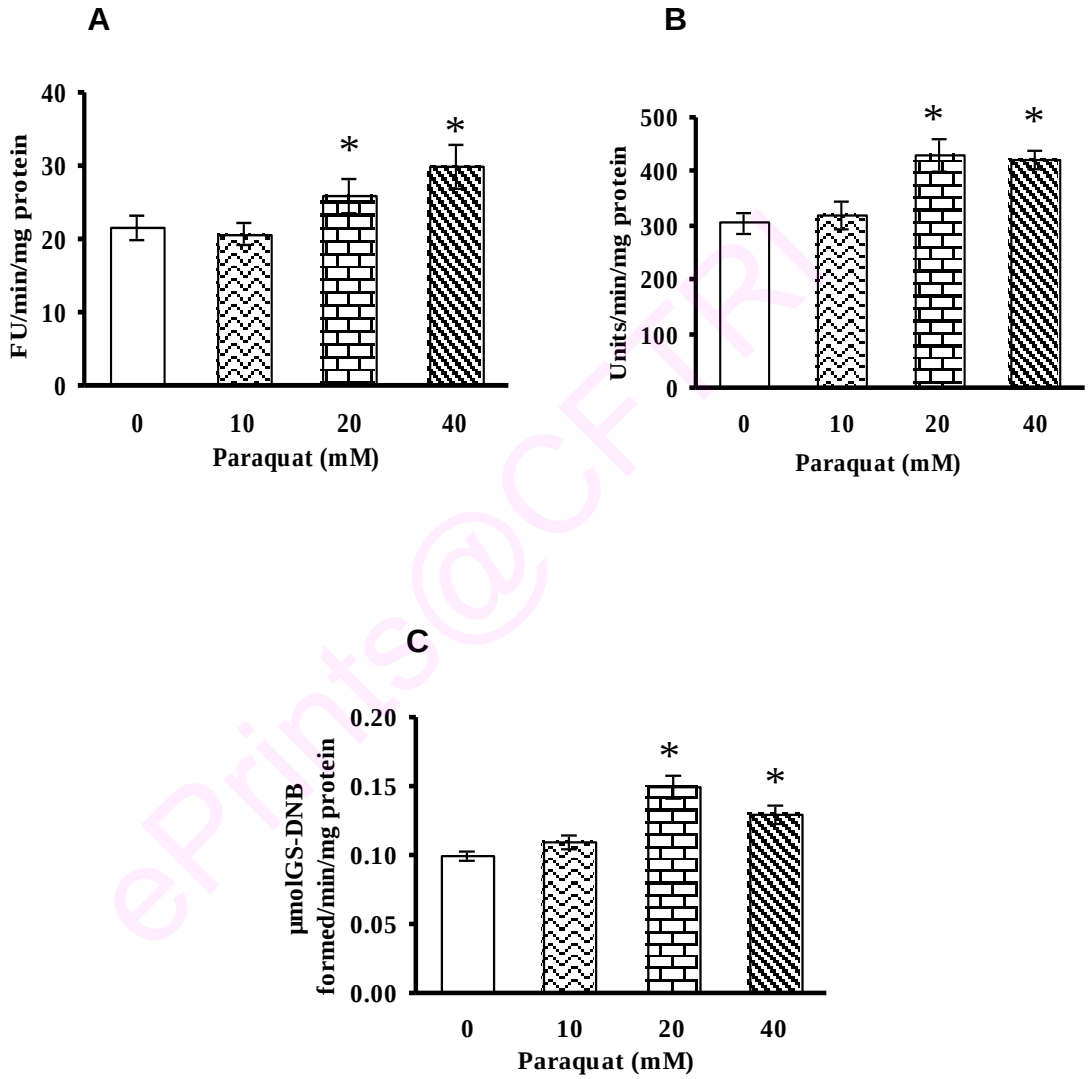
Parameter	Paraquat (mM)			
	0	10	20	40
MDA[1]	9.71±0.98	9.95±0.85	10.6±1.28	13.8±0.24*
ROS[2]	17.9±2.20	9.41±0.07	17.8±4.90	15.7± 2.70
GSH[3]	24.2±2.11	21.4±3.11	32±2.44*	33.2±2.08*
Iron level[4]	0.72±0.08	0.76±0.11	0.9±0.11*	0.85±0.01*

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Dunnet's test (* $p<0.05$)

[1]nmol MDA/mg protein
[2]pmol DCF formed/min/mg protein
[3]µg GSH/mg protein
[4]Absorbance/mg protein

Figure 3.4

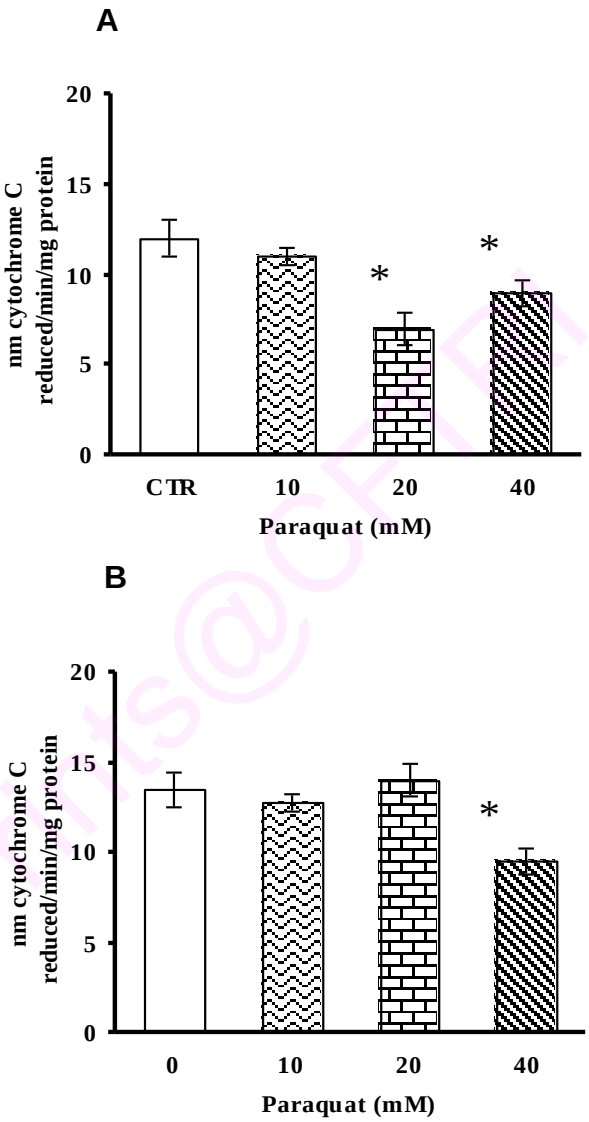
Effect of acute exposure of paraquat (PQ) on superoxide generation (A) and activities of Mn-SOD (B) and Glutathione-S-transferase (C) in whole body mitochondria isolated from *Drosophila*



Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Dunnet's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ groups (n=50 flies per replicate, three such replication used for assay).

Figure 3.5

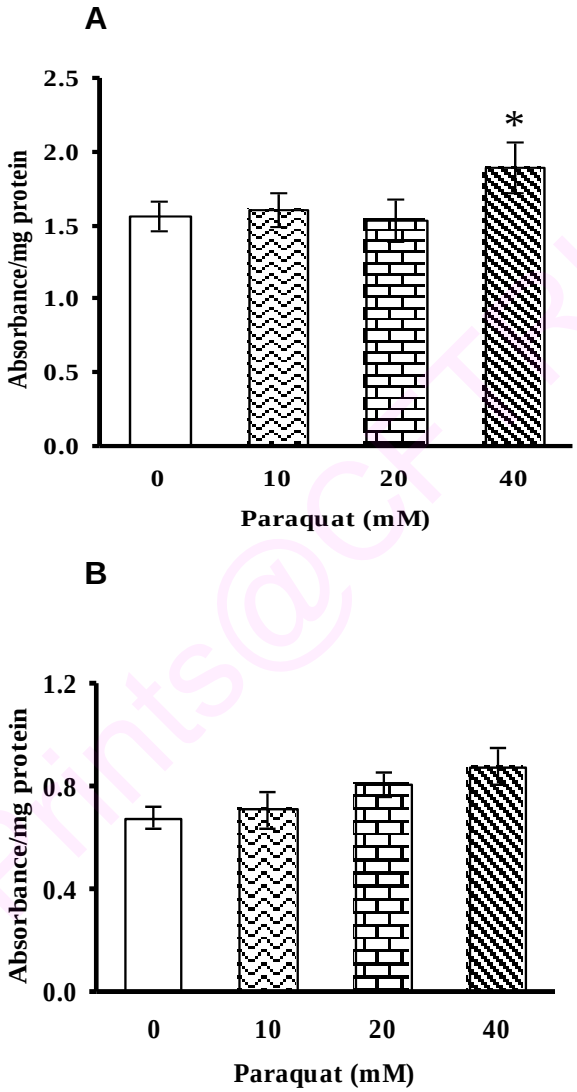
Effect of acute exposure of paraquat (PQ) on activities of complex I-III (A) and complex II-III (B) in whole body mitochondria of adult *Drosophila*



Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Dunnet's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.6

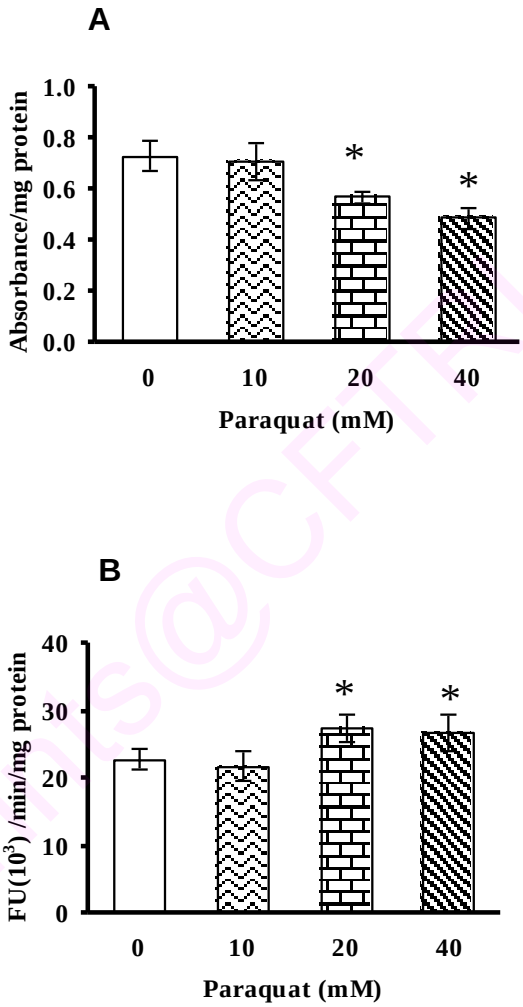
Effect of acute exposure of paraquat (PQ) on NADH dependent (A) and succinate dependent (B) MTT reduction determined in whole body mitochondria of *Drosophila*



Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Dunnet's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.7

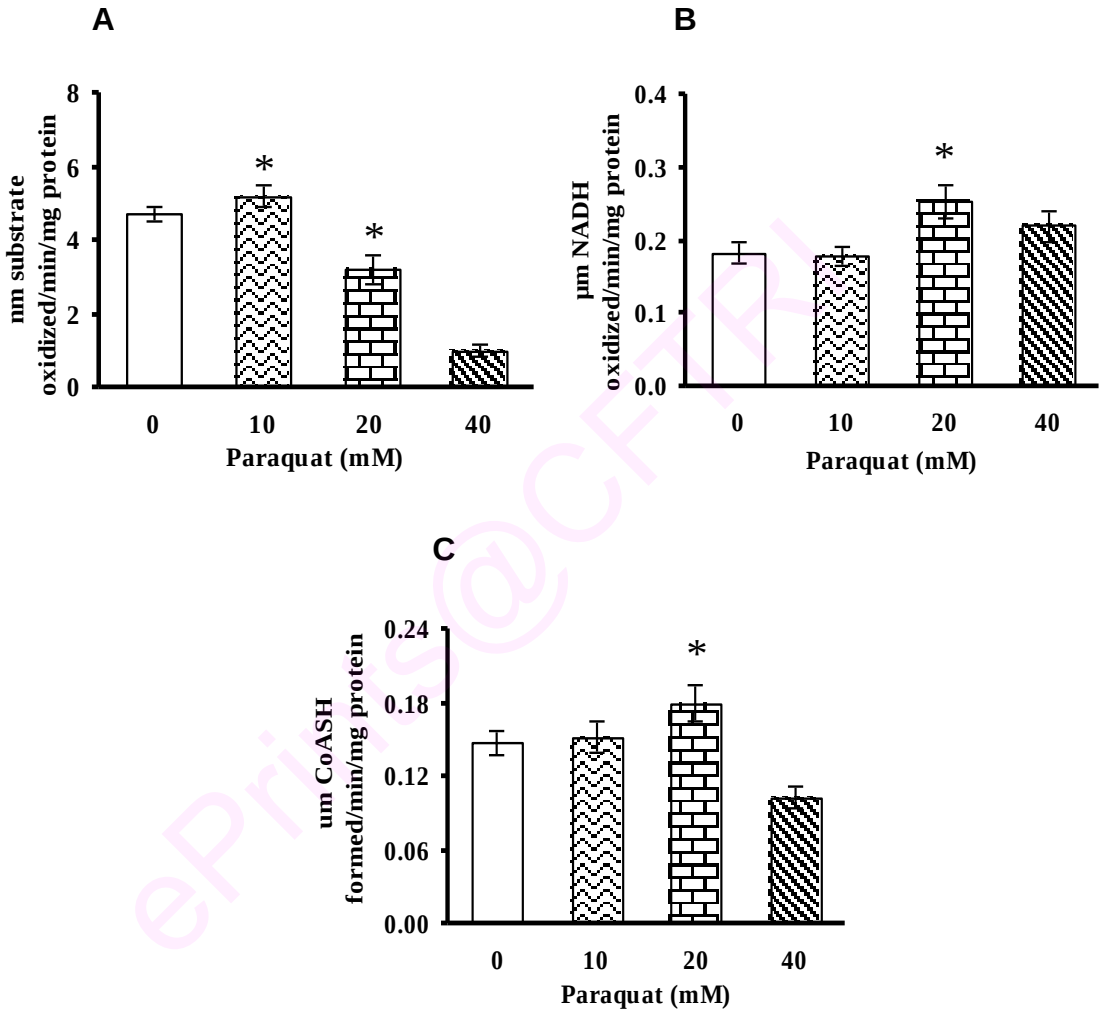
Effect of paraquat (PQ) exposure on Mg^{+2} ATPase activity (A) and membrane potential (B) in whole body mitochondria of *Drosophila*



Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Dunnet's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.8

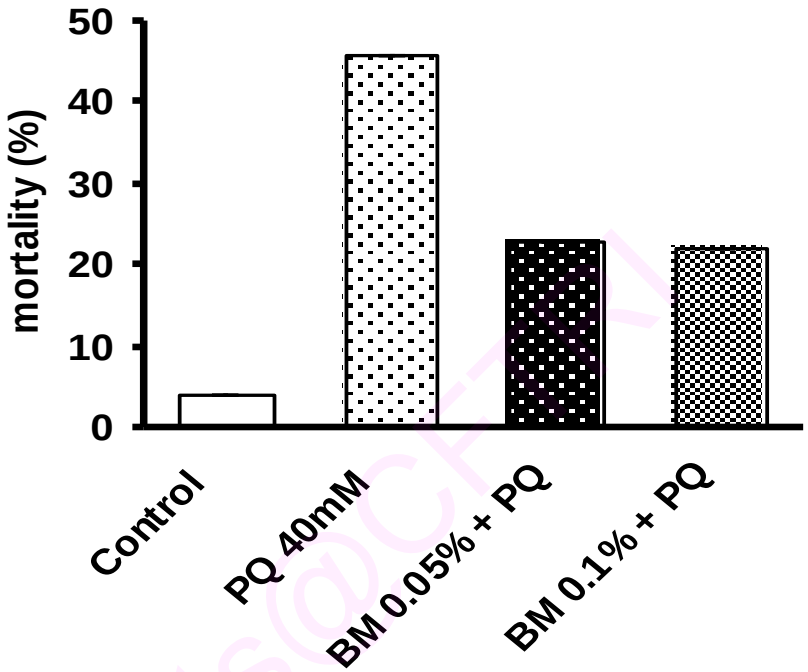
Effect of paraquat (PQ) exposure on the activities of Succinate dehydrogenase (A) Malate dehydrogenase (B) Citrate synthase (C) enzymes in whole body mitochondria of *Drosophila*



Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Dunnet's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.9

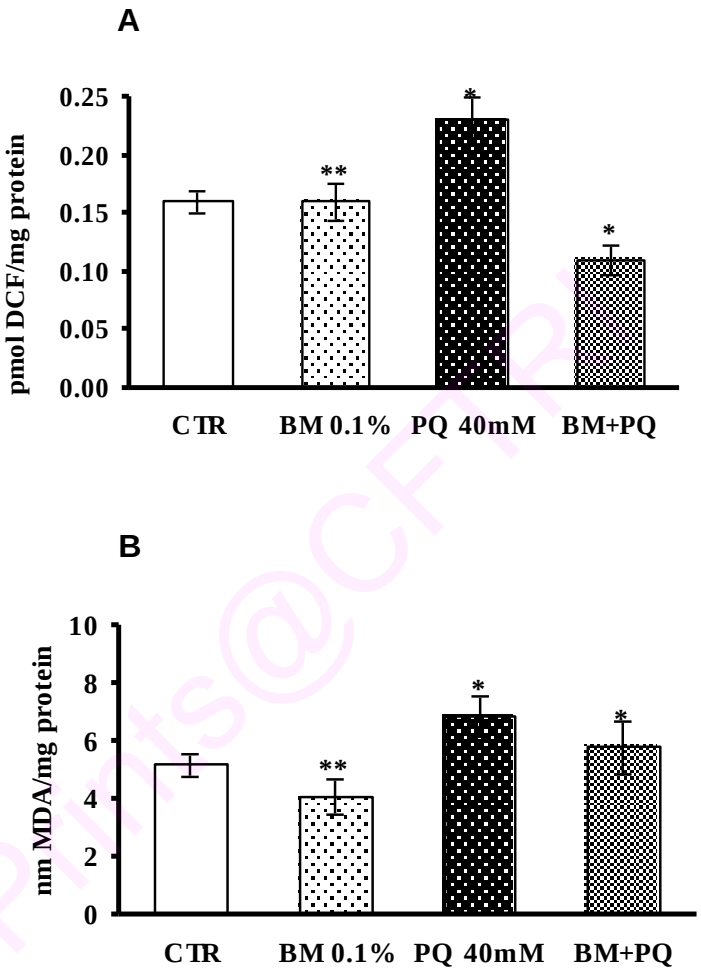
Modulatory effect of *Bacopa monnieri* (BM) prophylaxis on PQ induced mortality among adult *Drosophila*



Flies were given prophylactic treatment with BM for 7 days and subsequently exposed to 40 mM PQ for 48 h
n=50 flies per replicate, three such replication used for assay

Figure 3.10

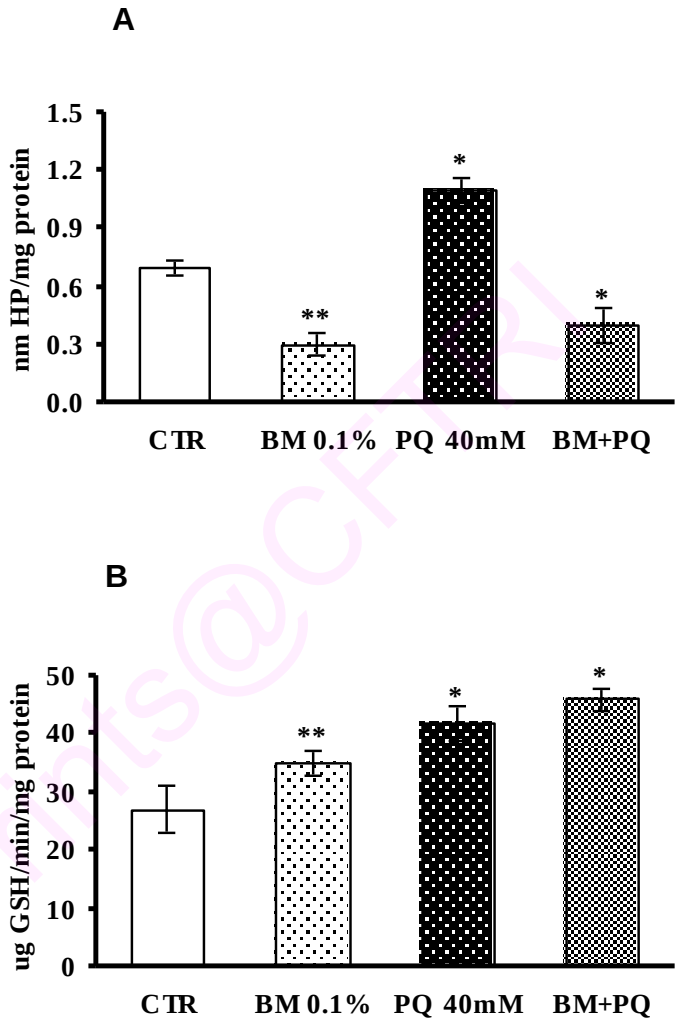
Modulatory effect of *Bacopa monneiri* prophylaxis on PQ induced generation of reactive oxygen species (ROS) (A) and malondialdehyde level (B) in whole body mitochondria of adult *Drosophila*



BM: *Bacopa monnieri*; PQ: Paraquat
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ. (n=50 flies per replicate, three such replication used for assay).

Figure 3.11

Modulatory effect of *Bacopa monneiri* prophylaxis on PQ induced impairment on hydroperoxide levels (A) and reduced glutathione (B) in whole body mitochondria of *Drosophila*

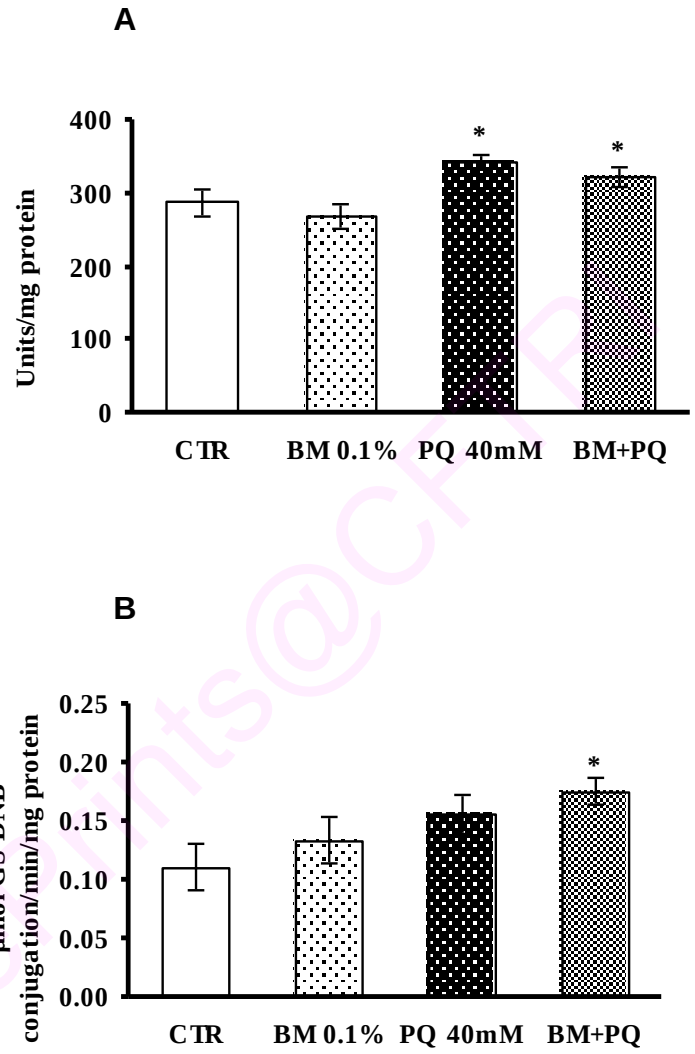


BM: *Bacopa monnieri*; PQ: Paraquat

Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.12

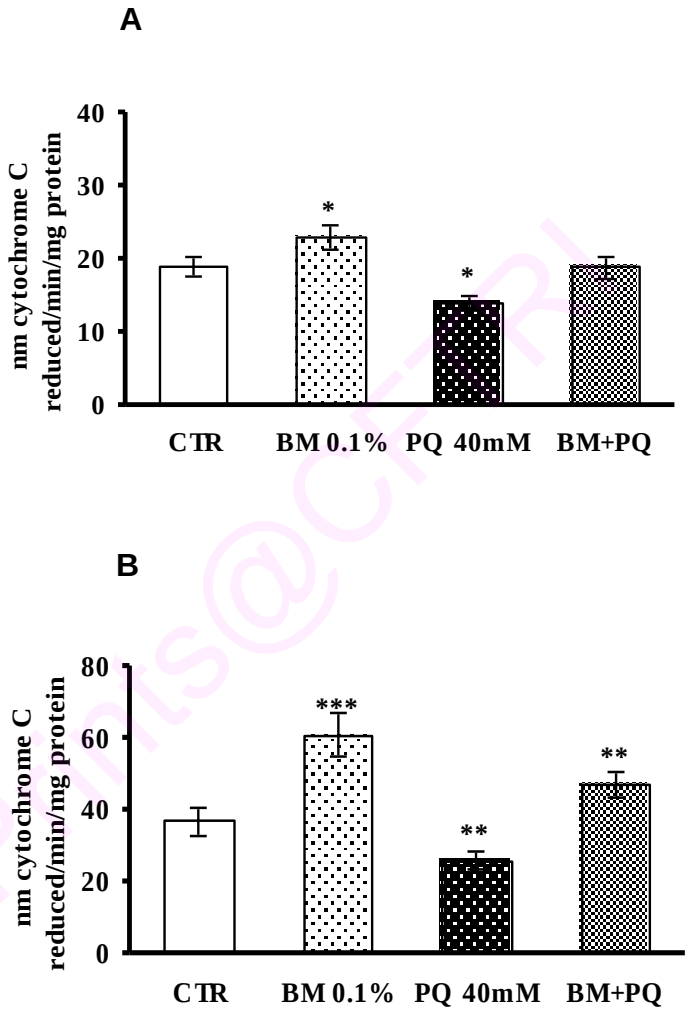
Modulatory effect of *Bacopa monneiri* (BM) prophylaxis on PQ induced alteration in the activities of SOD (A) and GST (B) enzymes in whole body mitochondria of adult *Drosophila*



BM: *Bacopa monnieri*; PQ: Paraquat
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.13

Modulatory effect of *Bacopa monneiri* prophylaxis on PQ induced alterations in ETC enzymes, NADH: cytochrome C reductase (complex I-III) (A) and Succinate: cytochrome C reductase (complex II-III) (B) in whole body mitochondria of *Drosophila*

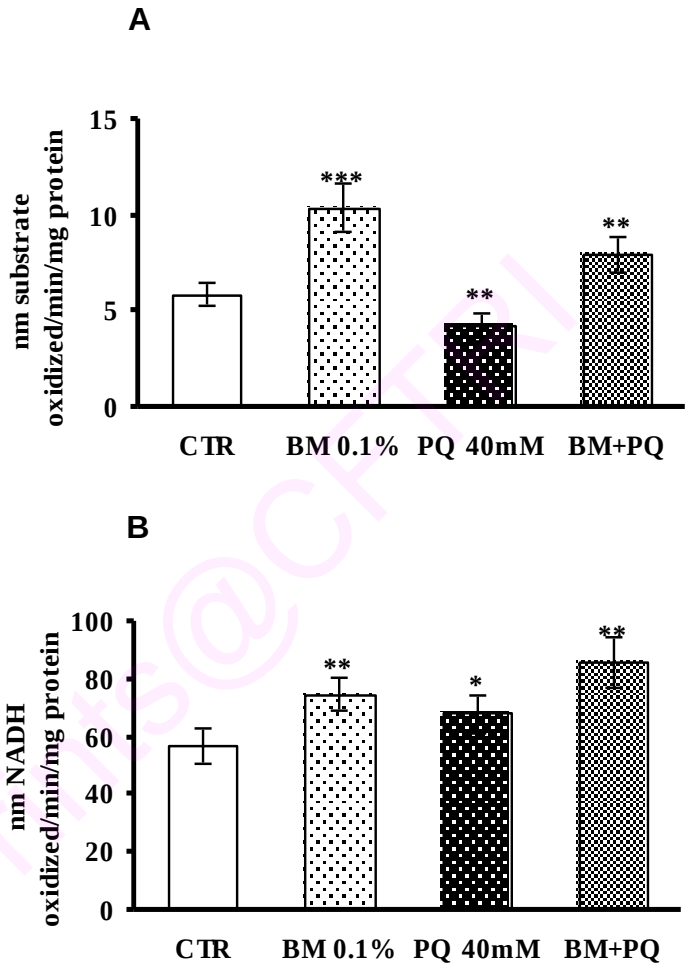


BM: *Bacopa monnieri*; PQ: Paraquat

Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

Figure 3.14

Modulatory effect of *Bacopa monneiri* prophylaxis on the activities of succinate dehydrogenase (SDH) (A) and malate dehydrogenase (MDH) (B) in mitochondria of *Drosophila* exposed to PQ



BM: *Bacopa monnieri*; PQ: Paraquat

Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey's test (* $p<0.05$). Significances were determined by making comparisons between control v/s PQ (n=50 flies per replicate, three such replication used for assay).

5.0 DISCUSSION

In the recent past, *Drosophila* has been widely employed to model environmental toxin-induced Parkinsonism (Coulom and Birman, 2004: Cauchi et al., 2006; Chaudhuri et al., 2007). The mechanisms underlying neuron death in PD are unknown, but both genetic defects and environmental factors are implicated in its pathogenesis (Saini et al., 2010). Both Rotenone and Paraquat have been commonly used to elucidate the pathophysiology of PD in wild and transgenic flies. While testing various pharmaceutical agents for their putative neuroprotection in the Drosophila model, researchers have developed an oxidative bioassay termed “PQ resistance test” (Bonilla et al., 2002; Vermeleun et al., 2005; Khazaeli et al., 2007; Choudhary etl ., 2007). This test basically involves determination of incidence of lethality among ‘untreated control flies’ and ‘drug-pretreated flies’ following exposure to an optimum PQ concentration for 48 h using the filter Disc method. Despite the wide usage of this test and usage of PQ as a model chemical to induce PD like symptoms in Drosophila, not many studies have addressed issues related to the biochemical consequences of PQ exposure in flies. More importantly the impact of PQ on mitochondrial functions in Drosophila has not been elucidated. In this context, the present study was conducted to examine the following aspects: i) the lethality response of flies to acute PQ exposure (48h) ii) oxidative stress induction in cytosol and mitochondrial oxidative dysfunctions following acute exposure and iii) the efficacy of BM prophylaxis to attenuate PQ induced lethality, oxidative dysfunction and neurotoxicity.

Initially, we established the lethality profile of PQ among adult flies following an acute exposure. PQ exposure caused concentration-dependent mortality at 72 hrs and the LC_{50} computed was 40 mM. These results are consistent with previously published toxicity values (Feany and Bender, 2000; Cooks-Weins and Grotewel, 2002; Bonilla et al., 2006; Chaudhary et al., 2007). The cellular pathology of PQ has been extensively investigated. PQ has been shown to undergo intracellular reduction to a free radical form, which then is reoxidized in the presence of oxygen to initiate an oxidative cascade that includes the generation of superoxide radicals that are converted by SOD to

hydrogen peroxide, which is then used as substrate for Catalase (Cocheme and Murphy, 2007). Our observation of elevated activities of both SOD and Catalase in whole body homogenates of flies exposed to PQ clearly suggests that flies were exposed to elevated oxidative stress *in vivo*. Further, evidences such as enhanced malondialdehyde and hydroperoxide levels (markers of oxidative stress) in mitochondrial fraction of PQ exposed flies also confirmed the induction of oxidative stress. These data corroborate with the recent findings wherein the cellular pathology of PQ in various PD- specific transgenic flies was reported (Chaudhary et al., 2007).

It is well known that, the mechanism of paraquat toxicity in dopaminergic neurons is most likely mediated *via* oxidative stress (Dinis-Oliveira et al., 2006). Paraquat is known to generate superoxide radicals by undergoing a NADH-dependent reduction to form a stable paraquat monocation radical that reacts very rapidly with oxygen to generate PQ^{2+} and superoxide radicals, which in turn produces hydrogen peroxide by either enzymatic or non-enzymatic dismutation reaction. Hence, any molecule or compound capable of blocking either superoxide radicals or hydrogen peroxide generation might have potential antioxidant activities. In recent times, various polyphenols have been shown to be neuroprotective in fly and rodent models (Ramaswamy, 2006; Jimenez-Del-Rio et al., 2010). Accumulation of free radicals and consequent neurodegeneration in specific brain regions has been proposed as causal factors in various neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Halliwell, 2006). This accumulation of oxidative free radicals is due to decreased activities of the free radical defense enzymes. Hence, a potential therapy which augments antioxidative defense system may prove beneficial under various neurodegenerative disease conditions. In view of this currently our laboratory is engaged in understanding the neuromodulatory role of phytochemicals such as *Bacopa monnieri* and *Centella asiatica* in Drosophila and mice model (Shinomol and Muralidhara, 2008, 2009, 2010; Shinomol et al., 2010; Hosamani and Muralidhara 2009, 2010a,b).

Human consumption of *Bacopa monnieri* is on the increase owing to its multiple beneficial effects (Anbarasi et al., 2005), which has been attributed mainly to the presence of characteristics saponins. In this regard, our laboratory

has been engaged in understanding the possible mechanism/s by which *Bacopa monnieri* offers protection against known neurotoxicant employing prepubertal mice(Shinomol, 2009; Shinomol and Muralidhara, 2010; Shinomol et al., 2010).

In a recent study, repeated exposure of *Drosophila* to sublethal doses of PQ was demonstrated to recapitulate the main symptomatic features of PD viz., a selective loss of dopaminergic neurons including locomotor defects (Chaudhuri et al., 2007). Based on these findings, we examined the neuroprotective efficacy of BM on PQ induced lethality, oxidative stress and mitochondrial dysfunction. In the present study, PQ at the exposed concentration induced a concentration related mortality and locomotor deficits in adult flies which are in agreement with previous reports (Botella, 2008). Although the precise mechanism of PQ action which leads to neurodegeneration is not well understood, PQ toxicity is attributed to specific sensitivity of dopaminergic neurons to reactive oxygen species and oxidative damage. Similar signs of oxidative damages have been detected frequently in dopaminergic neurons from PD patients, suggesting an implication of oxidative stress in this disease (Peng et al., 2005; Thiruchelvam et al., 2005: Chaudhuri et al., 2007). In a previous study, rotenone induced both locomotor deficits and Dopamine neuronal loss was attenuated by administration of Melatonin in *Drosophila,* suggesting the participation of oxidative stress mechanisms (Botella, 2008).

In the present study, PQ exposure caused significant diminution in the activity of ETC complexes (complex I-II and Complex II- III) clearly indicating mitochondrial oxidative stress. This is consistent with the hypothesis that interaction of PQ with mitochondria is an important component of its toxicity (Thiruchelvam et al., 2000; Cocheme and Murphy, 2008). Interestingly, among flies provided BM prophylaxis and PQ exposure, showed normal activity levels of both the enzymes suggesting the specific protective effect of BM in this PQ model. Although the precise mechanism/s by which BM rescues PQ mediated mitochondrial dysfunction is not clear, it is likely to be related to its antioxidative nature. Interestingly, the activity of both ETC enzymes was increased among BM only exposed flies which suggests a primary effect of BM. Further studies

are required to understand the precise mechanism/s by which bioactives of BM exert a specific effect on mitochondria. We have also obtained similar neuroprotective effects of BM prophylaxis in mitochondria of different brain regions of Rotenone administered prepubertal mice (Shinomol et al., 2010).

In conclusion, data obtained in the present study clearly indicate that Paraquat exposure at sub lethal concentration causes significant induction of early oxidative stress and mitochondrial dysfunctions among Drosophila which could be responsible for the subsequent development of neurotoxicity. More importantly, prophylactic treatment of flies with BM leaf powder for short duration has the propensity to modulate paraquat induced oxidative stress and neurotoxicity. BM Prophyalxis prevented the oxidative stress induction by PQ and restored the activities of ETC complexes, suggesting clearly its specific effects on the mitochondria. Although the precise mechanism/ s of action of BM needs further investigations, it may be related to its ability to enhance antioxidant defenses, and thus mitigate PQ-induced oxidative stress among flies.

6.0 SUMMARY

1. Adult flies exposed to paraquat (PQ) (10, 20, 30 and 40mM) (filter disc method) dose and time dependent mortality. The 48h LC 50 of PQ computed was 40mM.
2. Significant induction of oxidative stress was evident among adult flies exposed to PQ as evidenced by increased hydroperoxide levels, enhanced antioxidant enzymes (Catalase, SOD and GST) and redox status in cytosolic fraction.
3. PQ caused significant decrease in the activities of AChE and BChE enzymes at higher concentration indicating cholinergic dysfunction.
4. PQ exposure resulted in a marked concentration dependent elevation in lipid peroxidation, superoxide generation, enhanced GSH levels coupled with higher activities of Mn-SOD and GST suggesting that on going oxidative stress *in vivo* in mitochondrial fraction of adult flies. A further, increased free iron level indicates the possible role in the induction of oxidative stress.
5. Significant decrease in the activities of ETC enzymes viz., complex I-III and II-III, succinate dehydrogenase and Mg^{+2} ATPase coupled with altered MTT reduction clearly indicates mitochondrial dysfunction upon PQ exposure.
6. BM-enriched diet offered significant protection against PQ induced lethality as evidenced by lower incidence of mortality among BM prefed flies.
7. BM prophylaxis markedly attenuated PQ induced ROS generation, elevated levels of MDA, hydroperoxide and perturbations in enzymic antioxidant defenses.
8. BM prophylaxis offered significant protection against PQ induced attenuates in the activities of ETC and TCA cycle enzymes suggesting ability of BM to restore mitochondrial function.
9. Collectively these data suggest that PQ has propensity to induce early oxidative stress and associated mitochondrial dysfunctions, and BM prophylaxis has propensity to attenuate oxidative dysfunctions *in vivo*.

CHAPTER IV

Neuroprotective efficacy of *Bacopa monnieri* against paraquat intoxication: Validation in mice model

INTRODUCTION

In recent decades, the mechanisms, therapeutics targets and drug screening for PD are being studied in several model organisms including mice, flies, worms and yeast (Whitworth et al., 2006; Johnson and Cagan, 2010). Each of these systems has its pros and cons. For example, simpler eukaryotic-model systems offer potentially cheaper, faster and more powerful genetic approaches. To date, efforts to create genetic models of PD in simple systems have resulted in robust phenotypes. However, the relevance of some of these findings to PD, particularly those from evolutionarily distant model organisms such as yeast, has been challenged (Whitworth et al., 2006). By contrast, mouse system which offers most costly, time-consuming and genetic models of PD have largely failed to reproduce robust characteristics of PD (Whitworth et al., 2006). However, pharmacological studies in mice have contributed substantially to our understanding of the mechanisms responsible for PD. Perhaps greatest advantage of using mouse model is the striking similarity with human biology. Hence, findings in mice are potentially the most relevant to humans (Whitworth et al., 2006).

Earlier (Chapter 3) we demonstrated the neuroprotective efficacy of *Bacopa monnieri* against paraquat induced oxidative stress, neurotoxicity and associated mitochondrial dysfunctions in *Drosophila* model. In the present study, we set out to validate our findings in prepubertal mice model exposed to paraquat. The criteria for choosing prepubertal mice were the need and the importance of modulating adolescent neurotoxicity as emphasized in a recent review (Spear, 2007). In general 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 (Spear, 2000), there has been a growing interest in understanding whether this developmental transition is a vulnerable period for neurotoxicity.

Previous data from our laboratory has shown that dietary feeding of *Bacopa monnieri* to prepubertal mice modulates endogenous markers of

oxidative stress and neurotoxicity (Shinomol and Muralidhara, 2010). Further, the propensity of BM to modulate rotenone -induced oxidative stress, mitochondrial dysfunction and neurotoxicity was demonstrated (Shinomol, 2008). However, in this study the neuroprotective efficacy of BM was validated against paraquat (1,1'-dimethyl-4,4'-bipyridium) which is a widely used herbicide and a prototypic compound gaining increasingly importance as PD neurotoxin, known to exert its toxic effects *via* oxidative stress (Thiruchelvam et al., 2007; Bove et al., 2005). Systemic exposure of rodents to PQ has been shown to reproduce many of the pathological features of PD, including selective degeneration of dopaminergic neurons in the substantia nigra and presence of intracellular α-synuclein deposits (Manning-Bog et al., 2002; Thiruchelvam et al., 2003). Therefore, in recent years, PQ has become an increasingly popular model for studying the etiology of PD alone and also in combination with other environmental toxins (McCormack et al., 2002; Thiruchelvam et al., 2002).

Accordingly, in the current series of investigations, it was aimed to validate the data obtained in adult *Drosophila*. To achieve this, we have examined the neuroprotective efficacy of *Bacopa monnieri* against paraquat-induced oxidative stress, mitochondrial dysfunction and neurotoxicity in a prepubertal mice model intoxicated with PQ. Initial studies comprised of determination of acute toxicity of PQ in prepubertal mice and assessment of the potential of BM treatment on endogenous markers of oxidative stress in brain regions. Further, the propensity of BM prophylaxis to attenuate paraquat induced oxidative stress and mitochondrial dysfunctions in acute regimen and the efficacy of BM against chronic paraquat intoxication were investigated.

2.0 OBJECTIVE

The primary objective this investigation was to validate our findings in *Drosophila* in terms of the neuroprotective propensity of *Bacopa monnieri* in mice model. Two approaches viz., co-treatment and prophylactic protocol were employed to assess the modulatory effect of *Bacopa monnieri* against paraquat induced early oxidative stress and mitochondrial dysfunctions.

NEUROPROTECTIVE EFFICACY OF *BACOPA MONNIERI* AGAINST PARAQUAT INTOXICATION: VALIDATION IN MICE MODEL

3.0 EXPERIMENTAL DESIGN

Paraquat preparation

Paraquat was dissolved in double distilled water at a stock concentration of 5mg/ml was maintained in all the experiments.

I. <u>Paraquat (PQ) toxicity profile</u>

Acute toxicity profile

Groups of prepubertal mice (n=6) were administered with graded dosages of paraquat (10, 15 and 20mg/ kg bw i.p) and the incidence of mortality was recorded as and when it occurred during the experimental period of 7 days. The mortality data was expressed as percent mortality.

Chronic toxicity dose determination

In order to determine the chronic dose of PQ which would induce significant neurotoxicity and low or no mortality, prepubertal male mice were administered PQ in at selected dosages: 2.5, 5 and 10mg/kg bw, thrice a week for 4 weeks.

II. Effect of BM extract on endogenous markers of oxidative stress

Prepubertal male mice (n=6) were randomly assigned to control and treatment groups. Mice of both the groups were maintained on commercial pellet diet. Mice of treatment group were orally administered BM extract at a dosage of 200mg/kg bw/ d for 4 weeks. During the experimental period, known amount of diet was provided to the mice and the residual diet weighed in order to obtain the exact diet consumption by each group of mice. Body weights were recorded once a week. An interim sampling (after two weeks of feeding) was also carried out. Mice from control and BM groups were subjected to mild ether anesthesia and autopsied at the end of 4 weeks. Whole brain was excised and frozen immediately. The brain regions, cerebral cortex, cerebellum, hippocampus and striatum were subsequently dissected over ice. From each brain region cytosol and mitochondrial fractions were prepared and subjected to quantification of various biochemical parameters.

Determination of oxidative stress markers in brain regions

Status of oxidative damage was ascertained by measuring the extent of lipid peroxidation (quantified as malondialdehyde levels), generation of ROS (using dihydrodichlorofluorescein dye) and hydroperoxide levels (quantified using FOX reagent) in freshly prepared cytosolic fractions of the brain regions viz.,cortex, cerebellum, hippocampus and striatum.

Effect on mitochondrial functions

The activity of ETC enzymes viz., complex I-III and complex II-III were assayed in freshly prepared mitochondrial fractions of different brain regions. Similarly, the activity of citric acid cycle enzymes viz., succinate dehydrogenase SDH and MTT reduction were determined.

Effect on cholinergic functions

Studies were carried out to determine the effect of repeated BM administration on the activity of cholinergic enzymes such as AChE and BChE in cytosolic fractions of the brain regions.

III. Prophylactic neuroprotective efficacy of BM extract against acute PQ intoxication.

Dosages of paraquat and BM extract were selected on the basis of preliminary studies. Prepubertal male mice (4 wk old) were orally administered (200mg/kg bw) with BM extract for a period of 4 weeks (prophylaxis group). Both normal and mice given BM prophylaxis were challenged with an acute dose of PQ (i.p., 15mg/kg bw). Mice given physiological saline served as the normal controls. Both control and PQ challenged mice were sampled at the end of 48h. The induction of oxidative stress and mitochondrial dysfunctions were determined in cortex, cerebellum, hippocampus and striatum.

Ameliorative effect on oxidative markers

To assess the efficacy of BM prophylaxis against PQ-induced oxidative damage, biochemical assays viz., generation of ROS and lipid peroxidation (MDA levels) levels were carried out in all the regions of brain.

Ameliorative effect on the activities of ETC and TCA cycle enzymes

To assess the prophylactic effects of BM on paraquat –induced alterations in the activities of various ETC complexes, Complex I-III and Complex II-III were measured. SDH activity was measured to determine the extent of damage induced by paraquat and its amelioration by BM pretreatment. Further, MTT assay was done in mitochondria to determine the viability of mitochondrial dehydrogenase upon BM prophylaxis and challenged with paraquat.

Ameliorative effect on the striatal dopamine

The dopamine levels were quantified by HPLC-UVD (as described earlier) in striatal tissue to determine the ameliorative effect of BM extract on paraquat-induced perturbations.

IV. Ameliorative effect of BM extract on PQ induced oxidative stress in brain of prepubertal mice: Chronic paradigm

Dosages of paraquat and BM extract were selected on the basis of preliminary studies. For this study, prepubertal mice were randomly assigned into three groups. Three groups viz., Group I- control; group II- PQ (10mg/kg bw, i.p., thrice a week for 3wks), group III- PQ+BM (200mg/kg bw, oral for 3wks along with PQ). Body weight gain and food intake was monitored throughout the experimental period. At the end of 21 days, mice of all groups were sacrificed by cervical dislocation and brain was excised, blotted and weighed. Brain regions were dissected (cortex, cerebellum and striatum). Following biochemical investigations were carried out in both mitochondria and cytosol.

Effect on oxidative markers and activities of antioxidant enzymes

To determine the protective efficacy of BM extract on PQ-induced oxidative damage, biochemical assays viz., lipid peroxidation and hydroperoxide levels were carried out. Activity of selected antioxidant enzymes viz., catalase and glutathione-S-transferase (GST) were determined in cytosolic fractions of various brain regions.

Effect on SDH activity and MTT reduction

Activity of citric acid cycle enzymes were measured in terms of SDH activity. Further, MTT assay was done in mitochondria to determine the viability of mitochondrial dehydrogenase.

Effect on the activity of cholinergic enzymes

Studies were carried out to determine the effect of BM extract co-treatment on PQ-induced alteration in the activity of cholinergic enzymes (AChE and BChE) in cytosol of brain regions.

4.0 RESULTS

NEUROPROTECTIVE EFFICACY OF *BACOPA MONNIERI* AGAINST PARAQUAT INTOXICATION: VALIDATION IN MICE MODEL

I. Paraquat (PQ) toxicity profile

Administration of acute doses of PQ in prepubertal mice did not elicit any symptoms of poisoning at lower doses, and No mortality ensued at these doses during the experimental period **(Table 4.1)**. However, the highest dosage (20mg/kg bw) induced 50% mortality within 72h, while 100% mortality occurred at 96h. Hence the dosages of 10 and 15 mg/kg bw of PQ were used for further studies.

II. Effect of BM extract on endogenous levels of oxidative stress markers

Growth characteristics and food intake

No significant alterations were observed in daily food intake among mice given BM treatment. Further, no significant alterations in body weights and liver weights (data not shown) were noticeable among BM treated mice **(Table 4.2)**.

Determination of oxidative stress markers in brain regions

The brain regions of prepubertal mice administered BM for 4 wks showed significant decrease in endogenous levels of oxidative markers. Major changes consisted of significant decrease in ROS levels in striatum (26%), MDA levels in cortex (36%), cerebellum (28%) and striatum (31%). Further, significant and consistent diminution in hydroperoxide levels was observed in all the brain regions (cortex-21%; cerebellum-34%; hippocampus-40% and striatum- 35%) **(Table 4.3)**.

Effect on mitochondrial function

No significant change was evident in the activity of complex I-III among BM treated group in any of the regions except, in striatum where the activity was decreased (by 31%). Similarly the complex II-III activity was also diminished in

both hippocampus (21%) and striatum (37%). Significant decrease in SDH activity was observed in hippocampus (44%), while a significant (25%) increase in striatal region was evident among BM treated mice. BM group showed significant decrease in MTT activity in both cortex (33%) and cerebellar (50%) regions. However other regions showed no significant changes (**Table 4.4)**.

Effect cholinergic function

Significant decrease in acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evident in the brain regions of BM treated mice. A consistent reduction in the enzyme activity of AChE and BChE was observed in cortex (21-16%), cerebellum (21-19%) and hippocampus (19-16%) **(Table 4.5)**.

III. Prophylactic neuroprotective efficacy of BM extract against acute PQ intoxication.

Protective effect of BM prophylaxis on oxidative markers

In general PQ intoxication caused significant oxidative stress in specific brain regions of mice. ROS generation was significantly enhanced in cortex (32%), cerebellum (26%) and hippocampus (41%). In contrast, PQ challenged among 'mice given BM prophylaxis' failed to enhance the ROS levels in all the brain regions **(Figure 4.1A)**. Further, PQ intoxication among control mice caused enhanced MDA level only in hippocampus (21%). Interestingly, the MDA levels in hippocampus among BM-prophylaxis mice were normal. The endogenous MDA levels among BM only treated mice were significantly diminished (cortex-36%; cerebellum-28%; striatum-31%) **(Figure 4.1B)**

Protective effects of BM prophylaxis on the activities of ETC enzymes

Administration of PQ induced significant decrease in the activity of complex I-III in cortex (33%) and hippocampus (25%). Interestingly, among mice given BM-prophylaxis, PQ caused no appreciable alteration in the activity of complex I-III only in hippocampus. **(Figure 4.2A)**. Further, administration of PQ among *control mice* resulted in significant decrease in the activity of complex II-III in both hippocampus (22%) and striatum (37%).

Interestingly, with BM prophylaxis there was significant restoration in the activity in all regions **(Figure 4.2B)**.

Protective effects of BM prophylaxis on the activities of TCA cycle enzymes

Administration of PQ caused significant decline in SDH activity in hippocampus (56%) and striatum (50%). On the other hand, mice given BM-prophylaxis showed restored SDH activity partially in hippocampus (45%) and completely in striatum **(Figure 4.3A)**. Similarly, administration of PQ showed marked decrease in MTT reduction in cerebellum (27%) and hippocampus (34%). However, mice given BM-prophylaxis did not show any protection against MTT reduction **(Figure 4.3B)**.

Ameliorative effect on the striatal dopamine levels

Administration of PQ caused significant depletion in dopamine level in striatum (28%), interestingly, mice given BM-prophylaxis partially succeed to deplete striatal dopamine level indicating partially protection against PQ induced dopamine depletion (44% protection) in striatum **(Figure 4.4)**.

IV. Ameliorative effect of BM extract on PQ induced oxidative stress in brain of prepubertal mice: Chronic paradigm

Growth and food consumption

No significant changes were evident either in food intake or body weight gain among any of the treatment groups throughout the experiment period (Data not shown).

Modulatory effect on oxidative markers and activities of antioxidant enzymes

Administration of PQ to 'control mice' caused a significant induction of oxidative stress as evident by enhanced MDA levels in cerebellum (36%) and striatum (43%), although the levels in cortex were unchanged. Interestingly, PQ administration to 'BM-treated mice' failed to induce significant oxidative stress as the status of lipid peroxidation was normal in both regions **(Figure 4.5A)**.

Further, administration of PQ to 'control mice' caused marked elevation in hydroperoxide levels (48%) only in striatal region. In comparison hydroperoxide levels in striatum among 'BM-treated mice' were normalized **(Figure 4.5B)**.

Administration of PQ to 'control mice' resulted in marked elevation in the activity of catalase in cerebellum (42%) and striatum (67%). On the other hand, the activity of catalase among BM-treated mice was normal in both regions **(Figure 4.6A)**. Further Administration of PQ to 'control mice' resulted in marked Elevation in the activity levels of GST in cerebellum (38%) and striatum (86%). Interestingly, among BM-treated mice, PQ failed to induce any alteration in the activity of GST **(Figure 4.6B)**.

Ameliorative effect on SDH activity and MTT reduction

Administration of PQ to 'control mice' resulted in a significant decrease in SDH activity in cerebellum (29%) and striatum (24%), while the cortex showed normal activity. However, among BM-treated mice, PQ administration did not have any appreciable affect on SDH activity in cerebellum and striatum **(Figure 4.7A)**. In the MTT reduction assay PQ administration resulted in significant decrease only in striatal region among control mice. However striatum of BM-treated mice showed normal MTT reduction **(Figure 4.7 B)**.

Ameliorative effect activities on cholinergic function

Administration of PQ to control mice resulted in significant increase in AChE activity in striatal (59%) region only, where as among BM-treated mice, the enzyme activity was restored partially (33% protection) **(Figure 4.8 A)**. Likewise the activity of BChE in striatum (118%) of control mice was elevated on PQ administration. BM-treatment did not offer any significant protection as the activity levels was further increased in the striatum (171%) **(Figure 4.8 B)**.

Table 4.1

Mortality profile among prepubertal mice administered acute doses of paraquat

Dosage (mg/kg bw)	Incidence of mortality (%) at hrs			
	24	**48**	**72**	**96**
10	NM	NM	NM	NM
15	NM	NM	NM	NM
20	NM	16	50	100

***** Each treatment group consisted of 6 mice.

Table 4.2

Body weights of prepubertal male mice orally administered with *Bacopa monnieri* extract for 4 weeks.

Treatment	Body weights (g)		
	Week		
	0	**1**	**4**
Control	25.25 ± 1.5	26.47 ± 1.33	31.5 ± 1.0
BM	25.5 ± 1.0	27.02 ± 0.73	31.12 ± 1.65

BM: *Bacopa monnieri* (200mg/kg bw/d, oral for 4 wks).

Values are ± SE (n = 6). Data analyzed by student't' test.
No significant difference between control and treatment groups.

Table 4.3

Effect of *Bacopa monnieri* on endogenous oxidative markers in different brain regions of prepubertal mice

	Brain Regions			
	Cortex	**Cerebellum**	**Hippocampus**	**Striatum**
ROS[1]				
CTR	1.027±0.086	1.053±0.094	0.56±0.041	0.85±0.056
BM	0.94±0.075*	1.027±0.097	0.63±0.043	0.53±0.041*
MDA[2]				
CTR	24.56±1.80	34.91±2.10	34.1±2.20	76.5±6.10
BM	15.73±1.11*	25.23±2.01*	31.5±2.50	52.6±3.70*
HP[3]				
CTR	0.89±0.065	0.84±0.056	0.48±0.021	0.58±0.024
BM	0.70±0.056*	0.55±0.034*	0.29±0.018*	0.42±0.022*

BM: *Bacopa monnieri* (200mg/kg bw/d, oral for 4 wks)
ROS: Reactive oxygen species; MDA: Malondialdehyde; HP: Hydroperoxide

Values are mean ± SE (in triplicates). Data analyzed by one way ANOVA followed by 'Dunnet's' posthoc test (* $p < 0.05$).

[1] pmol DCF formed/min/mg protein
[2] p mol MDA/mg protein
[3] µmoles HP/mg protein

Table 4.4

Activities of various mitochondrial enzymes in brain regions of prepubertal male mice orally administered with *Bacopa monnieri* for 4wks

	Brain regions			
	Cortex	**Cerebellum**	**Hippocampus**	**Striatum**
Complex I-III [1]				
CTR	0.09±0.005	0.077±0.006	0.072±0.006	0.047±0.003
BM	0.08±0.004	0.067±0.005	0.050±0.005*	0.041±0.004
Complex II-III [2]				
CTR	0.065±0.005	0.060±0.004	0.056±0.005	0.057±0.005
BM	0.055±0.004	0.057±0.005	0.044±0.003	0.036±0.003*
SDH [3]				
CTR	7.1±0.62	5.0±0.45	9.0±0.75	3.0±0.28
BM	7.5±0.54	5.3±0.34	5.0±0.41*	4.1±0.34
MTT [4]				
CTR	29.1±1.8	34.0±3.1	21.97±2.1	10.36±0.98
BM	29.7±2.4	22.6±3.2	10.96±1.01	11.17±1.12

BM: *Bacopa monnieri* leaf powder (200mg/kg bw/d, oral for 4 wks).
Complex I-III: NADH cytochrome c reductase; Complex II-III: Succinate cytochrome c reductase; SDH: Succinate dehydrogenase; MTT: 3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide;

Values are mean ± SE (in triplicates). Data analyzed by one way ANOVA followed by 'Dunnet's' posthoc test (* $p < 0.05$).

[1, 2] ηmol/min/mg protein
[3] OD/mg protein
[4] OD/mg protein

Table 4.5

Effect of *Bacopa monnieri* on the activities of AChe and BChE in different brain regions of prepubertal mice

	Brain Regions			
	Cortex	**Cerebellum**	**Hippocampus**	**Striatum**
AChE [1]				
CTR	0.19±0.012	0.14±0.012	0.31±0.023	1.29±0.10
BM	0.15±0.012*	0.11±0.009*	0.25±0.019	1.26±0.10
BChE [2]				
CTR	0.018±0.001	0.021±0.002	0.019±0.001	0.028±0.002
BM	0.015±0.001*	0.017±0.001	0.016±0.001	0.002±0.002

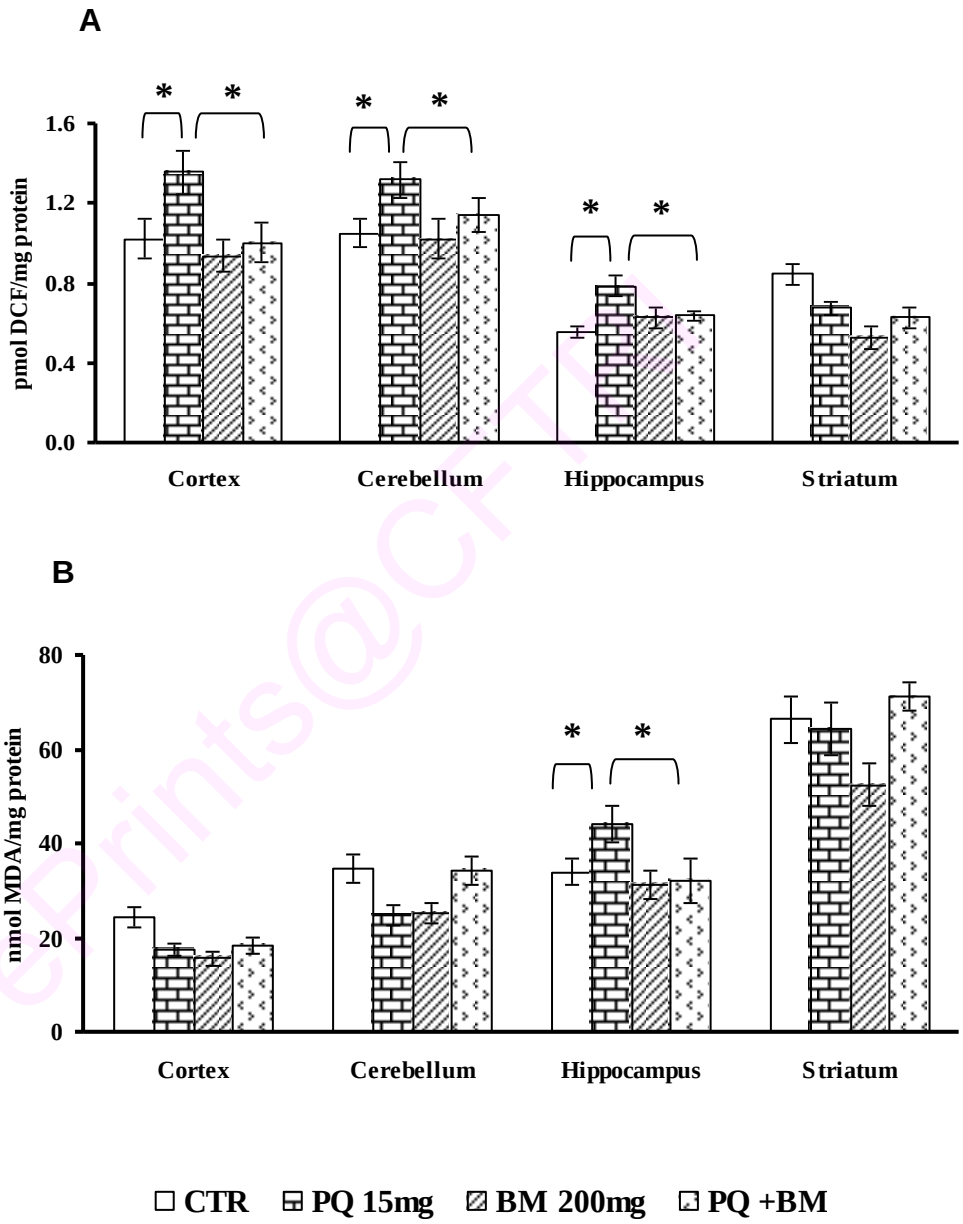
BM: *Bacopa monnieri* (200mg/kg bw/d, oral for 4 wks).
AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase:

Values are mean ± SE (in triplicates). Data analyzed by one way ANOVA followed by 'Dunnet's' posthoc test (* $p < 0.05$).

[1,2] ηmoles of substrate hydrolyzed/min/mg protein

Figure 4.1

Prophylactic neuroprotective efficacy of *Bacopa monnieri* against acute paraquat induced perturbations in oxidative stress markers viz., ROS (A) and MDA (B) in various brain regions of mice

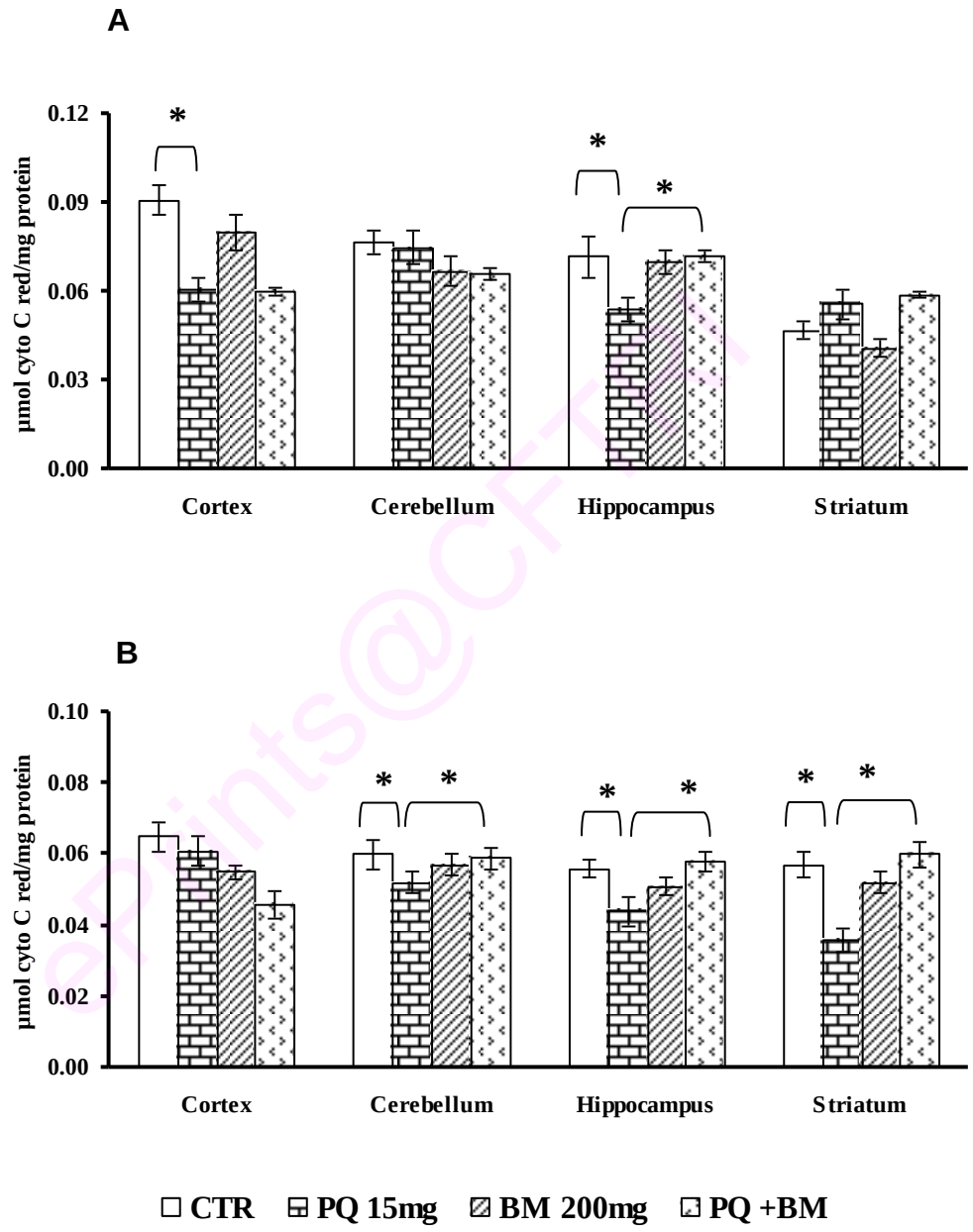


BM: *Bacopa monnieri* leaf powder (200mg/kg b.w/d for 4wks)
PQ: paraquat (15mg/kg bw, 48hrs)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$).

Figure 4.2

Modulatory potential of *Bacopa monnieri* on paraquat induced alterations in electron transport chain enzymes viz., complex I-III (A) and complex II-III (B) in brain regions of prepubertal mice

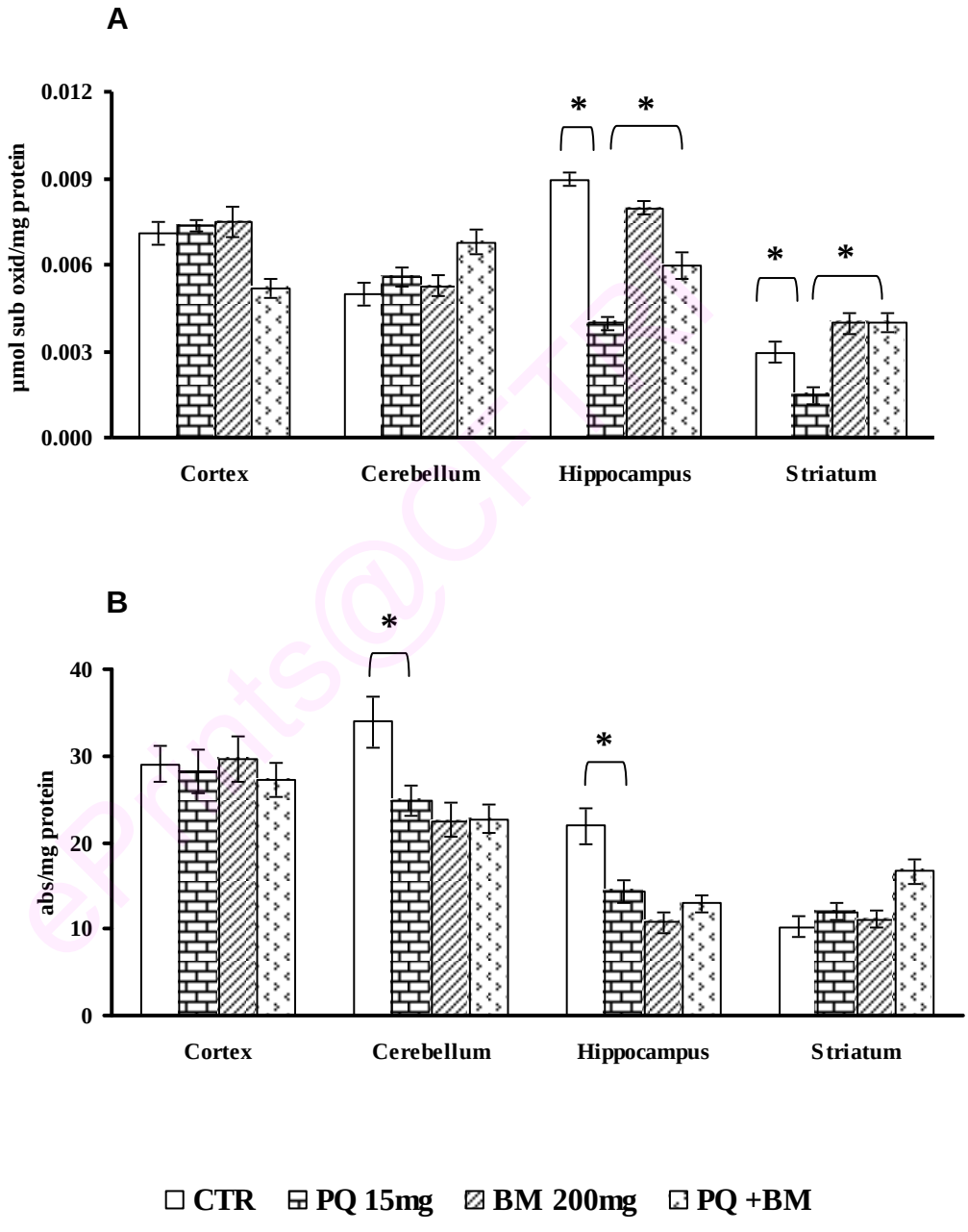


BM: *Bacopa monnieri* leaf powder (200mg/kg b.w/d for 4wks)
PQ: paraquat (15mg/kg bw, 48hrs)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$).

Figure 4.3

Effect of *Bacopa monnieri* prophylaxis on paraquat induced alterations in succinate dehydrogenase (A) and MTT assay (B) in brain regions of prepubertal mice

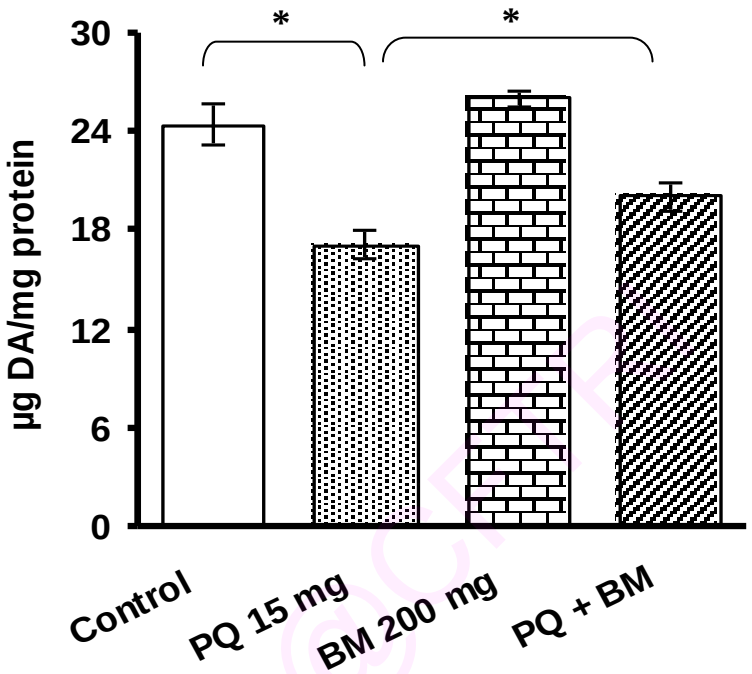


BM: *Bacopa monnieri* leaf powder (200mg/kg bw/d for 4wks)
PQ: paraquat (15mg/kg bw, 48hrs)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

Figure 4.4

Efficacy of *Bacopa monnieri* to modulate paraquat induced dopamine depletion in striatal region of mice brain

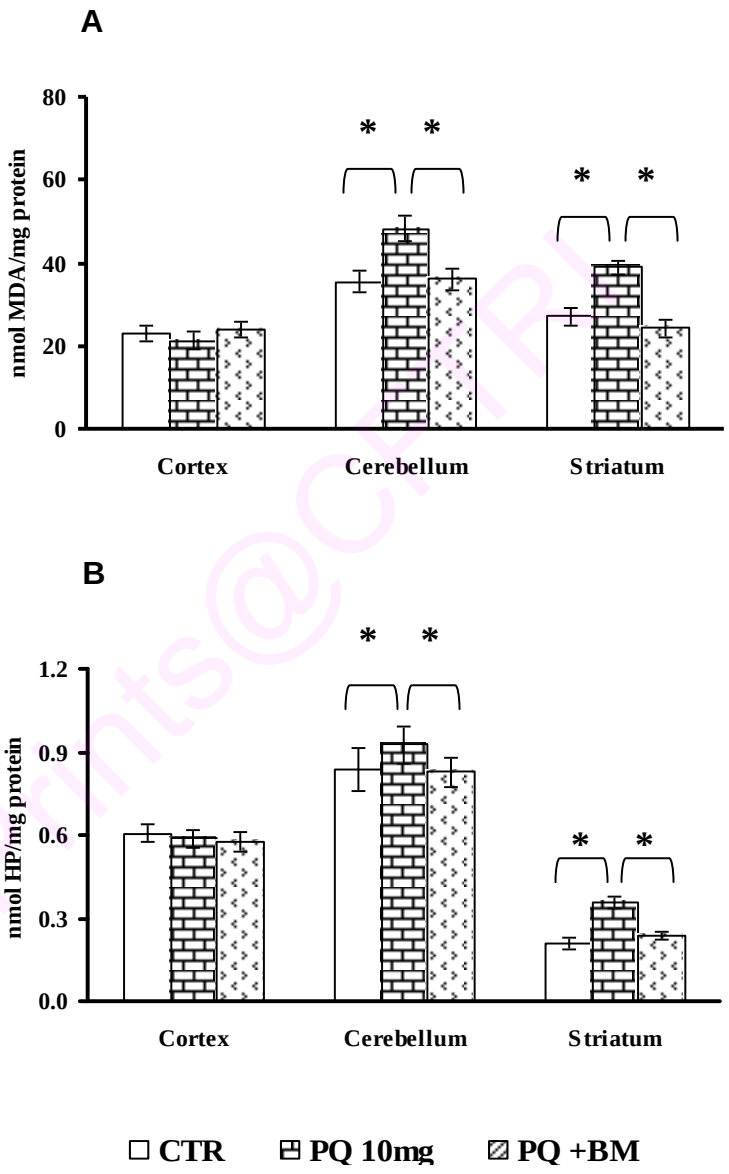


BM: *Bacopa monnieri* leaf powder (200mg/kg b.w/d for 4wks)
PQ: paraquat (15mg/kg bw, 48hrs)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

Figure 4.5

Ameliorative effect of *Bacopa monnieri* extract on paraquat induced alterations in oxidative stress markers viz., MDA (A) and hydroperoxide (B) levels in different brain regions of prepubertal mice

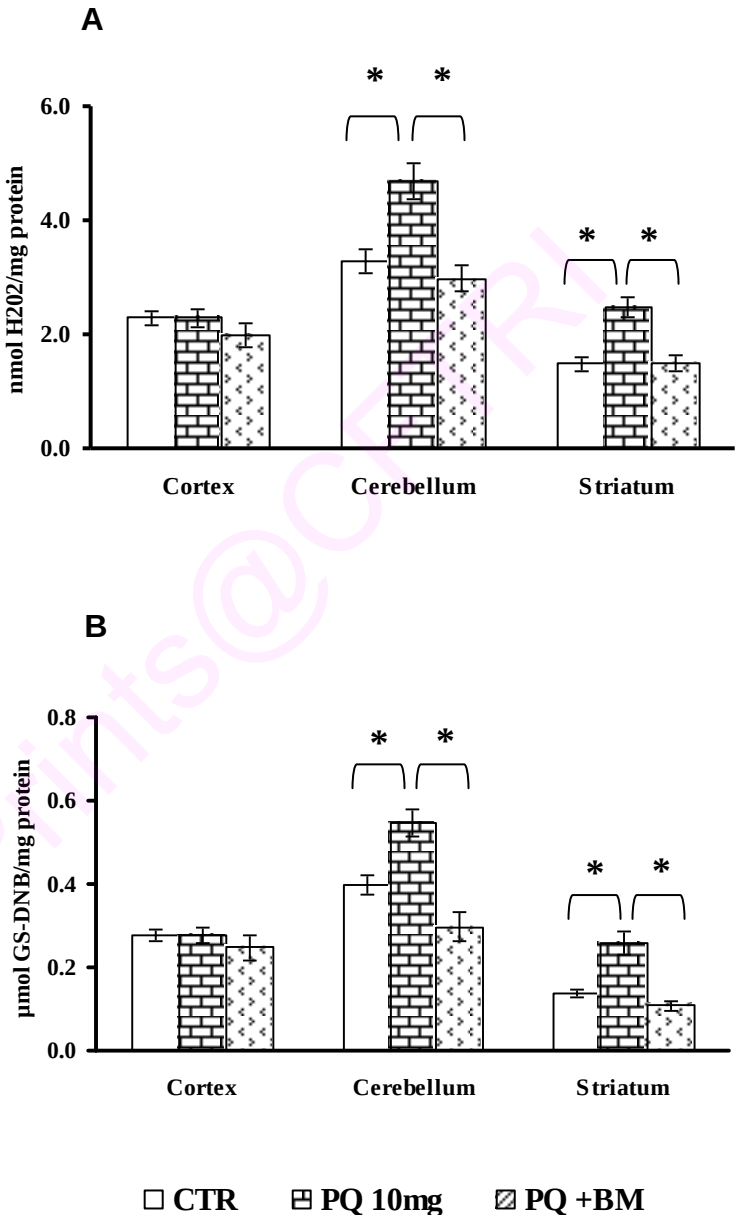


BM: *Bacopa monnieri* leaf powder (200mg/kg bw/d for 4wks)
PQ: paraquat (10mg/kg bw, i.p, thrice a wk for 4 wks)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

Figure 4.6

Ameliorative effect of *Bacopa monnieri* extract on paraquat induced alterations in the activities of catalase (A) and GST (B) enzymes in different brain regions of prepubertal mice

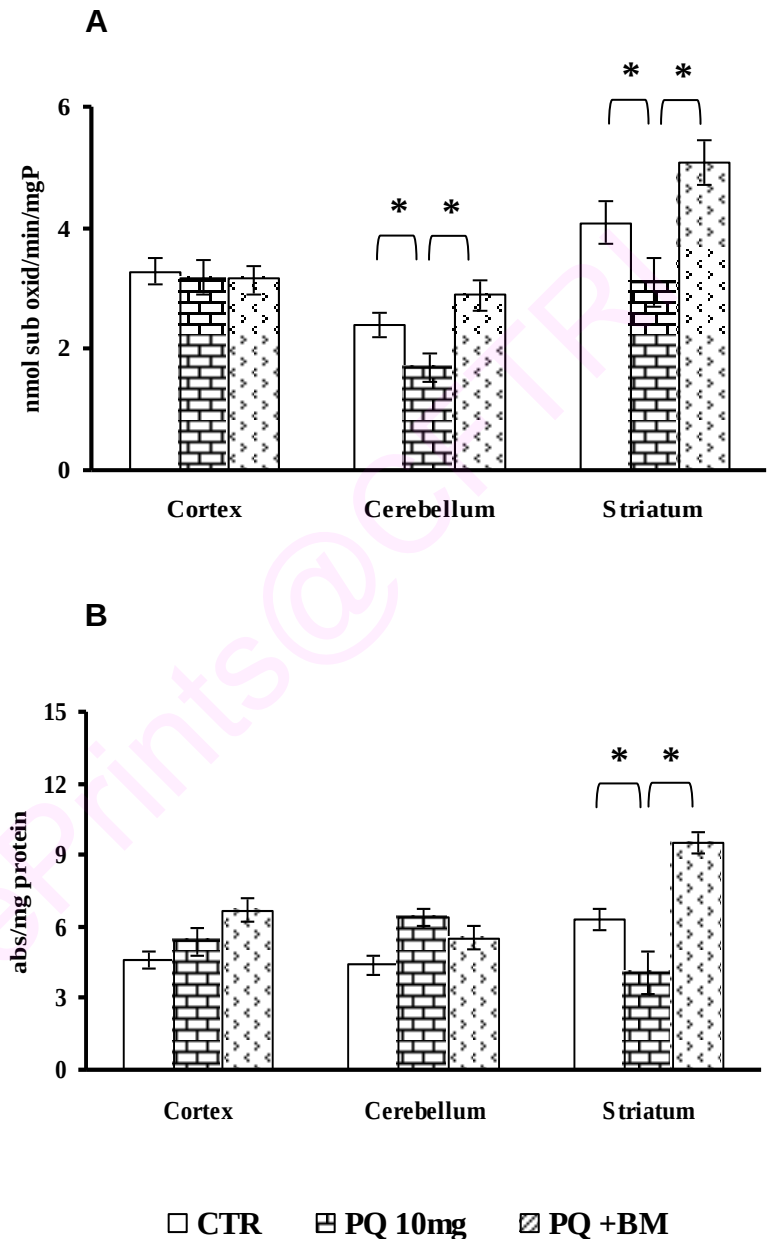


BM: *Bacopa monnieri* leaf powder (200mg/kg b.w/d for 4wks)
PQ: paraquat (10mg/kg bw, i.p, thrice a wk for 4 wks)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

Figure 4.7

Modulatory effect of *Bacopa monnieri* (BM) extract on the activity of succinate dehydrogenase (A) and MTT assay (B) in brain regions of prepubertal mice

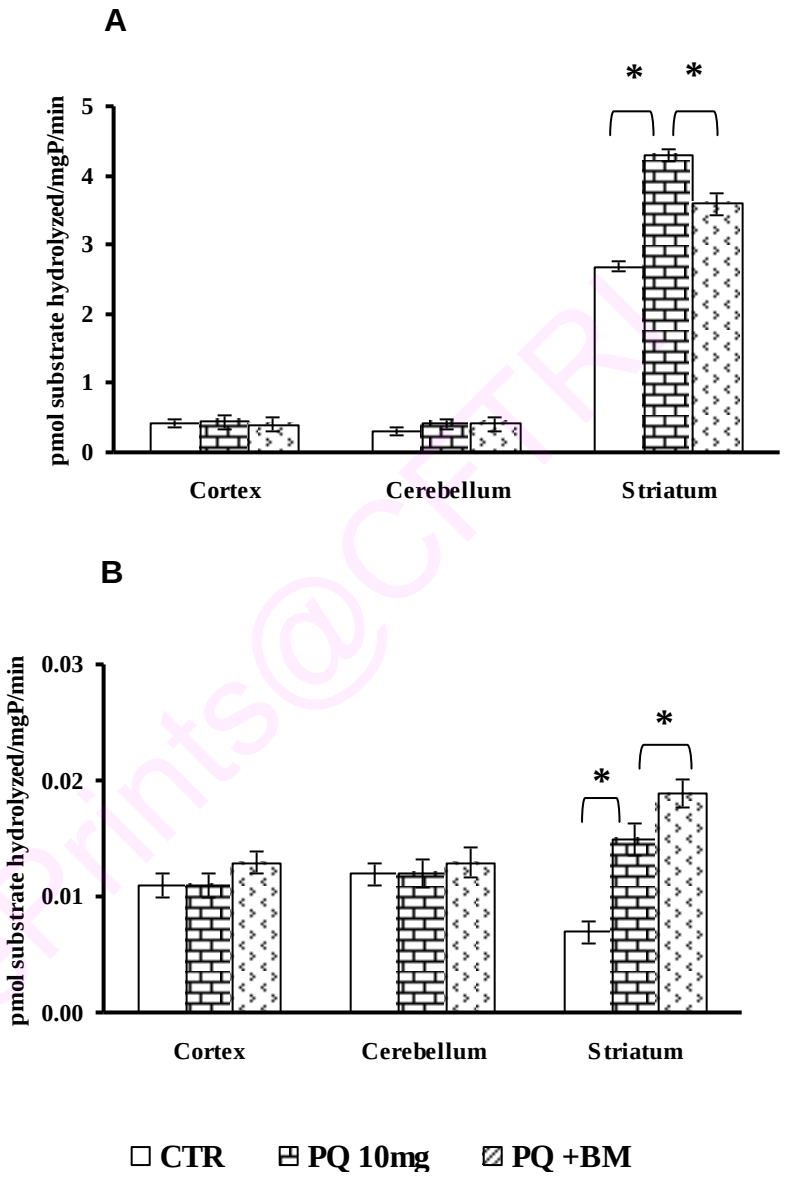


BM: *Bacopa monnieri* leaf powder (200mg/kg b.w/d for 4wks)
PQ: paraquat (10mg/kg bw, i.p, thrice a wk for 4 wks)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

Figure 4.8

Modulatory effect of *Bacopa monnieri* extract on paraquat induced alterations in the activities of AChE (A) and BChE (B) in brain regions of prepubertal mice



BM: *Bacopa monnieri* leaf powder (200mg/kg bw/d for 4wks)
PQ: paraquat (10mg/kg bw, i.p, thrice a wk for 4 wks)

Values are mean ± SE (in triplicates). Data analyzed by one-way ANOVA followed by post hoc 'Tukey' test. (* $p < 0.05$)

5.0 DISCUSSION

The primary objective of this study was to examine if neuroprotective effects of BM observed in the fly model can be reproduced in the mouse model *in vivo*. The relevance of using rodent models for assessment of neuroprotective ability of therapeutic agents subsequent to their screening in invertebrate models has been well emphasized. More importantly, the validation of findings in a mouse model would justify the use of *Drosophila* model to assess the neuroprotective efficacy of phytochemicals. Hence, to validate some of our findings on the neuroprotective efficacy of BM, paraquat was selected as a model chemical to induce oxidative stress, mitochondrial dysfunction and neurotoxicity in prepubertal mice. The compelling reasons for employing prepubertal mice for this study were: a) the brain in 4 week old mice is still in the process of development of new interneuronal connections and will continue during the postnatal development till the adult architecture is established by about 6 to 7 weeks (Spear, 2007). b) The prepubertal brain may be more responsive and c) The enhancement of memory and increased antioxidant levels are necessary in the growing stage.

Effects of BM extract on endogenous levels of oxidative stress markers

Initially, prepubertal mice were given daily oral supplementation of BM extract during a 4 week experimental period in order to assess its propensity to modulate the endogenous levels of oxidative stress markers in brain regions. Activities levels of antioxidant enzymes and redox status in different brain regions were also determined. At the tested dose, BM extract failed to affect the growth of mice and also did not have any significant effect on either body or liver weights. BM supplementation significantly diminished the basal levels of oxidative markers and the effect was differential in different brain regions. Although the MDA levels were consistently reduced in cortex, cerebellum and striatum, the ROS levels were diminished only in striatum. These data are consistent with our previous findings in prepubertal mice fed on BM-enriched diet (0.5 and 1.0% for 4 weeks) (Shinomol and Muralidhara, 2010). Dietary BM was shown to modulate both endogenous cytoplasmic and mitochondrial

markers in all brain regions which was accompanied with enhanced reduced GSH, thiols levels and elevated activities of antioxidant enzymes.

In the present study, the mitochondrial function measured in terms of the activities of complex enzymes revealed specific effect of BM in striatum. In the striatum, the activities of complex I-III and II-III were significantly lowered in mice fed BM, while hippocampus showed decreased complex II-III activity. Although the precise significance of these findings is not clear from the study, nevertheless the findings suggests that BM supplements were effective in reducing endogenous oxidative stress even in the mitochondria. This data corroborates with our earlier findings wherein dietary BM significantly enhanced the levels of both glutathione and thiol levels along with significant elevation in the activities of antioxidant enzymes GPx and SOD in cytosol and mitochondria of all the brain regions of prepubertal brain (Shinomol and Muralidhara, 2010). Hence, it was planned to study the protective effect of BM using both prophylaxis and co treatment protocol.

Prophylactic neuroprotective efficacy of BM extract: Acute PQ intoxication

To validate the neuroprotective efficacy of BM, we chose a mouse PQ model. PQ has been well demonstrated to preferentially kill dopaminergic neurons in SNpc in mice by various researchers. PQ also produces dopamine-mediated behavioral and biochemical changes in mice and rats (Di Monte et al., 2001; Thiruchelvam et al., 2003; Dinis-Olivera et al., 2006; Prasad et al., 2007). While the reasons for preferential dopaminergic neuronal loss is not well understood, a role for oxidative stress has been strongly emphasized based on several *in vitro* and *in vivo* evidences (Yumiko et al., 2002; McCormack et al., 2005; Prasad et al., 2007). Hence, we examined if BM prophylaxis renders neuroprotection effects against PQ-induced oxidative dysfunctions in the brain regions. The selection of PQ doses for acute as well as chronic treatment paradigm was based on our own preliminary findings and also published literature (Prasad et al., 2007). The dosage of BM extract and the duration of prophylaxis were also based on our preliminary findings.

With acute PQ administration, brain regions of untreated control mice (no prophylaxis) exhibited marked induction of oxidative stress measured at

48h post administration. This data on enhanced lipid peroxidation in different brain regions is consistent with previous reports of elevated ROS levels after single and repeated doses of PQ (McCormack et al., 2005; Thiruchelvam et al., 2005; Yang and Tiffany-Castiglioni et al., 2005). Interestingly, among mice given BM prophylaxis, PQ did not enhance the lipid peroxidation status in brain regions as measured by MDA and ROS levels, clearly suggesting its neuroprotective propensity. These findings are consistent with our earlier findings in which BM extract prophylaxis significantly protected prepubertal mice against 3-NPA induced early oxidative stress in striatum and other brain regions (Shinomol, 2008; Shinomol and Muralidhara, 2010).

PQ administration to untreated control mice (no prophylaxis) caused significant diminution in the activity of ETC complexes (Complex I-III and Complex II-III) clearly indicating mitochondrial oxidative stress. This is consistent with the hypothesis that interaction of PQ with mitochondria is an important component of its toxicity. Interestingly mice provided BM prophylaxis showed normal activities of both enzymes suggesting specific protective effect of BM in the PQ model. The principle effects of PQ on brain mitochondria among untreated control mice were: decreased activity of complex I-III in cortex and hippocampus, decline in SDH activity in hippocampus and striatum and MTT reduction in cerebellum/hippocampus. An increased ROS production in prepubertal mice induces mitochondrial permeability transition (Rizzuto et al., 2000). Further, it is also probable that the elevated hydrogen peroxide and ROS levels following PQ exposure may be the result of mitochondrial dysfunctions. Interestingly, among mice given BM prophylaxis the mitochondrial dysfunctions were not discernable indicating its potential to preserve the integrity of mitochondrial respiratory chain and thus energy metabolism. Although the precise mechanism/s by which BM rescues PQ mediated mitochondrial dysfunction is not clear, it is likely to be related to its antioxidative nature. Interestingly, the activities of both ETC enzymes were increased among BM *per se* treated mice which suggests the primary effect of BM. Further studies are required to understand the precise mechanism/s by which bioactives of BM exert specific effects on mitochondria.

Ameliorative effect of BM extract on chronic PQ intoxication

In this study, it was aimed to examine whether BM extract could ameliorate PQ induced oxidative stress and mitochondrial damage in a chronic treatment. Systemic administration of PQ has been employed by various workers to model PD in mice (Fernagut et al., 2007; McCormack et al., 2005). In the present model PQ at the administered dose (10mg/kg i.p., on alternate days for 4 wks) caused a significant increase in MDA and hydroperoxide levels clearly suggesting induction of oxidative stress in cerebellum and striatum. These observations are consistent with recent findings of differential induction of lipid peroxidation in ventral midbrain, striatum and frontal cortex of mice administered either single or multiple doses of PQ (Prasad et al., 2007). The persistence of PQ upto 28 days in brain regions was also demonstrated by these workers, although no specific reasons were ascribed.

Earlier, numerous workers have reported significant induction of oxidative stress in brain of different species of rodents (Di Monte et al., 2001; McCormack et al., 2005; Thiruchelvam et al., 2000, 2003, 2005). Enhanced ROS generation is well known to be potentially very damaging to the cells, leading 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 mitochondrial permeability transition pore (Cassarino and Benett, 1999; Cocheme and Murphy, 2008). The ability of PQ to induce superoxide reduction in mammalian mitochondria has been well demonstrated both *in vitro* and *in vivo* (Cocheme and Murphy, 2008). Interestingly daily supplements of BM extract offered in complete protection against PQ induced oxidative alterations in different brain regions. This data corroborates our earlier findings in which BM extract offered uniform and marked protection to prepubertal mice against rotenone – induced oxidative impairments in different brain regions such as cortex, cerebellum, hippocampus and striatum (Shinomol, 2008; Shinomol and Muralidhara, unpublished; Shinomol et al., 2010).

In the present study, there was significant increase in the activity of both AChE and BChE in striatal region of control mice (No BM treatment) administered PQ, clearly suggesting a specific effect of PQ on striatum. However, BM treatment significantly restored the activity levels of AChE (40% protection) in striatum. Although the reason for this specific effect is not clear, it may be related to the fact that striatal neurons are more susceptible to chronic PQ exposure and may be subjected to higher oxidative stress *in vivo*. BM treatment appears to protect striatal neurons of mice under toxicant induced oxidative stress. These findings are in agreement with earlier finding of our laboratory in which BM prophylaxis offered robust protection against the well known striatal toxicant, 3-NPA in *in vitro, in vivo* and *ex vivo* studies (Shinomol and Muralidhara, 2008; 2009; Shinomol et al., 2010).

Collectively, our findings in prepubertal mice clearly indicate that both BM prophylaxis and BM supplements significantly decreased the basal levels of several oxidative markers, increased thiol related antioxidant molecules and antioxidant enzymes suggesting its potential as a therapeutic antioxidant. Further, results obtained in the ameliorative study clearly demonstrate the neuroprotective efficacy of BM prophylaxis against PQ induced early oxidative stress and neurotoxicity. Findings in the mice model markedly corroborate our findings of the prophylactic neuroprotective property of BM extract in Drosophila. These *in vivo* evidences suggests that BM may be specifically employed as a therapeutic adjuvant in protecting the prepubertal brain against neurotoxicant associated oxidative insults and other NDD exhibiting elevated oxidative stress conditions.

6.0 SUMMARY

1. Administration (i.p) of PQ did not elicit any symptoms or mortality up to a dosage of 15mg/kg bw among prepubertal male mice.
2. Oral administration of BM extract (200mg/kg bw/d for 4 wks) resulted in significant diminution in the levels of endogenous oxidative markers (ROS, MDA and HP levels) in cytosolic fractions of different brain regions.
3. Differential effect was evident on the activity of ETC complex enzymes, while the activity of complex I-III was diminished only in striatum, the activity of complex II-III was reduced in hippocampus and striatum.
4. BM prophylaxis (for 4wks) markedly abrogated the induction of oxidative stress by acute PQ intoxication as evidenced by normalization of ROS levels in different brain regions (cortex/cerebellum/hippocampus), while the MDA levels were restored only in hippocampus.
5. BM prophylaxis significantly attenuated the activities of complex I-III in hippocampus and complex II-III in cerebellum/hippocampus/striatum Further SDH activity was also significantly protected.
6. The neuroprotective effects of BM against PQ intoxication were also discernable in a co-exposure chronic paradigm. BM treatment rendered significant protection against PQ induced oxidative stress as evidenced by normalized MDA/hydroperoxide levels coupled with restoration of activities of enzymic antioxidant defense in cerebellum and striatal region.
7. PQ induced diminution in the activity of SDH in cerebellum and striatum was completely restored by BM treatment, while decrease in MTT reduction was restored only in the striatum indicating a differential protective effect of BM.
8. BM treatment also offered significant protection against PQ induced perturbations in the activity of AChE only in the striatum.
9. Collectively, these findings in mice model clearly suggest that BM prophylaxis or BM treatment effectively rendered significant resistance to the brain of prepubertal mice against the neurotoxicant PQ. These findings are consistent with the findings in fly model of PQ intoxication.

CHAPTER V

Synergistic neuroprotective efficacy of *Bacopa monnieri* and creatine against rotenone in *Drosophila*

1.0 INTRODUCTION

In the quest to find pharmacologic treatments for human diseases, many combinatorial drug regimens are employed in the screening process, but in vivo validation of their efficacy remains very essential. Since NDD involve multiple cellular mechanisms, a combination of two or more potential compounds may yield additive or synergistic protective effects. Such attempts have been made recently in Drosophila system to treat Huntington's disease (Agarwal et al., 2005). Earlier several studies have demonstrated that combinatorial drug therapy was effective in the treatment of various human diseases such as cancers, AIDS and neurodegenerative diseases (Torrance et al., 2000; Johnson and Gerber, 2000; Zhang et al., 2003: Agrawal et al., 2005). For example combinations of compounds have been demonstrated to enhance antitumor activities leading to synergistic outcomes (Ikezoe et al., 2004). Additive neuroprotective effects have also been reported in a mouse model of ALS (Zhang et al., 2003). It is in this context the present study aimed to examine the possible synergistic neuroprotective efficacy of BM with a well known ergogenic nutritional supplement, creatine which is demonstrated to possess bio-energetic, anti-excitotoxic and antioxidant properties.

Creatine, a natural substance in the human body, is partly synthesized endogenously as well as ingested exogenously from food especially meat and fish. It plays a vital role in providing rapid energy during muscle contraction which involves the transfer of N-phosphoryl group from phosphorylcreatine (PCr) to ADP to regenerate ATP through a reversible reaction catalyzed by phosphorylcreatine kinase (PCK) (Gualano et al., 2010). Creatine, chiefly functions to transfer energy from mitochondria to cytosol. The PCr-PCK system serves as an energy buffer by connecting the mitochondrial sites of energy production with cytosolic sites of energy consumption in tissues with high energy demand such as brain and muscle (Wyss and Kaddurah, 2000). Of the various physiological functions assigned to the PCr-PCk system in *vivo*, prevention of oxidative stress *via* direct and indirect antioxidant action is one of the major roles (Greenhalf, 2001).

Currently creatine is widely used as an ergogenic nutritional supplement regularly by athletes to improve muscular performance (Froiland et al., 2004). Several studies have confirmed the beneficial effects of creatine supplementation in patients suffering from atrophy, muscle weakness, and metabolic dysfunctions. Recent investigations have revealed the potential therapeutic effects of creatine supplementation such as - improved brain function among young subjects and elderly people (Watanabe et al., 2002), attenuation of stress–induced cognitive impairment and reduction of mental fatigue. Several evidences indicate specific protective effects of creatine in various animal models of neurodegenerative diseases or ischemic stroke (Baker and Tornopolsky, 2003) (Bender et al., 2006; Bender et al., 2008). Clinical studies have demonstrated that creatine supplementation reduces oxidative stress (Hersch et al., 2006), glutamtate concentration(Bender et al., 2005) and improved clinical status in some patients with Huntington's disease (Tabrizi et al., 2005). Further several advances have been made employing creatine supplementation in patients with PD. This is basically due to the fact that exogenous creatine is able to cross the blood-brain barrier and increase the cerebral creatine concentration. Interestingly several neurological disorders are associated with redcued cerebral creatine content (Andres et al., 2008: Gualano et al., 2010). The neuroprotective effects of creatine has been ascribed to the buffering capacity of cellular ATP levels coupled with mitochondrial targeted antioxidant properties in cell and mammalian models (Andres et al., 2005; Lensman et al., 2006; Sestilli et al., 2006).

In this study, initially the ability of creatine to modulate endogenous levels of oxidative markers was assessed in the Drosophila system employing a dietary approach. Subsequently the neuroprotective action of creatine against rotenone induced mortality, mitochondrial oxidative stress and neurotoxicity in *Drosophila* were investigated in detail. Further, using a combination of BM extract and creatine, the possible synergistic effects against rotenone-induced oxidative stress and mitochondrial dysfunctions were also examined in the *Drosophila* model.

2.0 OBJECTIVE

The primary objective of this investigation was to establish the neuroprotective efficacy of creatine against rotenone-induced mitochondrial oxidative stress and neurotoxicity in the *Drosophila* system and examine the possible synergistic effects of creatine with Bacopa monnieri extract in the same model.

SYNERGISTIC PROTECTIVE EFFCTS OF *BACOPA MONNIERI* AND CREATINE AGAINST ROTENONE IN *DROSOPHILA*

3.0 EXPERIMENTAL DESIGN

I. Efficacy of creatine to modulate basal levels of oxidative stress

Creatine-enriched diet

Creatine was dissolved in double distilled water, Known concentration of 2, 5 and 10mM creatine was incorporated in 2ml semisolid diet.

Experimental protocol

Synchronized (8-10 days old) adult flies were allowed to feed on creatine-enriched diet (2, 5 and 10mM) for 7 days. Each group consisted of 50 flies, three such replicates were used. Flies were provided with fresh diet on alternative days. Terminally whole body homogenate was prepared and cytosolic fraction was used for various biochemical assays.

Effect of creatine on endogenous oxidative markers

Adult flies supplemented with creatine at 2, 5 & 10mM were homogenized and assayed for oxidative markers viz., MDA (using TBA) and hydroperoxide levels (FOX reagents). Similarly redox status measured in terms of reduced glutathione and thiols status as total thiols in whole body homogenates.

Effect of creatine on activities of antioxidant enzymes and thiol status

Effect of creatine supplementation on activities of selective antioxidant enzymes viz., catalase and superoxide dismutase were determined in whole body homogenates prepared from adult flies.

Effect of creatine on cholinergic function

The activities of acetylcholinesterase and butyrylcholinesterase were assayed among creatine fed flies as a marker of cholinergic function.

II. <u>Neuromodulatory effects of creatine in rotenone-model of neurotoxicity</u>

Modulation of rotenone-induced lethality

For mortality modulatory studies we selected only two concentration of creatine (5 & 10mM) against one concentration of rotenone (500 µM). Adult flies (n=50, triplicates) were exposed to diet containing both creatine and rotenone for 7 days (co-exposure protocol). Every, 24hrs mortality was counted till the end of the observation period.

Protective effects of creatine against rotenone-induced locomotor deficits

For locomotor assessment studies also we used same concentration of creatine (5 & 10mM) and rotenone (500µM) for 7 days. At the end of the experiment flies were quantified for locomotor deficits by visual counting method employing negative geotaxis assay. Data was expressed as percent flies escaped beyond minimum of 6cm in 60 seconds of interval. 20 adults per replication were used for each assay and the assay was repeated three times. The score for each replication was an average of three such trials for each group of flies including control.

Modulatory effect against rotenone-induced dopamine depletion

For this study only one concentration of creatine (10mM) was used. Homogenates from head and rest of the body region of adult flies co-exposed with creatine and rotenone were isolated separately. Further, dopamine (DA) depletion was quantified by using HPLC.

III. Protective effect of creatine against rotenone-induced mitochondrial oxidative dysfunctions

Synchronized (8-10 days old) adult flies were assigned to four groups viz., group I: control (untreated); group II: Creatine (10mM); group III: Rotenone (500µM) and group IV: creatine + rotenone (Co-exposure group, 10mM+500µM). Each group consisted of 50 flies, three such replicates were used. While group I flies were maintained on normal diet, flies of group II were fed with creatine-enriched diet. Flies of group III were exposed to rotenone alone in the diet, while flies of group IV were exposed to rotenone along with creatine. The duration of experimentation was 7 days and flies were provided with fresh respective diet on alternative days. Terminally whole flies were homogenized in sodium phosphate buffer 0.1M pH 7.0 and mitochondrial fraction was isolated by differential centrifugation. Following biochemical investigations were made.

Ameliorative effects on ROS generation and GSH levels

Whole body mitochondrial fraction was isolated from adult flies co-treated with creatine (10mM) and rotenone (500µM) for 7 days. Generation of reactive oxygen species (ROS) and reduced glutathione status were quantified by using DCF-DA and OPT respectively.

Ameliorative effect on the Mn-SOD activity

Since paraquat is known to induce superoxide generation, in the present study it is planned to understand mitochondrial Mn-SOD activity among flies co-treated with creatine and rotenone.

Ameliorative effect on the Nitric oxide levels

Effect of creatine on rotenone-induced nitrosative oxidative stress was measured in terms of nitric oxide levels by using greiss reagent.

Ameliorative effects on the activities of ETC enzymes

Activities of NADH-cytochrome C reductase (complex I-III) and succinate-C reductase (complex II-III) of electron transport chain enzymes were monitored for 3 min among flies supplemented with creatine, rotenone *per se* and in combination of both.

IV. Paraquat resistance bioassay

Creatine prophylaxis confers resistance against PQ toxicity

Adult flies maintained on creatine-enriched diet (10mM for 7 days) were challenged with PQ (20 and 40mM in 5% sucrose solution) for 48h using filter disc procedure. Mortality was recorded among both control (creatine untreated flies) and creatine pretreated flies at 24 and 48h. Before paraquat exposure, treated flies were starved for 3hrs to avoid the interference of diet and mortality was monitored for every 24hrs till the end of the experiment.

V. Synergistic effect of *BM* extract and creatine in the rotenone model

Experimental protocol

To examine whether a combination of *Bacopa monnieri* extract and creatine could result in synergistic protective effect, a separate study was conducted with lower concentrations of both modulants, the concentrations selected for the synergistic study were: BM-0.01% and creatine-2.5mM. Both the modulants given per se or alone in a co-exposure regimen offered only marginal protection against rotenone induced mortality. Hence these concentrations were selected for the determinative study.

Synchronized adult flies were assigned to following seven groups.

Group I: control (untreated); group II: Rot (500µM); group III: BM (0.01%);

Group IV: Creatine (2.5mM); group V: BM (0.01%) + Rot (500µM);

Group VI: Creatine (2.5mM) + Rot (500µM) and

Group VII: BM (0.01%) + Creatine (2.5mM) + Rot (500µM).

Each group consisted of 50 flies, three such replicates were used. While group I flies were maintained on normal diet, flies of other groups were fed with respective modulant enriched diet with or without rotenone. The duration of

experimentation was 7 days and flies were provided with respective fresh diet on alternative days. Using this protocol the following measurements were made.

Modulatory effect of BM and creatine on rotenone-induced lethality

Employing the above protocol the incidence of mortality was recorded daily among various treatments during an experimental period of 7 days.

Protective effects of BM and creatine against rotenone-induced locomotor deficits

In another study, to assess the effects of modulants either alone or in combination, with or without rotenone, flies of various groups were subjected to negative geotaxis assay as described earlier.

Biochemical measurements

Employing the above protocol terminally the following biochemical investigations were made in flies of various treatment groups as described earlier.

Ameliorative effect on ROS generation and GSH levels

Generation of reactive oxygen species (ROS) and reduced glutathione status were quantified by using DCF-DA and OPT respectively.

Ameliorative effect on the activities of Mn-SOD and complex I-III

Activities of Mn-SOD activity and NADH-cytochrome C reductase (complex I-III) and were monitored for 3 min.

4.0 REUSLTS

SYNERGISTIC PROTECTIVE EFFECTS OF *BACOPA MONNIERI* AND CREATINE AGAINST ROTENONE IN *DROSOPHILA*

I. Efficacy of creatine: Effects on basal markers of oxidative stress

A concentration related diminution in both MDA and hydroperoxide levels was observed in whole body homogenates of adult flies fed with creatine supplemented diet for 7 days. The reduction in MDA levels were more marked (17-44%) compared to the reduction in HP levels (17-21%). However, creatine did not have any effect on GSH and total thiols content in whole body homogenates **(Table 5.1)**.

Likewise, the activities of antioxidant enzymes *viz*., Catalase and Superoxide dismutase also showed no significant alterations. The activities of both acetylcholinesterase and butyrylcholinesterase enzymes among flies fed with creatine for 7 days remained unchanged at all creatine concentrations tested **(Table 5.2)**.

II. Protective effect of creatine against rotenone-induced neurotoxicity

Modulatory effect of creatine against rotenone induced lethality

Exposure of adult flies to rotenone resulted in a concentration dependent lethality over a 7d experimental period. Neurotoxicant induced deaths occurred between 4-7d and terminally the cumulative percent lethality was: 250µM- 14%; 500µM -55%; 1000µM -78%. In order to examine the modulatory effect of creatine, we used only one concentration of rotenone (500µM) and two concentrations of creatine. Interestingly, co–exposure of flies with creatine (5 and 10mM) resulted in lower incidence of mortality among rotenone exposed flies. The degree of protection was robust (74% and 52%) **(Figure 5.1A)** indicating its ability to protect against rotenone induced lethality.

Creatine protects rotenone-induced locomotor deficits

Data obtained in the negative geotaxis assay in flies exposed previously to rotenone for 7 d revealed concentration dependent locomotor dysfunctions. At higher concentrations of rotenone, more number of flies had a tendency to stay at the bottom of vial. Among untreated controls, more than 94% flies were able to reach at the top of the vial within a minute, while rotenone exposed flies exhibited significant decrease in climbing ability with increasing concentration of rotenone (250µM, 44%; 500µM, 19% and1000µM, 15%) clearly suggesting the induction of locomotor deficits. In a parallel experiment, we also measured the modulatory effect of creatine on rotenone induced locomotor deficits in a similar paradigm. Rotenone treated flies exhibited severe locomotor impairments, while co-exposure with creatine significantly improved (35% and 45%) the performances of flies **(Figure 5.1B)** in the negative geotaxis test. In general, co-exposed flies appeared to be more active than rotenone alone treated flies. Further, creatine at both the tested concentration *per se* had no significant effect on locomotor behavior among flies.

Modulatory effect against rotenone-induced dopamine (DA) depletion

Creatine alone treatment did not significantly affect the DA levels measured either in head or body homogenates of flies. In contrast, rotenone caused robust DA depletion in both the regions (head, 41%; body region, 70%). Interestingly, the degree of rotenone induced DA depletion was less robust among flies co-exposed to creatine. Restoration of DA levels affected by creatine treatment was 50% in head and 28% in rest of the body region of flies **(Figure 5.2)**.

III. <u>Protective effect of creatine against rotenone-induced mitochondrial oxidative dysfunctions</u>

Ameliorative effect on ROS generation and GSH levels

Flies fed with creatine *per se* for 7d showed marginal (14%) decrease in endogenous ROS levels. On exposure to rotenone, mitochondria obtained from whole body homogenate of flies revealed marked (43%) increase in ROS

levels. Interestingly, co-exposure with creatine completely abrogated the rotenone induced

ROS generation as revealed by near normal levels of ROS (nearly 90% protection) **(Figure 5.3A)**. While rotenone only treated flies showed significant (31%) depletion of GSH levels, flies co exposed to creatine exhibited normal GSH levels. Further, GSH levels among creatine *per se* treated flies remained unaltered **(Figure 5.3B)**.

Ameliorative effect on the activity of Mn-SOD

Creatine treatment *per se* did not alter the activity levels of Mn-SOD enzyme measured in mitochondria. However, rotenone alone treated flies showed elevated (by 19%) SOD activity. Surprisingly, co-exposed (rotenone + creatine) flies showed normalized activity compared to rotenone treated group **(Figure 5.4A)**.

Ameliorative effect on nitric oxide (NO) levels

Flies exposed to creatine (10mM) *per se* showed significantly decreased endogenous nitric oxide levels (by 33%). However, rotenone exposure resulted in significant increase in nitric oxide levels (29%). Interestingly, creatine supplementation resulted in complete restoration of NO to normal levels **(Figure 5.4B)**.

Ameliorative effect on the activities of ETC enzymes

Creatine *per se* fed flies showed no change in the complex I-III activity compared to control. However, significant inhibition (29%) was evident among rotenone only treated flies. Interestingly, the activity levels of complex I-III were completely restored among creatine co-exposed flies **(Figure 5.5A)**. Further, creatine per *se* fed flies showed no change in the activity level of complex II-III enzyme. Furthermore, rotenone *per se* and co-treatment with creatine also failed to modulate the activity level of complex II-III **(Figure 5.5B)**.

IV. Paraquat resistance bioassay

Creatine prophylaxis confers resistance against paraquat toxicity

Initially, we determined the LC_{50} concentration of paraquat among adult flies. At 24hrs in both the tested concentration no significant mortality was recorded. However, significant mortality was evident at 48hrs and 72hrs. The incidence of mortality was: 15, 42% at 48hrs and 39 & 95% at 72hrs at both the concentration respectively. Interestingly, creatine supplemented flies were able to survive for longer time compared to rotenone alone treatment at both observation times. The percent protection at 48hrs was - 33 & 52% and at 72hrs; 49 & 47% at both 20 and 40mM of PQ respectively **(Figure 5.6).**

V. Synergistic effect of BM extract and creatine in rotenone model

Modulatory effect of BM + Creatine against rotenone induced lethality

Both the modulants at the tested concentration when given alone did not induce any mortality among flies. Terminally, among rotenone *per se* group significant (65%) mortality occurred. Co-exposure of flies with either BM or creatine along with rotenone resulted in lower incidence of mortality (~40%). The percent protection offered by each of the modulants was nearly 30%. Interestingly the modulants in combination provided robust protection against rotenone induced lethality clearly indicating synergistic interaction of the modulant **(Figure 5.7A)**.

Protective effect of BM + Creatine against rotenone-induced locomotor deficits

Both the modulants at the tested concentrations when given alone did not have any effect on fly movement. However, flies exposed to rotenone *per se* as anticipated exhibited severe locomotor deficits, Co-exposure of flies with either BM or creatine along with rotenone resulted in marginal improvement in the performances of flies in the negative geotaxis test. Interestingly, flies among synergistic group (BM+Creatine) showed robust recovery against rotenone induced locomotor deficits as evidenced by marked improvement in the climbing behavior and scored better in the negative geotaxis assay **(Figure 5.7B)**.

Ameliorative effect on ROS generation and GSH levels

On exposure to rotenone, mitochondria obtained from whole body homogenate of flies revealed marked (47%) increase in ROS levels, while co-exposure with BM/Creatine separately offered significant protection (BM, 49% and Creatine, 60%). Interestingly, flies among synergistic group (BM+Creatine) showed a complete attenuation against rotenone induced ROS generation as revealed by normalized ROS levels **(Figure 5.8A)**.

Similarly, rotenone only treated flies showed significant (40%) depletion of GSH levels, while, flies co exposed to BM/Creatine separately offered significant restoration of GSH (BM, 50%; Creatine, 40%). However, among flies fed combination of BM and Creatine (synergistic group, BM+Creatine) showed complete restoration of GSH, indicating complete protection against rotenone induced GSH depletion **(Figure 5.8B).**

Ameliorative effect on Complex I-III and Mn-SOD activty

Significant inhibition (35%) of complex I-III was evident among rotenone only treated flies, while the activity levels of complex I-III were moderately restored among BM/Creatine co-exposed flies (BM, 62.8%; Creatine: 46% protection). Interestingly, flies among synergistic group (BM+Creatine) exhibited complete restoration of complex I-III activity **(Figure 5.9A)**.

Rotenone alone treated flies showed elevated (by 37%) SOD activity, while co-exposed flies with BM/Creatine showed normalized activity compared to rotenone treated group. Surprisingly, flies among synergistic group (BM+Creatine) also showed restored activity of SOD **(Figure 5.9B)**.

Table 5.1

Effect of dietary creatine supplementation on endogenous markers of oxidative stress and redox status in adult *Drosophila melanogaster*

Parameters	***Creatine concentration (mM)***			
	0	***2***	***5***	***10***
Oxidative markers				
Malondialdehyde[1]	3.51± 0.32	2.91±0.23	2.26±0.11*	1.98±0.36*
Hydroperoxide[2]	0.52±0.08	0.42±0.009	0.43±0.019*	0.41±0.062*
Redox status				
Glutathione[3]	31.33±0.89	34.34±2.14	36.09±1.02	32.30±2.27
Total thiols[4]	0.07±0.005	0.064±0.004	0.076±0.003	0.067±0.006

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Tukey' test (* $p<0.05$)

[1]ηmol MDA/mg protein
[2]ηmol HP/mg protein
[3]µg GSH/mg protein
[4]mmol DTNB/min/mg protein

Table 5. 2

Effect of dietary creatine supplementation on the activities of antioxidant enzymes and cholinergic enzymes in adult *Drosophila melanogaster*

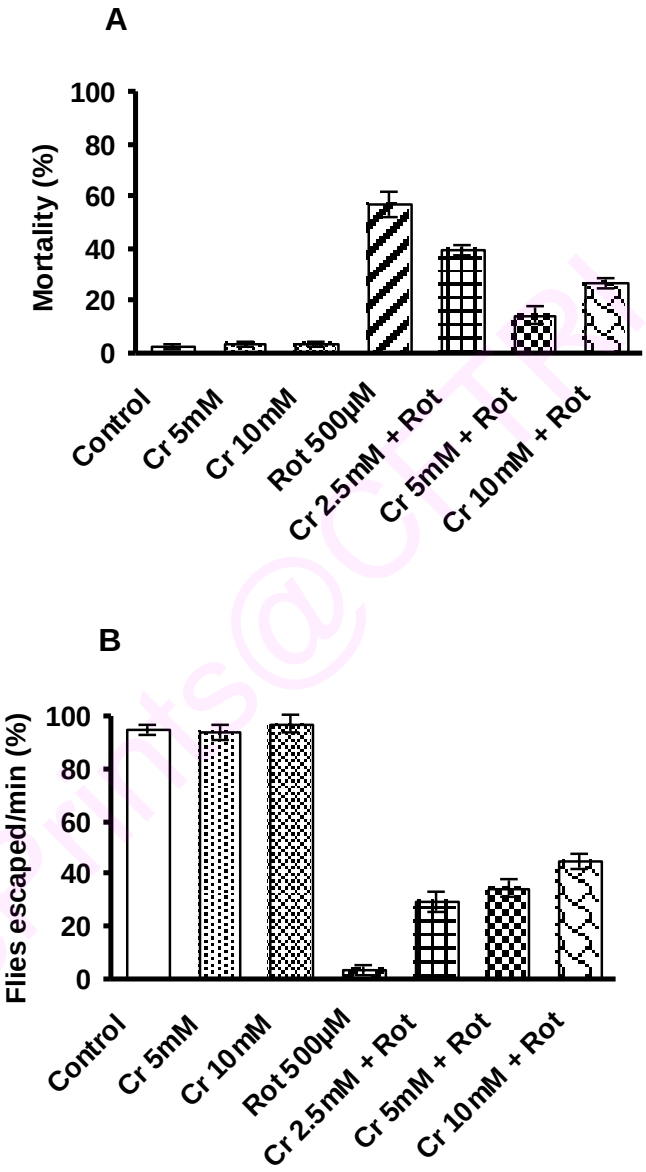
Parameters	***Creatine (mM)***			
	0	***2***	***5***	***10***
Antioxidant enzymes				
Catalase[1]	0.174±0.01	0.178±0.003	0.171±0.01	0.136±0.005
SOD [2]	204.2±1.73	244.1±18.2	253.2±38.0	238.2±28.3
Cholinergic enzymes				
AChE [3]	0.080±0.001	0.084±0.005	0.083±0.003	0.076±0.009
BChE[4]	0.041±0.002	0.041±0.002	0.043±0.002	0.038±0.004

Values are mean ±SE (Three sets of flies). Data analyzed by one way ANOVA followed by post hoc 'Tukey' test (* $p<0.05$)

[1]µmol H_2O_2 hydrolyzed/min/mg protein
[2]Units /min/mg protein
[3]ρmol DTNB /min/ mg protein

Figure 5.1

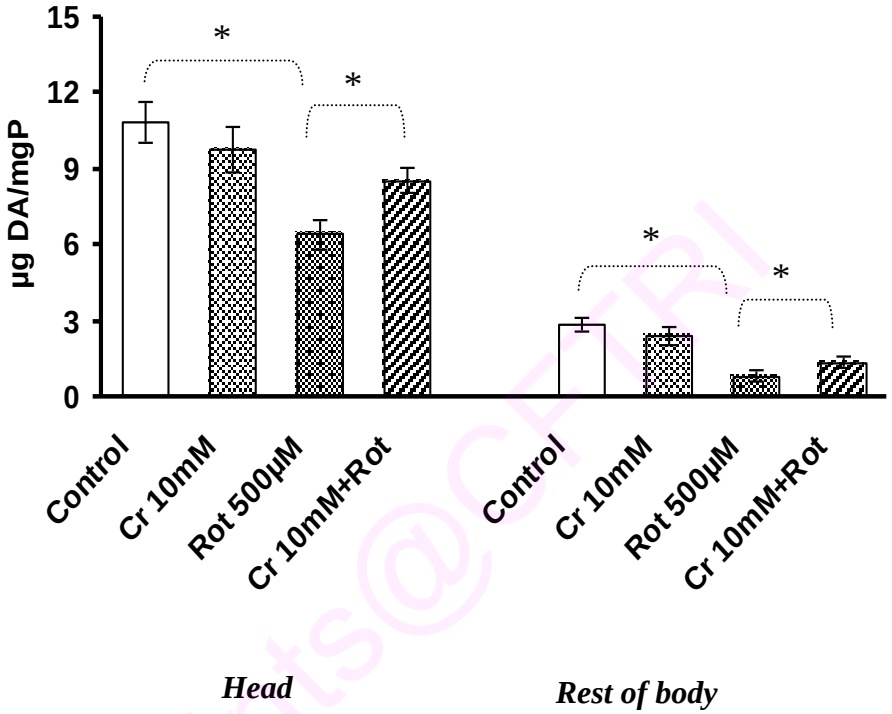
Modulation of Rotenone (500µM) induced mortality (A) and locomotor deficits (B) among flies given creatine supplementation



Rot: Rotenone (500µM); Cr: Creatine (2.5, 5, 10mM)
n=50 flies per replicate, three such replication used for assay

Figure 5.2

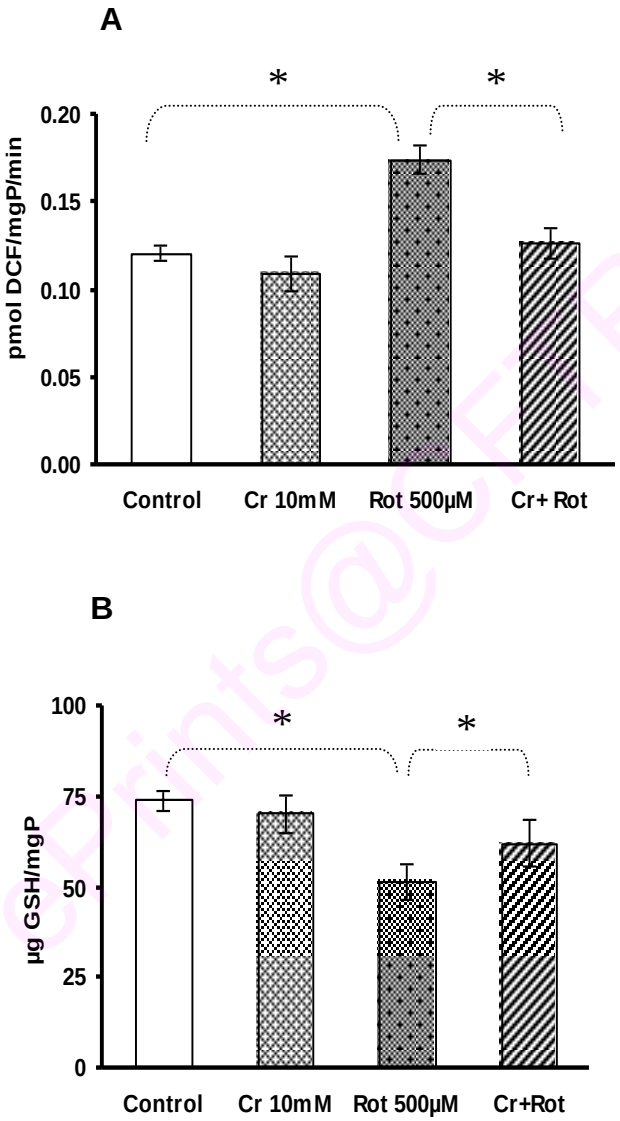
Modulatory effect of creatine (Cr) supplementation on Rotenone (Rot) - induced dopamine depletion among *Drosophila melanogaster*



Rot: Rotenone (500µM); Cr: Creatine (10mM)
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control v/s Cr, Rot; Rot v/s Rot + Cr.
n=50 flies per replicate, three such replication used for assay

Figure 5.3

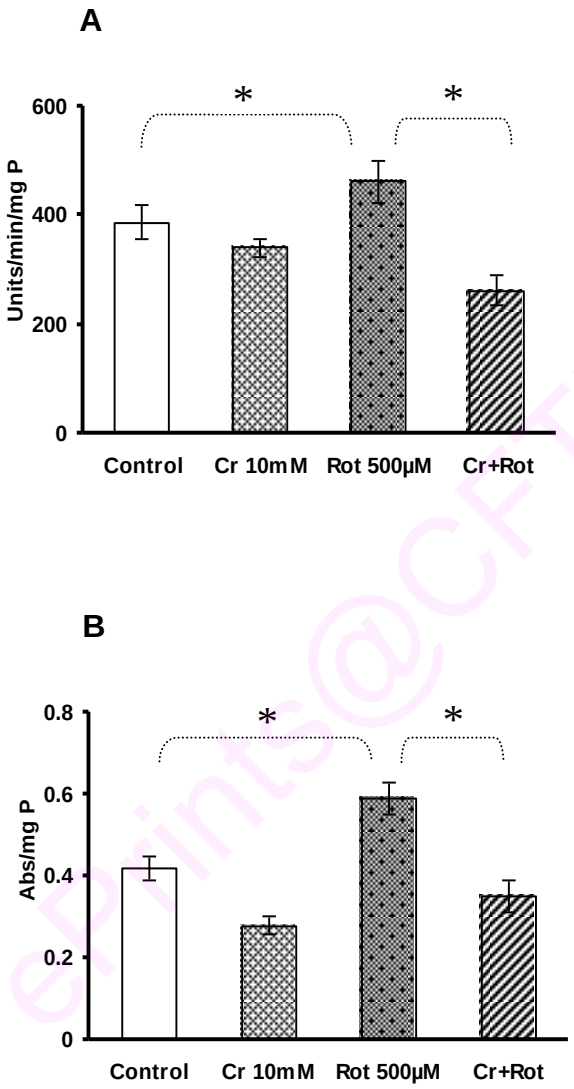
Modulatory effect of creatine supplementation on rotenone-induced oxidative stress measured as reactive oxygen species (ROS) (A) and glutathione (GSH) (B) levels in whole body mitochondria of *Drosophila*



Rot: Rotenone (500µM); Cr: Creatine (10mM)
Values are mean ± SE. Data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significance was determined by making comparisons between control v/s Cr, Rot; Rot v/s Rot + Cr.
n=50 flies per replicate, three such replication used for assay

Figure 5.4

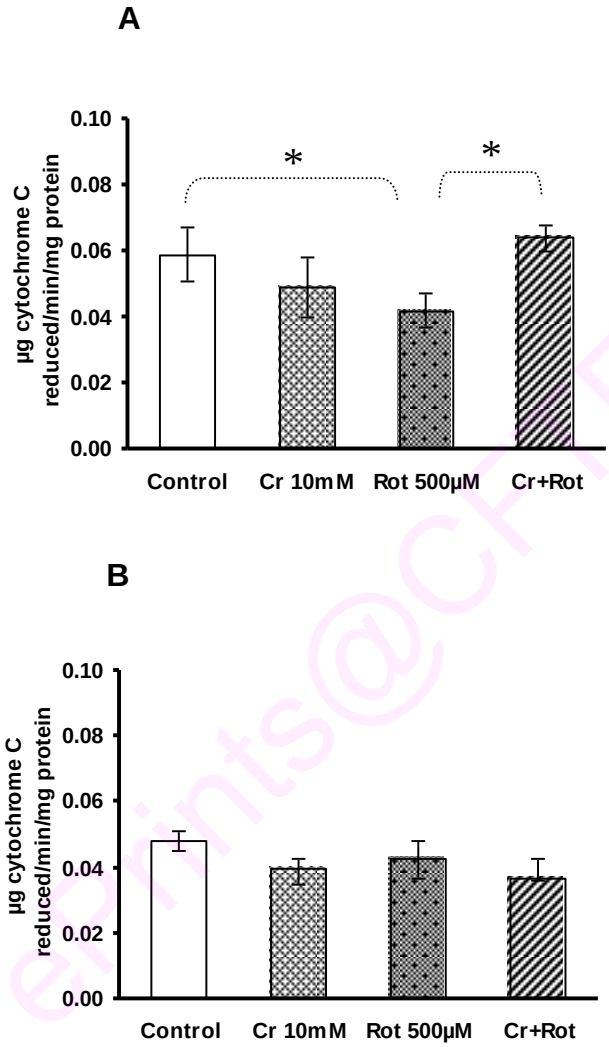
Modulatory effect of creatine supplementation on mitochondrial superoxide dismutase activity (Mn-SOD) (A) and nitric oxide (NO) levels (B) in whole body mitochondria of *Drosophila* exposed to rotenone



Rot: Rotenone (500µM); Cr: Creatine (10mM)
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control v/s Cr, Rot; Rot v/s Rot + Cr.
n=50 flies per replicate, three such replication used for assay

Figure 5.5

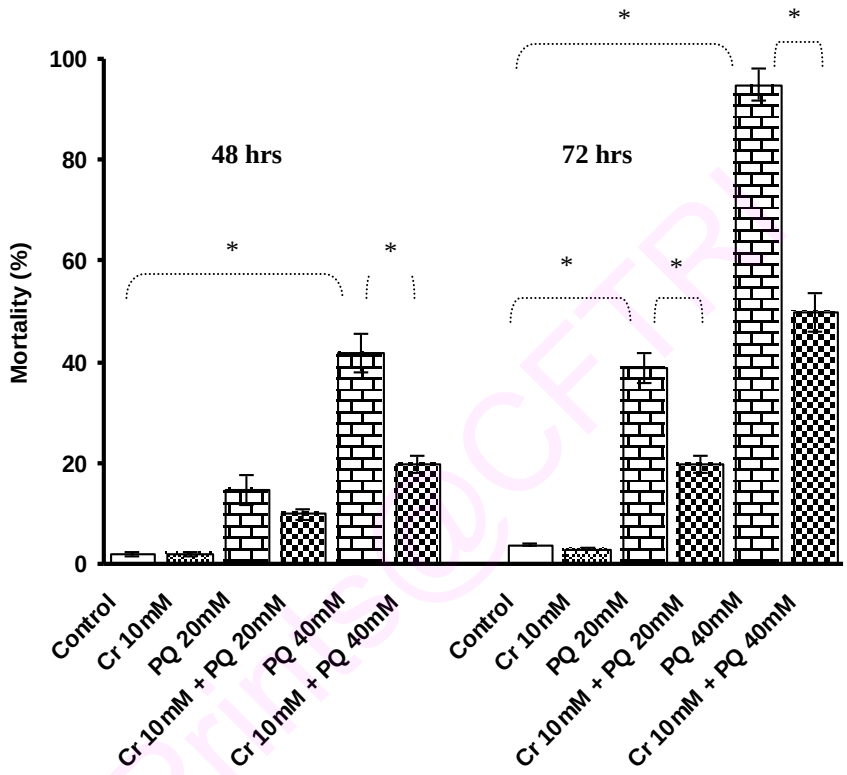
Modulatory effect of creatine supplementation on the activities of complex I-III (A) and Complex II-III (B) in whole body mitochondrial fractions of *Drosophila* exposed to Rotenone



Rot: Rotenone (500µM); Cr: Creatine (10mM)
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control v/s Cr, Rot; Rot v/s Rot + Cr.
n=50 flies per replicate, three such replication used for assay

Figure 5.6

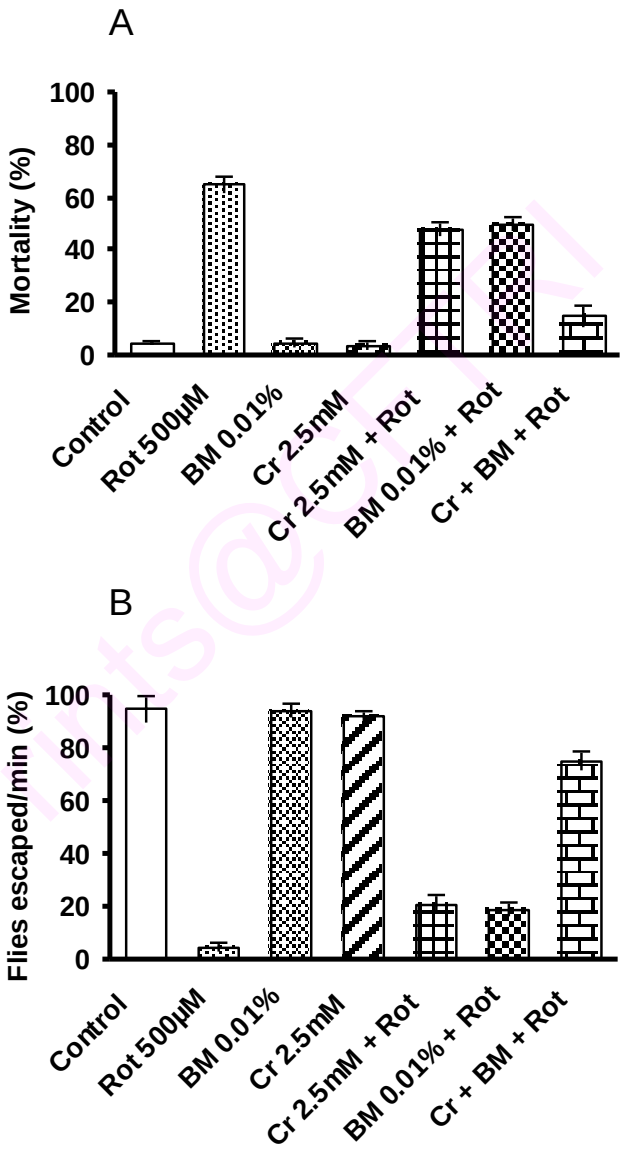
Modulatory effect of creatine prophylaxis against paraquat-induced mortality response in *Drosophila melanogaster*



PQ: Paraquat (20, 40mM); Cr: Creatine (10mM)
Values are mean ± SE (in triplicates), data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significances were determined by making comparisons between control v/s Cr, PQ; PQ v/s PQ + Cr.
n=50 flies per replicate, three such replication used for assay

Figure 5.7

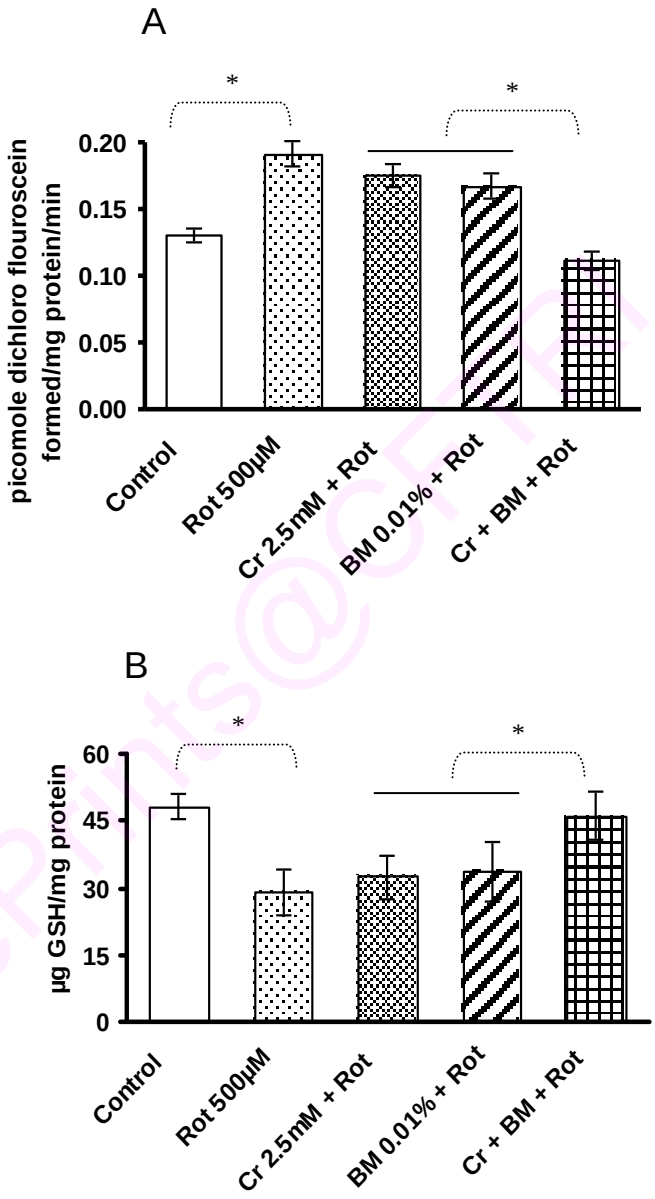
Synergistic protective effect of *Bacopa monnieri* and creatine against rotenone (500µM) induced mortality (A) and locomotor deficits (B) among adult *Drosophila*



Rot: Rotenone (500µM); BM: *Bacopa monnieri* (0.01%); Cr: Creatine (2.5mM) n=50 flies per replicate, three such replication used for assay.

Figure 5.8

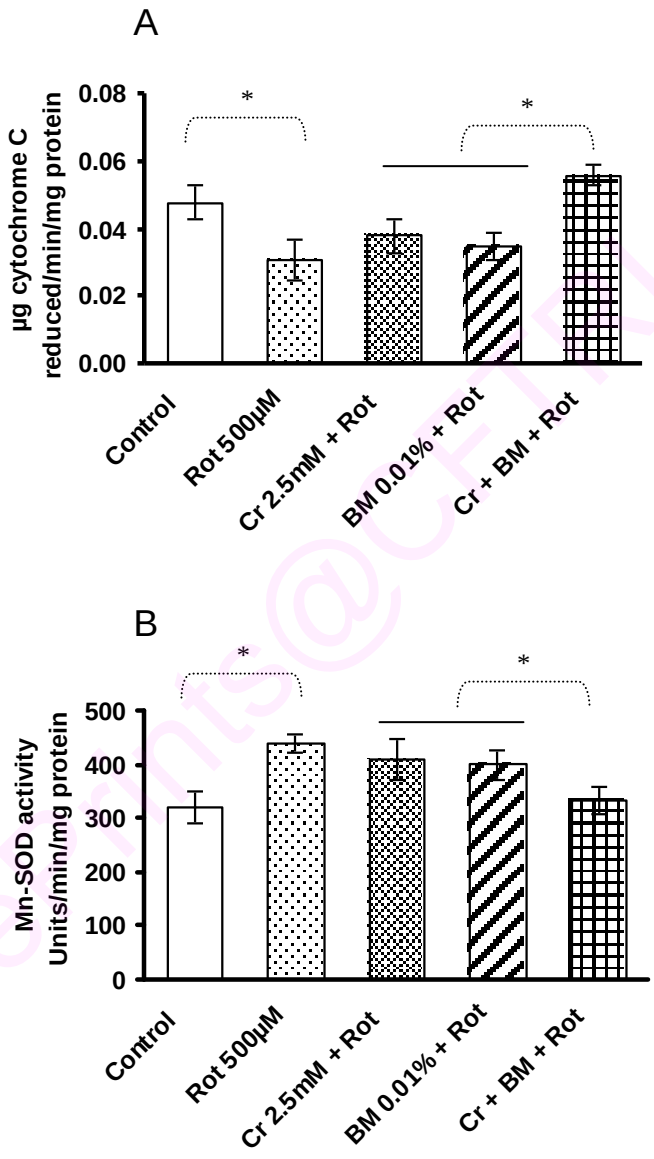
Synergistic protective effect of *Bacopa monnieri* and creatine against rotenone (500µM) induced reactive oxygen species (ROS) (A) and reduced glutathione (GSH) (B) levels in whole body mitochondria of *Drosophila*



Rot: Rotenone (500µM); BM: *Bacopa monnieri* (0.01%); Cr: Creatine (2.5mM)
Values are mean ± SE. Data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significance was determined by making comparisons between control v/s Rot; Rot v/s Rot + Cr, Rot + BM, Rot + Cr + BM. n=50 flies per replicate, three such replication used for assay.

Figure 5.9

Synergistic protective effect of *Bacopa monnieri* and creatine against rotenone induced alterations in the activities of complex I-III (A) and Mn-SOD (B) in whole body mitochondria of *Drosophila* exposed to rotenone



Rot: Rotenone (500µM); BM: *Bacopa monnieri* (0.01%); Cr: Creatine (2.5mM)
Values are mean ± SE. Data was analyzed by one way ANOVA followed by post hoc Tukey test (* $p<0.05$). Significance was determined by making comparisons between control v/s Rot; Rot v/s Rot + Cr, Rot + BM, Rot + Cr + BM. n=50 flies per replicate, three such replication used for assay

5.0 DISCUSSION

Although there is lack of certainty on the pathophysiology of neurodegenerative mechanisms, it is well accepted that energy depletion, oxidative stress and mitochondrial dysfunctions are vital factors associated with most of these disorders (Dawson and Dawson, 2003). *Drosophila* is widely used as a model to understand the pathophysiology of several NDD. In recent times it is also effectively serving as an easy platform to screen several phytochemicals for their neuroprotective properties and is anticipated to be highly valuable for first-step drug screen. Previously, using *Drosophila* we demonstrated the protective effects of *Bacopa monnieri* against rotenone induced neurotoxicity (Hosamani and Muralidhara, 2009). In this study we tested the hypothesis that creatine supplementation can significantly offset rotenone induced mitochondrial oxidative stress in *Drosophila* and rescue the flies from the neurotoxic consequences. Further, the possible synergistic effects of a combination of creatine and BM extract were also studied.

Modulatory potency of creatine against rotenone neurotoxicity

Creatine has been widely used as an ergogenic aid to improve exercise performance in humans and also to ameliorate oxidative stress mediated diseases. However, the underlying mechanism/s of its action are less well understood *in vivo* (Wyss and Schulze, 2002; Persky and Brazeau, 2001) (Andres et al., 2008). In the present study, flies provided with creatine supplements exhibited significant reduction in the endogenous levels of oxidative markers such as malondialdehyde and hydroperoxide. While the lowest concentration of creatine had no appreciable effect, at higher concentrations significant diminution of MDA and hydroperoxide levels in whole body homogenates suggested its potential to modulate endogenous levels of oxidative markers. These findings of diminished levels of oxidative markers were associated with enhanced GSH levels in whole body homogenates clearly suggest the antioxidative effects of creatine. Owing to higher reduction in MDA levels observed with 10mM creatine, we employed this concentration for our further experimentation. The observed effects in flies are consistent with previous reports on the direct antioxidant properties of creatine both under *in*

vitro and *in vivo* conditions. Previously the potential of creatine to scavenge ABTS $^{+}$, superoxide anions and peroxynitrite radicals were demonstrated *in vitro* (Lawler et al., 2002). Studies in cell models also demonstrated the direct antioxidant activity of creatine *via* a scavenging mechanism in oxidatively injured mammalian cells and its DNA protective action against oxidative attack (Guidi et al., 2008; Azzi et al., 2004). Further long term creatine supplementation in aged mice was reported to diminish the ROS levels, decrease the accumulation of lipofuscin pigments in brain and extend longevity (Bender et al., 2008). In a recent study, creatine supplementation was shown to reduce endogenous lipid peroxidation biomarkers in rats suggesting a protective role against oxidative damage (Deminice et al., 2009).

The impact of Rotenone at the concentration tested in our model has been discussed in detail in Chapter 2. In the present model, rotenone induced a concentration related mortality and locomotor deficits in adult flies, data which is consistent with previous reports (Celetto and Palladino, 2005; Chaudhuri et al., 2007). While the exact mechanism of rotenone action leading to neurotoxicity is not well understood, in the insect model, rotenone action is attributed to specific sensitivity of dopaminergic neurons to ROS and oxidative damage (Sherer et al. 2003). Earlier findings in *Drosophila* viz., prevention of rotenone induced locomotor deficits and DA neuronal loss by the antioxidant melatonin clearly suggest the involvement of oxidative stress mechanisms (Tan et al., 2002). Interestingly, similar signs of oxidative damages have been reported frequently in dopaminergic neurons from PD patients, suggesting implication of oxidative stress in this disease (Coto-montes and Hardeland, 1999; Beal, 2003). In the present study, rotenone induced significant oxidative markers as evidenced by enhanced MDA and hydroperoxide levels, elevated activities of antioxidant enzymes (Catalase and SOD). These findings are consistent with our own findings in *Drosophila* (Ravikumar and Muralidhara, 2009) and previous reports in rats exposed to rotenone (Jennar, 2003; Bove et al., 2005). Severe depletion in cellular GSH levels upon rotenone exposure in *Drosophila* adds further evidence that a state of oxidative stress exists *in vivo* which may lead to mitochondrial damage and increase in free radical generation.

Creatine supplementation restored the depleted GSH pool and normalized the ROS levels in rotenone treated flies. This protective action may be chiefly attributable to the direct quenching of free radicals or alternatively due to the up regulation of antioxidative defenses. While the primary effect of creatine supplementation on GSH synthesis in flies needs further investigations, it may be the result of *de novo* synthesis of GSH and phase II detoxification in response to rotenone induced altered redox status. The lower incidence of rotenone induced mortality response among flies maintained on creatine supplemented diet clearly suggests the protective action of creatine. Furthermore, rotenone induced neurotoxicity was clearly discernible by the high rate of locomotor deficits measured in the negative geotaxis assay. Flies with locomotor deficits have tendency to stay at the bottom of vial and do not appear to coordinate their legs in normal fashion. The development of this phenotypic expression of among flies exposed to rotenone is speculated to be due to the deficits in high energy levels of ambulatory and flight muscles which are rich in mitochondria. Hence it is likely that uncoupled mitochondrial machinery may be responsible of the induced locomotor dysfunctions as evidenced by severe complex I inhibition. Another possible reason might be due to the differential and significant depletion of dopamine pool as observed in both head and body homogenate. Interestingly creatine supplementation was able to rescue the flies significantly from deteriorating locomotor dysfunctions indicating its potential to restore dopamine pool and protect the mitochondrial function. This thinking is consistent with earlier findings which suggest significant correlation between locomotor dysfunction and dopamine deficiency (Celetto and Palladino, 2005). Although speculative, the better performance resulting from creatine supplements among rotenone exposed flies could be partly related to its well known physiological role in the maintenance of ATP/ADP ratio resulting in enhanced mitochondrial respiration (Gualano et al., 2010). However, further investigations are necessary to delineate the direct effects of creatine on flight muscles among flies exposed to rotenone.

Employing a prophylactic approach, we obtained further evidence that creatine has the potential to confer protection against paraquat induced oxidative insult. Paraquat, a well known herbicide is demonstrated to cause

selective degeneration of dopaminergic neurons in substantia nigra pars compacta in animal models (Dupuis et al., 2004). The biochemical consequences of acute paraquat intoxication in *Drosophila* have been discussed in detail earlier *(Please see chapter 3*). Briefly, it is known to induce oxidative stress in mammals and in *Drosophila* (Peng et al., 2007; Cocheme and Murphy, 2008; Hosamani and Muralidhara, 2010). Data obtained in our bioassay revealed significant reduction in the degree of paraquat-induced lethality among flies given creatine prophylaxis clearly indicating that flies were able to successfully mitigate the PQ induced oxidative stress resulting in better survival rate. Further investigations on the mechanism by which creatine protects flies against PQ induced oxidative stress and neurotoxicity is underway.

Synergistic neuroprotective effects of BM and Creatine

There is substantial evidence that mitochondrial dysfunction and bio-energetic abnormalities play a role in the pathogenesis of NDD (Lin and Beal, 2006). Hence it is possible that agents which improve mitochondrial and cellular bioenergetics are likely to effective in the treatment of NDD. Earlier findings have demonstrated that both Coenzyme-Q10 and creatine exert neuroprotective effects both *in vitro* and *in vivo* in animal models of NDD (Beal and Shults, 2003) . This has led to clinical trials in both PD and HD (Shults et al., 2002). Hence it is opined that combinations of agents targeting different disease mechanism may show improved efficacy and allow agents to be utilized at lower doses to minimize side effects (Yang et al., 2009). Since it is less expensive and relatively easy to experiment, many workers have studied several combinatorial drug regimens for their efficacy to alleviate disease processes in the *Drosophila* system (Agrawal et al., 2005; yang et al., 2009). Hence it was hypothesized a combination treatment of flies with BM extract and creatine may provide a higher degree of protection under neurotoxicant exposure. Our hypothesis was based on the following considerations : i) BM enriched diet significantly modulated the oxidative stress, locomotor deficits ad neurotoxicity under rotenone exposure ii) BM prophylaxis provided marked resistance against paraquat induced oxidative stress (in cytosol) and

mitochondrial oxidative dysfunctions; iii) creatine supplementation markedly protected against rotenone induced oxidative stress, locomotor deficits and neurotoxicity. However, these effects were not evident at lower concentration of these modulants. Hence, in the combination dietary regimen, these were employed at lower conc. Viz., BM extract (0.01 %) and creatine (2.5 mM).

Creatine exerts neuroprotective effects both *in vitro* and *in vivo.* It protects against both glutamate and beta-amyloid toxicity in rat hippcampal and strait neurons (Brewer and Wallimann, 2000; Brustovetsky et al., 2001), against NMDA and ibotenic acid straital excitotoxic lesions in vivo (PenaAltamira et al 2005). Several workers have reported additive effect of creatine with C0Q10 (Ferrante et al., 2002) creatine in combination with cyclooxygenase 2 inhibitors exerts additive neuroprotective effects against MPTP neurotoxicity and in a transgenic mouse model of ALS (Klivenyi et al., 2003) and with minocylcine in a mouse model of ALS (Zhang et al., 2003). In the present study we obtained significant neuroprotective effect of BM and creatine at lower concentrations and these effects were evident in terms of reduced lethality, oxidative stress induction in both cytosol and mitochondria. However further studies are required to understand the precise mechanism of synergistic effect of BM with creatine in the rotenone model of *Drosophila*.

In conclusion, the neuroprotective efficacy of creatine enrichment in *Drosophila* was demonstrable by its ability to modulate endogenous oxidative markers, propensity to mitigate rotenone induced mitochondrial oxidative stress, restore dopamine levels and neurotoxicity. Additional evidence of its neuroprotective action was exemplified by the enhanced resistance to Paraquat. We hypothesize that the protective effects of creatine may be due to the combined effects of its antioxidative action and direct physiological effects on mitochondria. This was more exemplified in the combination treatment with BM extract. More importantly, these results confirm the utility of *Drosophila* as a primary tool to rapidly screen compounds either alone or in combination which are suspected to possess neuropharmacological properties prior to their testing in mammalian models and further therapeutic use in humans.

6.0 SUMMARY

1. Adult flies fed on creatine-enriched diet for 7 days showed significantly diminished levels of MDA and hydroperoxide levels.
2. The incidence of rotenone induced mortality was markedly reduced among flies maintained on creatine -supplemented diet
3. Flies maintained on creatine-supplemented diet and exposed to rotenone exhibited marked improvement in climbing behavior suggesting significant attenuation of rotenone effects on locomotor deficits
4. Rotenone exposure caused significant depletion in dopamine level in both head and body region as measured by HPLC analysis. The neuroprotective effect of creatine was clearly discernible as evident by significant restoration of dopamine levels.
5. Creatine supplementation completely offset rotenone induced oxidative stress as evidenced by the normalized ROS generation and Nitric oxide levels accompanied by restoration of glutathione and Mn-SOD activity.
6. Creatine supplementation offered significant protection against rotenone induced reduction in the activity of complex I-III suggesting its potential to restore mitochondrial function.
7. Flies given creatine prophylaxis (10mM for 7 d) showed marked resistance to acute paraquat (filter disc method) challenge as evident by lower incidence of mortality.
8. In combination, BM extract and creatine (at lower concentrations) offered significantly higher degree of protection against rotenone -induced lethality and locomotor deficits suggesting a synergistic effect.
9. In combination, the modulants completely abrogated the rotenone – induced oxidative stress as evident by the normalization of ROS, GSH levels and activity of Mn -SOD in whole body homogenates.
10. In conclusion, a combination of BM extract and creatine at lower concentrations appeared to completely offset rotenone induced effects suggesting a higher potency, and their possible usage in combination for therapeutic purposes.

Conclusions

CONCLUSIONS

1. Exposure of adult male *Drosophila* to Rotenone (250-1000µM in the diet) resulted in significant concentration dependent lethality.
2. BM-enriched diet offered robust protection against rotenone induced lethality.
3. Among the tested phytochemicals, BM offered significant protection against all the tested neurotoxicants in adult flies.
4. Feeding of BM-enriched diet (0.05 and 0.1% for 7 d) to adult flies caused significant diminution in the levels of endogenous oxidative markers viz., ROS generation , malondialdehyde(MDA) and hydroperoxide levels and was accompanied with reduced protein carbonyls and enhanced glutathione clearly suggesting its antioxidative activity *in vivo.*
5. The incidence of rotenone (500 µM) induced mortality was significantly reduced among flies fed with BM-enriched diet suggesting its ameliorative effect under co-exposure regimen.
6 Co-exposure with BM completely offset rotenone induced oxidative stress as evidenced by the normalized MDA/hydroperoxide/ protein carbonyl levels which was accompanied by restoration of GSH in whole body homogenates. Rotenone induced alterations in the activities of antioxidant enzymes (catalase, SOD and GST) among flies fed on BM-enriched diet adult flies were significantly ameliorated.
7. Rotenone induced neurotoxicity was clearly evident among flies as measured by severe locomotor deficits. Interestingly, flies fed BM –enriched diet marked improvement as their performance was better in the negative geotaxis assay. Rotenone exposure caused significant depletion in dopamine levels in both head and body region as measured by HPLC analysis. The neuroprotective effect of BM was clearly discernible as evident by the significant restoration of dopamine levels.
8. Exposure of adult flies to paraquat (PQ) (10- 40mM) (filter disc method) resulted in a concentration related mortality. Further PQ exposure caused significant induction of oxidative stress as evidenced by increased hydroperoxide levels, enhanced antioxidant enzymes and redox status in cytosolic fraction.

9. PQ exposure resulted in a marked concentration dependent elevation in lipid peroxidation, superoxide generation, enhanced GSH levels coupled with higher activities of Mn-SOD and GST suggesting marked induction of oxidative stress *in vivo* in mitochondria of adult flies. Interestingly, increased free iron level in whole body homogenates suggested its possible role in PQ associated oxidative stress.
10. Significant decrease in the activities of ETC enzymes viz., complex I-III and II-III, succinate dehydrogenase and Mg^{+2} ATPase coupled with altered MTT reduction clearly indicated that acute PQ exposure causes mitochondrial dysfunction
11. BM-Prophylaxis offered significant protection against PQ induced lethality as evidenced by lower incidence of mortality, marked abrogation of PQ induced ROS generation, elevated levels of MDA, hydroperoxide and perturbations in enzymic antioxidant defenses.
12. BM prophylaxis offered significant protection against PQ induced perturbations in the activities of ETC and TCA cycle enzymes suggesting ability of BM to restore mitochondrial function.
13. Oral administration of BM extract (200mg/kg bw/d for 4 wks) to prepubertal mice resulted in significant diminution in the levels of various endogenous oxidative markers in cytosolic fractions of brain regions.
14. BM prophylaxis (for 4wks) markedly abrogated the induction of oxidative stress by acute PQ intoxication as revealed by normalization of ROS levels in different brain regions (cortex/cerebellum/hippocampus), while the MDA levels were restored only in hippocampus. BM prophylaxis significantly attenuated the activities of complex I-III in hippocampus and complex II-III in cerebellum/hippocampus/striatum.
15. The neuroprotective effects of BM against PQ intoxication in prepubertal mice were also discernable in a co-exposure chronic paradigm. BM treatment rendered significant protection against PQ induced oxidative stress in different brain regions as evidenced by normalized MDA/hydroperoxide levels coupled with restoration of activities of enzymic antioxidant defense in cerebellum and striatal region. PQ induced diminution in the activity of SDH in cerebellum and striatum was

completely restored by BM treatment, while decrease in MTT reduction was restored only in the stratum indicating a differential protective effect of BM. BM treatment also offered significant protection against PQ induced perturbations in the activity of AChE only in the striatum.

16. Adult flies fed on creatine-enriched diet for 7 days showed markedly diminished levels of MDA and hydroperoxide levels. The incidence of rotenone induced mortality was markedly reduced among flies maintained on creatine -supplemented diet. Flies maintained on creatine-supplemented diet and exposed to rotenone exhibited marked improvement in climbing behavior suggesting significant attenuation of rotenone effects on locomotor deficits
17. Rotenone exposure caused significant depletion in dopamine levels in both head and body region and the neuroprotective effect of creatine was clearly discernible by the significant restoration of dopamine levels.
18. Creatine supplementation completely offset rotenone induced oxidative stress as reflected by the normalized ROS generation and nitric oxide levels accompanied by restoration of glutathione and Mn-SOD activity. Creatine supplementation offered significant protection against rotenone induced reduction in the activity of complex I-III suggesting its potential to restore mitochondrial function.
19. In combination, BM extract and creatine (at lower concentrations) offered significantly higher degree of protection against rotenone -induced lethality and locomotor deficits suggesting a synergistic effect. Further, the modulants completely attenuated the rotenone-induced oxidative stress. In conclusion, a combination of BM extract and creatine at lower concentrations appeared to completely offset rotenone induced effects suggesting a higher potency, and their possible usage in combination for therapeutic purposes.

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LIST OF PUBLICATIONS

I. Paper published / to be published

1) **Ravikumar Hosamani and Muralidhara 2009.**
Neuroprotective efficacy of *Bacopa monnieri* against rotenone induced oxidative stress and neurotoxicity in *Drosophila melanogaster. NeuroToxicology 30; 977-985*
(Cited among TOP 25 hottest articles by Science direct Oct-Dec 2009)

2) **Ravikumar Hosamani, Saraf R Ramesh and Muralidhara 2010.**
Attenuation of rotenone-induced mitochondrial oxidative damage and neurotoxicity in *Drosophila melanogaster* supplemented with creatine. *Neurochemical Research 35; 1402-1412*

3) **Ravikumar Hosamani and Muralidhara 2010.**
Paraquat induced oxidative perturbations and lethality in *Drosophila melanogaster* are mitigated by prophylactic treatment with *Bacopa monnieri*. *Indian Journal of Biochemistry and Biophysics 47; 75-82*

4) **George K Shinomol, Ravikumar H and Muralidhara 2010.**
Prophylaxis with *Centella asiatica* confers protection to prepubertal mice against 3-nitropropionic-acid-induced oxidative stress in brain. *Phytotherapy Research 24:885-892*

5) **Ravikumar Hosamani and Muralidhara 2010.**
Paraquat induced early oxidative stress and mitochondrial dysfunctions in adult *Drosophila melanogaster*
(Communicated: Journal of insect physiology)

6) **Ravikumar Hosamani and Muralidhara**
Prophylactic *Bacopa monnieri* treatment ameliorates Paraquat induced oxidative stress and mitochondrial dysfunctions in prepubertal mice brain
(Under preparation)

II. Papers presented at National and International symposia

1) **Ravikumar Hosamani**, Gokul K, Muralidhara, 2011
Validation of neuroprotective effect of Bacopa monnieri prophylaxis against paraquat induced neurotoxicity in prepubertal mice.
International Conference on Recent Trends in Therapeutic Advancement of Free Radical Science & 10th Annual Meeting of the Society for Free Radical Research (SFRR), 9-11 January 2011, Chennai, India.

2) **Ravikumar Hosamani**, Lalith kumar V and Muralidhara, 2010,
Paraquat induced oxidative perturbations and mitochondrial dysfunctions in adult *Drosophila melanogaster* ***(Best poster presentation award)*** *International Conference: On Advances in Free radical Research & Natural Products, Antioxidants and radio protectors in health & 9th Annual Meeting of the Society for Free Radical Research –India 11-13 January 2010, Hyderabad, India*

3) **Ravikumar Hosamani**, Gokul K and Muralidhara, 2009,
Chronic exposure to paraquat causes oxidative stress induction and altered cholinergic function in brain regions of prepubertal mice*:*
Indian Convention of Food Scientists and Technologists (ICFOST-2009) 21-23 December 2009, NIMHANS,, Bangalore, Karnataka, India

4) **Ravikumar Hosamani**, Ramesh SR and Muralidhara, 2009,
Paraquat induced mitochondrial dysfunctions in *Drosophila*: attenuating effect of *Bacopa monnieri* standard extract.
International Conference: On Advances in Free radical Research & Natural Products, Antioxidants and radio protectors & 8th Annual Meeting of the Society for Free Radical Research –India 19-21 March 2009, Lucknow, India

5) **Ravikumar Hosamani**, Shinomol GK, Ramesh SR & Muralidhara, 2008,
Drosophila as model system to assess neuromodulatory potential of phytochemicals*:*
Genomics, Model Organism & Disease, satellite meeting of 13th Human Genome Organization conference, 1-2 October 2008 NCBS Bangalore

6) **Ravikumar Hosamani**, Chandrasekhar KN and Muralidhara, 2008, Neuroprotective effect of creatine against rotenone induced neurotoxicity and mitochondrial dysfunction in *Drosophila melanogaster* ***(Pre-doctoral travel grant award)****:*
12th European Drosophila Neurobiology Conference, NEUROFLY 2008" Sept 6th- 10th 2008 Wurzburg, Germany ***(Published in Journal of Neurogenetics special issue, 2009; vol, 23(1): S1- S102***

7) **Ravikumar Hosamani**, Chandrasekhar KN, Ramesh SR and Muralidhara, 2008, (oral presentation)
Antioxidant action of eugenol in *Drosophila melanogaster* and mice: modulatory effects on endogenous oxidative markers and/against rotenone induced oxidative stress*:*
5th Drosophila meeting March 28-29: Department of Science & Technology, New-Delhi. Unit on Evolution and Genetics DOS in Zoology, University of Mysore, Manasagangotri, Mysore. Karnataka

8) **Ravikumar Hosamani**, Ramesh SR and Muralidhara, 2008, Neuromodulatory effects of *Bacopa monnieri* ethanolic extract and Brahmi capsule TM in *Drosophila melanogaster*: A comparative approach*:*
International Conference: On "Free radicals & Natural Products in Health" & 7th Annual Meeting of the Society for Free Radical Research –India (SFRR-India), 14-16 February 2008, Jaipur, India

9) **Ravikumar Hosamani**, K.N Chandrashekar and Muralidhara 2007. Protective efficacy of *Bacopa monnieri* extract against rotenone induced neurotoxic effects in *Drosophila*.
24th *International Neurotoxicology conference November 11- 14, 2007 San Antonio, Texas, USA*
(Published in NeuroToxicology special issue, 2008; vol, 29(5): 753-910)

10) KN Chandrashekar, **Ravikumar Hosamani** and Muralidhara, 2007. Oxidative perturbations in immature rat testis following multiple administration of D- Aspartic acid *in vivo.*
International Symposia: Third Biennial Meeting of the Society for Free Radical Research -Asia (SFRR-Asia)& Sixth Annual Meeting of the Society for Free Radical Research –India (SFRR-India), 8-11 January Lonavala, Mumbai

11) K Shinomol George, K N Chandrashekar, **Ravikumar Hosamani** and Muralidhara.
Centella asiatica significantly mitigates endogenous oxidative biomarkers in brain regions of mice and experimentally induced oxidative stress *Indian Convention of Food Scientists and Technologists (ICFOST)-2006, Hyderabad*

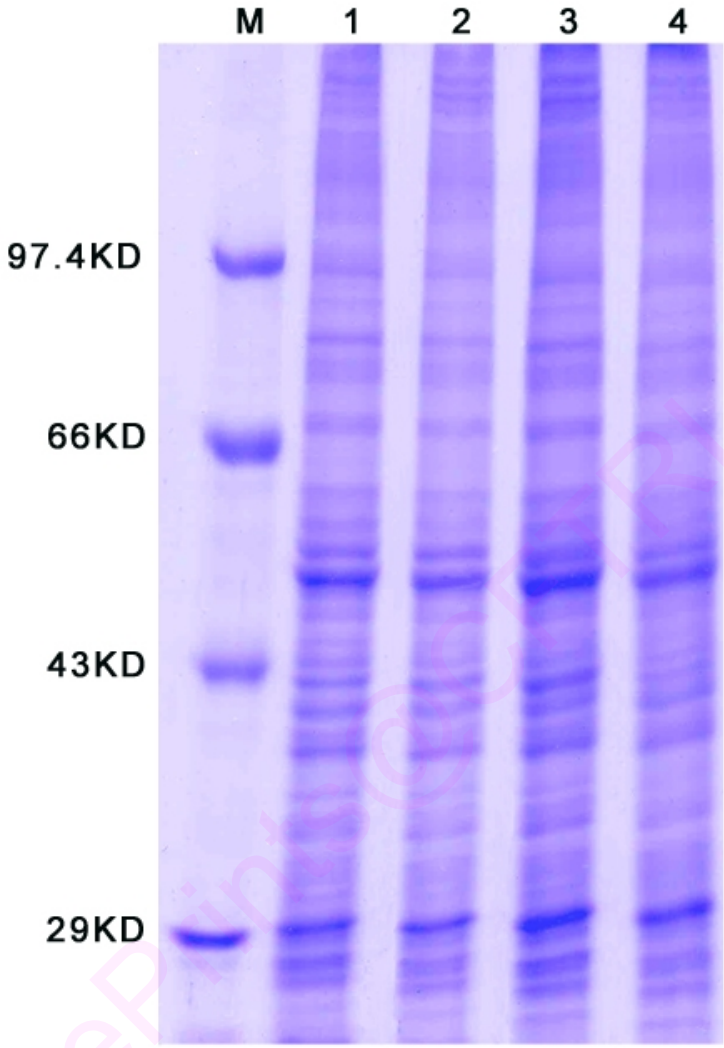
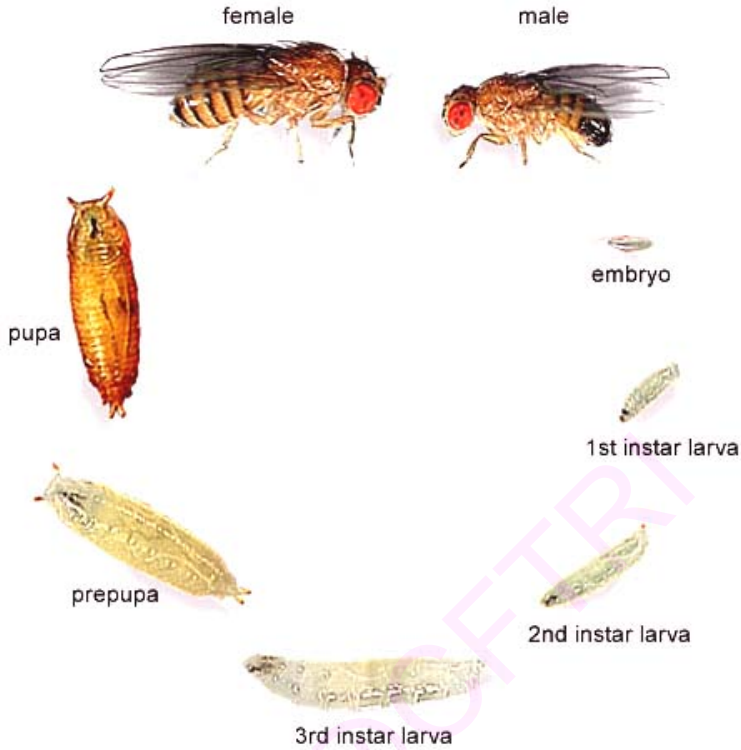
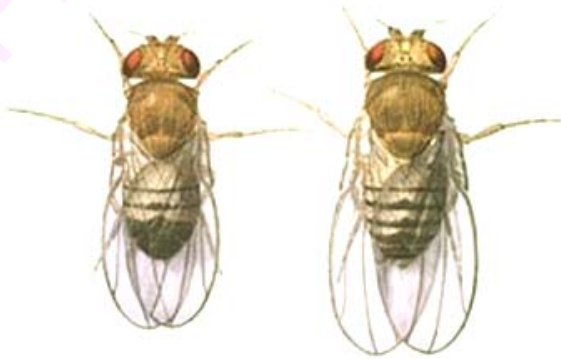


Plate 1: 5-15% SDS-PAGE protein profile of the *Drosophila* adult brain

M) Marker protein
1) Control
2) Rotenone 500µM
3) *Bacopa monnieri* 0.1%
4) Rotenone + *Bacopa monnieri*



Life cycle of *D. melanogaster*



Adult male and female *D. melanogaster*