CHAPTER III

Validation of Neuromodulatory efficacy of *Withania somnifera* in mice model and its synergistic effects with Ferulic acid
Preface

Having established the neuroprotective potential of WSE in the fly model, as a proof or principle, its neuromodulatory efficacy was investigated employing a rotenone model of neurotoxicity in mice. This model is often employed to induce Parkinson's like symptoms in rodent models *in vivo*. Further, the efficacy of Ferulic acid (FA) and a combination of WSE and FA was also investigated in the Rotenone model.
Chapter 3

1.0 INTRODUCTION

Withania somnifera commonly known as ‘Ashwagandha’ or ‘Winter cherry’ is widely used in the traditional Indian medicine as a nerve tonic and memory enhancer. It is being used more than 100 formulations in various system of medicine such as Ayurveda, Siddha and Unani; WSE is therapeutically considered as Indian ginseng (Singh et al., 2001). It possesses anti-aging, anti-stress, immune-modulatory and antioxidant properties (Dhuley, 1998; Archana and Namasivayam, 1999; Ziauddin et al., 1996). Various parts of the plant have been in use for centuries to treat a variety of ailments and constantly new biological activities are being ascribed from time to time (Mishra et al., 2000; Gupta and Rana, 2007; Kulkarni and Dhir, 2008). Phytochemically, it is described to possess a diverse set of withanolides (modified steroidal molecules), with the major bioactive constituent being glycowithanolides. WSE has been extensively used as an antioxidant, liver tonic, adaptogen, anti-inflammatory agent, astringent and to treat ulcers, bacterial infections, venom toxins and senile dementia (Bhatnagar et al., 2009).

Several studies have shown the beneficial effects of Withania somnifera root and some of the withanolide derivatives in particular promote dendrite formation in human neuroblastoma cells in vitro (Tohda et al., 2000). Further, withanolide A and withanosides IV/ VI extend axons and dendrites in vitro and in vivo. In an animal model of dementia and spinalcord injury, withanolide A, withanoside IV, and withanoside VI were demonstrated to restore pre- and post-synapses (Kuboyama et al., 2005). WSE root extracts and withanolides have been shown to inhibit the activity of acetylcholinesterase both in vitro and in vivo (Choudhary et al., 2005). WSE root extracts were demonstrated to significantly reduce the number of hippocampal degenerating cells in the brains of stressed rodents, improve antioxidant status and provide neuroprotection (Jain et al., 2001).

Various neurotoxins are considered as etiological factors in the development of neurodegenerative diseases (NDD). Rotenone (ROT), a complex ketone derived from the roots of Lonchocarpus species, commonly used natural pesticide is a classic high affinity specific inhibitor of mitochondrial complex I and is capable of causing mitochondrial perturbations that mimics
PD. This lipophilic compound freely crosses cell membranes and accesses cytoplasm and mitochondria. Rotenone (ROT) is a well-characterized pesticide implicated in motor impairment and behavioral deficits (Subramaniam and Chesselet, 2013; Jin et al., 2013). ROT causes mitochondrial dysfunction, oxidative stress and consequent neurodegeneration (Graham, 1978; Jenner, 2003; Andersen, 2004). Although most of the studies regarding ROT have concentrated on age-related neurodegeneration and PD and the role of oxidative damage in neurotoxicity, studies relevant to children and preadolescents and in adult are limited. To the best of our knowledge, susceptibility of prepubertal (PP) and adult brain to ROT–induced oxidative dysfunction and associated neurotoxicity has not been investigated earlier.

The beneficial effect of polyphenol compounds in the treatment of various neurodegenerative diseases is receiving wide attention (Gilgun-Sherki et al., 2001). There is increasing suggestive evidence with regard to the beneficial effects of phytochemicals on human brain function and polyphenols which are abundant in plants, fruits and vegetables are demonstrated to exert significant neuromodulatory effects. Various polyphenols exhibit a wide spectrum of biological effects viz., antioxidant, anti-inflammatory, anti-proliferative and anti-apoptotic properties (Fang et al., 2001; Folz et al., 1997). Ferulic acid (FA) a ubiquitous phenolic compound present in wide range of staple foods such as grain bran, whole grain foods, citrus fruits, banana, coffee, orange juice, eggplant, bamboo shoots, beetroot, cabbage, spinach and broccoli. It is estimated that a meal containing whole grains, vegetables and phenolic–rich beverages could supply nearly 500-1000mg of hydroxycinnamic acids and more importantly FA is bioavailable, as its metabolites have been detected in humans (Bourne and Rice-Evans, 1998). FA exhibits a wide range of in vivo and in vitro therapeutic effects which are attributed to its antioxidant properties. Owing to its excellent capacity to scavenge free radicals, several countries have approved its use as a food additive to prevent lipid peroxidation (Bourne and Rice-Evans, 1998). FA is demonstrated to possess strong anti-inflammatory, anti-diabetic and anti-apoptotic property (Cheng et al., 2008; Koh, 2012). Studies in mice have shown the modulatory effects of FA on β-amyloid peptide-induced
microglial activation and its potential to improve cognitive functions (Mamiya et al., 2008).

In view of the above, the primary focus of the study was to examine the hypothesis that WSE and FA prophylaxis or in combination is likely to render protection against neurotoxicant insult/ challenge. The hypothesis was tested in a ROT model of neurotoxicity employing both prepubertal and adult mice. The neuroprotective propensity of WSE or FA or WSE+FA supplements was investigated in terms of their potency to attenuate ROT–induced oxidative stress in cytosol/ mitochondria, antioxidant defenses, cholinergic functions and mitochondrial dysfunctions in cortex (Ct), cerebellum (Cb), hippocampus (Hc) and striatum (St).

2.0 OBJECTIVE

The primary objective of the investigation was to determine the neuroprotective efficacy of WSE and FA individually and also the synergistic modulatory effect in combination against ROT (a mitochondrial toxin)–induced oxidative stress and neurotoxicity mice.

3.0 EXPERIMENTAL DESIGN

SECTION- A

3.1 Rotenone mediated systemic effects in mice

3.1.1 Criteria of ROT dosage

The criteria of dosage selection were based on the previous findings from our laboratory (Shinomol and Muralidhara, 2012; Girish and Muralidhara, 2012). We have employed ROT at the dosages of 0.5 and 1mg/kg b.w/d. Since ROT caused higher mortality at a dosage of 1mg/kg bw, the dosage of 0.5mg/kg b.w/d was administered to mice intraperitoneally (i.p) for 7 consecutive days. At this dosage, ROT did not induce mortality in both growing and adult male mice.
3.1.2 Preparation of Rotenone

Rotenone (ROT), 1mg/2.5mL was dissolved in dimethyl sulphoxide (DMSO) and was prepared fresh each day. Suitable aliquots were administered (i.p) to respective mice according to their body weights.

3.1.3 Rotenone (ROT) treatment protocol

In order to determine the sublethal dosage of ROT, age synchronized prepubertal (4wk old) male mice (n=6, 3 replicates) were exposed to graded dosages of ROT (0.1-2mg/kg body weight, 7days) maintaining a DMSO control. The incidence of lethality was recorded at 24h intervals. Both control and ROT-mice were maintained on pellet diet with tap water. The concentration determinative study was performed separately three times and the mortality response was pooled and sublethal value was computed.

3.1.4 Effect of ROT per se on oxidative markers

Prepubertal male mice (n=6, 3replicates) were administered with ROT 0.5mg/kg b.w (for 7days) and DMSO to the control mice. Terminally, mice were anesthetized, sacrificed, dissected brain and subdissection was done to separate cortex, cerebellum, hippocampus and striatum; and homogenized in buffer B (as expalained in meterials and methods), centrifuged to obtain cytosol and mitochondrial samples and used for the biochemical investigations. The following biochemical measurements were made:

Oxidative markers: Markers of oxidative stress viz., reactive species (ROS), hydroperoxides (HP) and protein carbonyls (PC) were determined in all part of the brain regions.
Antioxidant defenses: The redox status was assessed by determining the levels of antioxidants – reduced glutathione (GSH) and activities of antioxidant/detoxifying enzymes viz., SOD, CAT and GST in the cytosol and mitochondria. Further, activity levels of AChE, BChE, Dopamine, SDH, MTT and ETC complex were measured.

3.2 Effect of *Withania somnifera* per se on endogenous oxidative markers in brain regions

3.2.1 Preparation of WSE

The standard powder of WSE was dissolved in distilled water at a concentration of 100mg/mL and used throughout the study.

3.2.2 Oral administration of WSE and its effect on oxidative markers

Prepubertal male mice (n=6, 3 replicates) were orally administered with WSE 100-400mg/kg b.w (4weeks) and distilled water to the control mice. Terminally, mice were anesthetized, sacrificed, dissected brain and subdissection was done to separate cortex, cerebellum, hippocampus and striatum; and homogenized in buffer B, pH 7.4 (as expalained in materials and methods), centrifuged to obtain cytosol and mitochondrial samples and used for the biochemical investigations. The following biochemical measurements were made:

Oxidative markers: Markers of oxidative stress viz., reactive species (ROS), hydroperoxides (HP) and protein carbonyls (PC) were determined in cytosol and mitochondria of different brain regions.

Antioxidant defenses: The redox status was assessed by determining the levels of antioxidants – reduced glutathione (GSH), total thiols and activities of...
antioxidant/ detoxifying enzymes *viz.*, SOD, CAT, GPx and GST in the cytosol and mitochondria.

*Mitochondrial Impairments*: Markers of Neurotoxicity: Further, activity levels of AChE, BChE, Dopamine, SDH, MTT and ETC complex were measured.

### 3.3 Protective effect of WSE prophylaxis against ROT –induced neurotoxicity

Prepubertal male mice (*n*=6, 3 replicates) maintained on pellet diet with tap water. For the first group only distilled water was administered for the second and third group WSE (400mg/kg b.w, oral, 4weeks) and ROT (0.5mg/kg bw, i.p, 7days) were administered respectively. For the forth group co-treatment with WSE and ROT (as explained in material method). Terminally, the mice were anaesthetized, sacrificed, dissected brain and subdissection was done to separate cortex, cerebellum, hippocampus and striatum; and homogenized in buffer B, pH 7.4 (as expalained in materials and methods), centrifuged to obtain cytosol and mitochondrial samples and used for the biochemical investigations. The following biochemical measurements were made:

**Oxidative markers**: Markers of oxidative stress *viz.*, reactive species (ROS), hydroperoxides (HP) and protein carbonyls (PC) were determined in cytosol and mitochondria of different brain regions.

**Antioxidant defenses**: The redox status was assessed by determining the levels of antioxidants – reduced glutathione (GSH), total thiols and activities of antioxidant/ detoxifying enzymes *viz.*, SOD, CAT, GPx and GST in the cytosol and mitochondria.

**Mitochondrial functions**: Further, activity levels of SDH, MTT and ETC complex were measured.

**Neurochemical markers**: The activity of acetylcholinesterase (AChE), butrylcholinesterase (BChE) enzymes and the levels of dopamine (DA) were determined in the cytosolic fractions of different brain regions of prepubertal male mice homogenates.
SECTION- B

3.4 Implications of Ferulic acid (FA) on endogenous oxidative markers

3.4.1 Preparation of stock of FA

FA was prepared as a suspension in distilled warm water at a concentration of 12.5mg/mL and used throughout the study.

Prepubertal male mice (n=6, 3 replicates) maintained on pellet diet with tap water. For the first group only distilled water was administered for the second and third group FA (25 and 50mg/kg b.w, oral, 2 weeks) and ROT (0.5mg/kg bw, i.p, 7 days) were administered respectively. For the forth group co-treatment with FA and ROT (as explained in material method). Terminally, the mice were anaesthetized, sacrificed, dissected brain and subdissection was done to separate cortex, cerebellum, hippocampus and striatum; and homogenized in buffer B, pH 7.4 (as explained in materials and methods), centrifuged to obtain cytosol and mitochondrial samples and used for the biochemical investigations. The following biochemical measurements were made:

Oxidative markers: Markers of oxidative stress viz., reactive species (ROS), hydroperoxides (HP) and protein carbonyls (PC) were determined in cytosol and mitochondria of different brain regions.

Antioxidant defenses: The redox status was assessed by determining the levels of antioxidants – reduced glutathione (GSH), total thiols and activities of antioxidant/ detoxifying enzymes viz., SOD, CAT, GPx and GST in the cytosol and mitochondria. Further, activity levels of SDH, MTT, AChE, BChE, Dopamine and ETC complex were measured.
3.5 Synergistic protective effects of WSE and FA: Rotenone model

3.5.1 Synergistic protective effects in growing male mice

For this study, growing male mice were assigned to five groups viz., control (untreated), ROT (ROT control), WSE, FA and WSE+FA prophylaxis group (n=6). While the first group mice were not provided WSE/ FA/ WSE+FA or ROT (controls), the second, groups (ROT treatment), third (WSE for 30days), fourth (15days) and fifth group of mice were daily supplemented (as illustrated in the experimental design) with WSE/ FA at the dosage of 400mg/kg b.w/d and 25mg/kg b.w/d for 4 weeks and 2 weeks respectively. On the day 23, both control and prophylaxis groups (n=6) were intraperitoneally administered with ROT at the dosage of 0.5mg/kg b.w/d for 7 consecutive days. DMSO was administered to the control mice. Mice of prophylaxis group continued to receive WSE or FA or WSE+FA during the duration of ROT treatment. Animals were subjected to routine physical examination. Recording the daily food intake, weekly body weights, the animals were subjected to necropsy on day 31st and the excised brain was frozen immediately until further biochemical analysis. The following biochemical measurements were made:

Oxidative markers: Markers of oxidative stress viz., reactive species (ROS), hydroperoxides (HP) and protein carbonyls (PC) were determined in cytosol and mitochondria of different brain regions.

Antioxidant defenses: The redox status was assessed by determining the levels of antioxidants – reduced glutathione (GSH), total thiols and activities of antioxidant/ detoxifying enzymes viz., SOD, CAT, GPx and GST in the cytosol and mitochondria. Further, activity levels of SDH, MTT, AChE, BChE, dopamine and ETC complex were measured.
4.0 RESULTS

SECTION- A

4.1 Rotenone mediated systemic effects in mice

4.1.1 Food Intake and Growth

Administration of ROT at both dosages did not caused significant effect either daily food intake or in the body weights (Table 3.1).

4.1.2 Effect on oxidative markers in cerebellum (Cb) and striatum (St)

In general, administration of ROT caused significant induction of oxidative stress in both Cb and St regions of cytosol and mitochondrial fractions. Data on oxidative induction pattern measured in terms of ROS, MDA and HP levels are presented in the Fig. 3.1 and Fig. 3.2. In the cytosol the ROS levels were markedly elevated in Cb (51%) and St (30%). However, the enhancement in MDA levels were relatively higher in St compared to Cb (22%). On the other hand the HP levels were consistently elevated in both the regions (Cb: 13% and St: 22%). Likewise, the ROS levels in the mitochondria were also significantly elevated in the both the brain regions (Cb: 38%; St: 14%). Further, in mitochondria both MDA and HP levels were relatively more elevated in St region compared to the Cb region.

4.1.3 Effect on GSH, total thiols and activities of antioxidant enzymes

At the lower dose, ROT caused a significant depletion in GSH levels in the cytosol (Cb: 83%; St: 13%), while the total thiol content was marginally elevated (Fig. 3.3 A and B). Data on the the activity levels of antioxidant levels were determined in the Cb and St are presented in Fig. 3.4 A-C. Marked elevation in the activity levels of SOD in both the regions (Cb: 38%; St: 40%). Further, ROT caused robust increase in the activity levels of catalase in both the regions (Cb: 58%; St: 208%). However, the activity levels of GPx were not affect in the both the regions (Fig. 3.4 C). Further, GST levels were marginally elevated in both Cb and St regions.
4.1.4 Effect on the activity of acetylcholinesterase enzyme

ROT administration caused significant enhancement in the activity levels of acetylcholinesterase in both brain regions only at lower dose (Cb: 33%; St: 21%) (Table 3.2 and Fig. 3.5).

4.1.5 Effect on the activities of mitochondrial complex enzymes

ROT administration caused significant decrease in the activity level of NADH-cytochrome-C reductase (Cb: 19%; St: 22%) and succinate-cytochrome-C reductase in both the brain regions (Fig. 3.6 A and B). Further, the activity levels of SDH were diminished significantly only in the striatum (22-31%), while MTT reduction was reduced markedly at the higher ROT dose in both the brain regions (Fig. 3.6 C and D).

4.2 Modualtory effect of Withania somnifera per se on endogenous oxidative markers in brain regions

4.2.1 Effect on food consumsion and growth

Oral supplements of WSE among mice did not significantly affect either food intake or gain in the body weight at any of the dose employed (data not shown).

4.2.2 Effect on oxidative markers in cerebellum and striatum

Mice given oral supplements of WSE exhibited significant decrease in the endogenous levels of oxidative markers in both regions (Table 3.3). While WSE at the lower dose caused a marginal increase in the oxidative markers, at higher dose significant reduction was evident in cytosolic levels of ROS (Cb: 15%; St: 24%), MDA (Cb: 20%; St: 20%) and hydroperoxides (Cb:13%; St: 30%). Further, the nitric oxide levels were also diminished at the higher doses.

4.2.3 Effect on GSH, total thiols and antioxidant enzymes levels

WSE prophylaxis at the highest dose significantly enhanced the levels of reduced glutathione (Cb: 25%; St:51%) and total thiol content (Cb: 14%; St: 18%) (Table 3.3). Data on the activity levels of antioxidant enzymes and GST in Cb and St regions is presented in Table 3.4. WSE caused consistant elevation
in the activity levels of SOD, CAT and GPx in both regions. Clearly suggesting a
global increase in the enzymic antioxidant levels. However, only a marginal
elevation was evident in the activity levels of GST (Table 3.4).

4.3 Prophylactic effects of WSE against ROT –induced oxidative stress
and neurotoxicity

4.3.1 Protective efficacy of WSE on ROT –induced motor dysfunction

ROT exposure caused a significant alteration in the motor behavior as
assessed by Stride length (SL) and Landing foot spread distance (LFSD). While
the SL among ROT mice was diminished by 15%, the LFSD increased by 24%.
However, mice that received WSE prophylaxis exhibited no reduction in SL and
showed nearly normal LFSD measurements suggesting total protection against
ROT mediated motor dysfunctions (Data not shown).

4.3.2 Modulatory effect of WSE on ROT –induced oxidative Stress

For this study mice were provided with WSE prophylaxis at the dosage of
400mg/kg b.w/day. Mice administered with ROT exhibited marked elevation in
ROS levels in both Cb (50%) and St (30%). Similarly, the cytosolic MDA levels
were also significantly elevated in both regions Cb (22%) and St (35%), while
HP levels were marginally elevated. Intrestingly WSE prophylaxis was found to
offer robust protection against ROT mediated oxidative stress induction, since
both brain regions exhibited nearly normal levels of oxidative markers (Fig. 3.7
A-C). Likewise, ROT exposure also caused significant elevation in the ROS (Cb:
38%; St: 15%), MDA (Cb: 15%; St: 42%) and HP levels (Cb: 13%; St: 22%) in
mitochondria (Fig. 3.8 A-C). In contrast, WSE prophylaxis restored the ROT –
induced increase in the levels of oxidative markers (Fig. 3.8 A-C). Further, ROT
administration resulted in significant elevation in NO levels in cytosol and
mitochondria in both Cb and St regions, which were completely normalized in
cytosol Cb and St, and mitochondria St region (Fig. 3.9 A and B).
4.3.3 Effect on reduced glutathione, total thiols and antioxidant enzymes

ROT exposure caused significant diminution of glutathione levels, which were markedly restored by WSE prophylaxis in both cerebellum and striatum of cytosol and mitochondria of the male mice brain. However, ROT exposure resulted in elevation in the total thiol content in cytosol and mitochondrial fractions and WSE prophylaxis caused further enhancement in the total thiols content (Fig. 3.10 A-D). Data on the modulatory effect of WSE prophylaxis on the activity levels of antioxidant enzyme is presented in the Table 3.5. Among ROT administered mice, both the brain regions showed enhanced activity levels of SOD (Cb: 28%; St: 30%), CAT (Cb: 60%; St: 78%), GPx (Cb: 10%; St: 25%) and GST (Cb: 10%; St: 20%). Interestingly, WSE prophylaxis restored the activity levels to normalcy (Table 3.5).

4.3.4 Effect on ROT–induced changes in neuronal function markers

ROT administration significantly elevated the activity levels of acetylcholinesterase enzyme (Cb: 33%; St: 21%) and WSE prophylaxis restored the same. Further, only the St DA levels were significantly (St 28%) diminished among mice given ROT, while the levels remained unaltered among mice given WSE prophylaxis (Table 3.6).

4.3.5 Effect on the activities of ETC enzymes and MTT in brain regions

ROT exposure resulted in significant perturbation in the activity levels of mitochondrial ETC enzymes. Significant diminution in the activity levels of Complex-I (Cb: 17%; St: 22%) and Complex-II (Cb: 19%; St: 20%) caused by ROT exposure, where as unaltered among mice receiving WSE prophylaxis (Fig. 3.11 A and B). Likewise, WSE prophylaxis also significantly ameliorated MTT (Cb: 12%; St: 32%) reduction and the activity levels of SDH (Cb: 17%; St: 27%) (Fig. 3.11 C and D).
SECTION – B

4.4 Modulatory effects of ferulic acid on endogenous oxidative markers

4.4.1 Effect on body weights

FA prophylaxis for 15 days, did not significantly affect the food intake pattern and the body weights of male mice (Data not shown).

4.4.2 Effect on oxidative markers in cerebellum and striatum

Mice given oral supplements of FA exhibited significant decrease in the endogenous levels of oxidative stress markers in all the regions of the brain examined. Both the doses of FA caused diminished levels of ROS, MDA and HP levels (Table 3.7 and 3.8)

4.4.3 Effect on GSH and antioxidant enzymes levels

FA prophylaxis significantly enhanced the levels of reduced glutathione in all region of the brain (Ct: 12-15%; Cb: 26-34%; Hc: 18-28% and St: 22-25%) (Table 3.9). Further, FA prophylaxis also caused enhanced activity levels of antioxidant enzymes viz., SOD and CAT (Fig. 3.12 A, B and C). The effect of FA was dose dependent. Likewise the activity of GST were also enhanced cosistancely except hippocampus.

4.5 Prophylactic efficacy of FA against ROT -induced oxidative stress and neurotoxicity

4.5.1 FA prophylaxis causes amelioration of oxidative impairments

Mice administered with ROT displayed varing levels of oxidative stress in different brain regions as evident by increased levels of MDA levels, ROS and HP levels (Fig. 3.13 A-F). The affect of ROT appeared to be more pronounced in Cb and St. Minimal changes were evident in Ct and Hc regions. In general mice given FA prophylaxis restored the levels of oxidative markers to normalcy in both Cb and St clearly suggesting its neuroprotective action.

Further, administration of ROT also caused significant oxidative stress in mitochondrial fractions as evident by increasedin the levels of MDA (Cb- 15%; St- 42%), reactive oxygen species (Cb- 38%; St- 14%) and hydroperoxide (Cb-
13%; St- 22%) as depicted in Fig. 3.14 A-F. Interestingly, the FA prophylaxis significantly restored the levels of oxidative markers in mitochondria

4.5.2 Effect on reduced GSH and enzymic antioxidant defenses

ROT administration caused a significant diminution in the GSH levels in cerebellum and striatum which were markedly restored among mice given FA treatment (Table 3.10). However, the glutathione levels were not affected in Ct and Hc. Significant perturbations in the activity levels of antioxidant enzymes were evident in Cb and St (Table 3.10). FA prophylaxis caused varying degree of restoration in the activity levels of antioxidant enzymes. Further, ROT caused significant increase in the levels of GST in St, while FA prophylaxis normalized the activity levels.

4.5.3 Effect of FA prophylaxis on protein carbonyls and nitric oxide levels

ROT administration induced a significant elevation in protein carbonyl levels in brain regions (Ct- 16%; Cb- 18%; St- 27%). Interestingly, FA administered mice showed significant reduction in the levels of protein carbonyls suggesting robust protection (Fig. 3.15 A and B). Likewise, ROT administration also resulted in significantly elevated NO levels (Ct- 32%; Cb- 16%; St- 27%) which were restored among mice given FA supplements (Fig. 3.15 C and D).

4.5.4 Effect on AChE activity and dopamine levels in striatum

ROT treatment caused significant elevation in the activity levels of AChE in brain regions (Cb- 34%; St- 26%). However, FA treatment restored ROT altered activity levels to normalcy (Table 3.11). Further, ROT administration caused significant (28%) depletion DA levels in only in the striatum. However, the depleted DA levels were restored among the mice provided FA prophylaxis (Table 3.11).

4.5.5 Effect of FA prophylaxis on mitochondrial functions

ROT administration caused a significant decrease in the activity levels of succinate dehydrogenase enzyme in striatal region (St- 27%). However, the altered activity levels were restored to the normalcy by the FA prophylaxis.
Further, ROT administration also induced marked decrease in MTT reduction in both Cb and St regions (Cb- 12%; St- 32%) of the brain. Interestingly, the altered levels were significantly restored by the FA treatment (**Fig.3.16 A and B**). Further, it is also evident that the ETC enzymes activities were significantly reduced (complex I: Cb- 21%; St- 24% and complex II: Cb- 19%; St- 20%) up on the administration of the rotenone. However, the ROT altered activity levels were restored near normalcy among mice given FA supplements (**Fig. 3.17 A and B**).

4.6 Synergistic neuroprotective efficacy of WSE+FA against Rotenone induced neurotoxicity

4.6.1 Effect of WSE+FA in modulating ROT–induced oxidative impairments

Administration of ROT caused significant increase in the levels of oxidative markers in both cb and st regions ROS (Cb: 51%; St: 30%), LPO (Cb: 22%; St: 35%) and HP (Cb: 16%; St: 17%). Interestingly, WSE+FA co-treatment significantly abrogated the ROT –induced oxidative impairments as evidenced by significant decrease in the ROS (Cb: 21%; St: 49%), LPO (Cb: 31%; St: 44%), and HP (Cb: 32%; St: 47%) (**Fig. 2.18 A- C**).

4.6.2 Effect of WSE+FA on nitric oxide and total thiols

Administration of ROT to the prepubertal male mice caused significant increase in the levels of NO (Cb: 16%; St: 27%) and total thiols (Cb: 16%; St: 22%) in both the regions (**Table 3.12**). However WSE+FA co-treatment significantly restored the ROT altered levels to normalcy.

4.6.3 Effect on reduced glutathione and antioxidant enzymes levels

Administration of ROT to the prepubertal male mice caused significant decrease in the levels of GSH in both the regions (Cb: 83%; St: 13%) (**Fig. 3.26**). However WSE+FA co-treatment significantly restored the ROT depleted levels of GSH (**Table 3.13**).

ROT exposure with similar paradigm caused significant elevation in the levels of SOD (Cb: 28%; St: 30%), CAT (Cb: 58%; St: 74%) (**Table. 3.13**) and GPx(Cb: 10%; St: 20%). Interestingly, WSE+FA co-treatment significantly up-
regulated levels of SOD (Cb: 28%; St: 43%) and CAT (Cb: 32%; St: 33%), however the levels of GST were significantly abrogated and given significant protection against Rotenone (Cb: 31%; St: 42%) (Table 3.13).

4.6.4 Effect of WSE+FA on cholinergic function

ROT administration with similar paradigm caused significant enhancement in the activity levels of AChE in Cb and St (Cb: 50%; St: 27%). However WSE+FA oral prophylaxis caused significant decrease in the ROT induced perturbations in both the regions of Cb and St (Cb: 37%; St: 28%) (Table 3.14 and Fig. 3.19).

4.6.5 Effect of WSE+FA on mitochondrial functions

ROT administration caused significant inhibition in the activity levels of complex I–III (Cb: 17%; St: 22%) and complex II–III (Cb: 19%; St: 20) in both the Cb and St regions of the mice brain. However, prophylactic co-administration with WSE+FA combination attenuated ROT –induced alterations and offered significant protection (complex I-III: Cb: 17%; St: 22%; complex II-III: Cb: 32%; St: 27%) (Fig. 2.20 A and B).

4.6.6 Synergistic effect of WSE+FA on SDH and MTT

Administration of ROT caused significant decrease in the activity levels of SDH (Cb: 22%; St: 31%) and MTT (Cb and St: 10%). However, the oral administration of WSE+FA rendered significant protection against ROT – induced alteration in the both the region of Cb and St (SDH: Cb: 45%; St: 43%; MTT: Cb: 26%; St: 42%) (Table 3.15).
Table 3.1

Effect of Rotenone administration on body weights and food intake of male mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CTR</th>
<th>ROT1</th>
<th>ROT2</th>
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<td>Body weights (g)</td>
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<td>Final</td>
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<td>Food Intake (g)</td>
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<td>3.3 ± 0.02</td>
<td>3.2 ± 0.03</td>
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<tr>
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<td>3.2 ± 0.02</td>
<td>3.2 ± 0.02</td>
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</table>

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Figure 3.1

Effect of Rotenone administration on oxidative markers in cytosolic fraction of cerebellum and striatum of male mice: Reactive oxygen species (A); Malondialdehyde (B) and Hydroperoxides (C)

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Figure 3.2

Effect of Rotenone administration on oxidative markers in mitochondrial fraction of cerebellum and striatum of male mice; Reactive oxygen species (A); Malondialdehyde (B) and Hydroperoxides (C)

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Figure 3.3

Effect of Rotenone (ROT) administration on the status of reduced Glutathione and total thiols in cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnet’s test’ (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Figure 3.4

Effect of Rotenone administration on the activity levels of antioxidant enzymes: SOD (A); CAT (B) and GPx (C) in cytosolic fraction of cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc 'Dunnett's test' (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Table 3.2

Effect of Rotenone (ROT) administration on the activity levels of Acetylcholinesterase in cerebellum and striatum of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>AChE (pmol DTNB hydrolyzed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cerebellum</td>
</tr>
<tr>
<td>Control</td>
<td>10.80 ± 0.43</td>
</tr>
<tr>
<td>Rotenone1</td>
<td>16.20 ± 0.45*</td>
</tr>
<tr>
<td>Rotenone2</td>
<td>10.14 ± 0.34</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Figure 3.5

Effect of Rotenone (ROT) administration on the activity levels of Acetylcholinesterase in cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Rotenone (ROT): ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Figure 3.6

Effect of Rotenone administration on the activities of Complex-I-III (A), Complex -II-III (B), SDH (C) and MTT (D) in cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

**Rotenone (ROT):** ROT1: 0.5mg/kg b.w/d; ROT2: 1mg/kg b.w/d, i.p.
Table 3.3

Effect of *Withania somnifera* extract (WSEE) on oxidative stress markers, reduced glutathione and total thiols of cytosolic fraction of male mice brain

<table>
<thead>
<tr>
<th>Groups</th>
<th>Withania somnifera (mg/kg b.w/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ROS¹</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>11.73 ± 0.72</td>
</tr>
<tr>
<td>St</td>
<td>9.18 ± 0.58</td>
</tr>
<tr>
<td>MDA²</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>15.69 ± 0.96</td>
</tr>
<tr>
<td>St</td>
<td>20.84 ± 0.84</td>
</tr>
<tr>
<td>HP³</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>7.79 ± 0.33</td>
</tr>
<tr>
<td>St</td>
<td>7.53 ± 0.53</td>
</tr>
<tr>
<td>NO⁴</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>7.04 ± 0.46</td>
</tr>
<tr>
<td>St</td>
<td>6.34 ± 0.22</td>
</tr>
<tr>
<td>GSH⁵</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>30.69 ± 1.66</td>
</tr>
<tr>
<td>St</td>
<td>28.09 ± 1.72</td>
</tr>
<tr>
<td>TSH⁶</td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>12.95 ± 0.55</td>
</tr>
<tr>
<td>St</td>
<td>17.92 ± 0.82</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

ROS¹: Reactive oxygen species- pmol DCF/min/mg protein
MDA²: Malondialdehyde- nmol MDA/mg protein
HP³: Hydroperoxides- nmol hydroperoxides/mg protein
NO⁴: Nitric oxide- Absorbance/mg protein
GSH⁵: Reduced glutathione- µg GSH/mg protein
TSH⁶: Hydroperoxides- nmol hydroperoxides/mg protein
Table 3.4

Activity levels of antioxidant enzymes in cerebellum and striatum of male mice given oral supplements of *Withania somnifera* extract for 4 weeks

<table>
<thead>
<tr>
<th>Groups</th>
<th>Withania somnifera (mg/kg b.w/d)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD¹</td>
<td>Cb</td>
<td>12.56 ± 0.62</td>
<td>13.56 ± 0.71</td>
<td>14.35 ± 0.55*</td>
<td>15.01 ± 0.73*</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>19.62 ± 0.68</td>
<td>19.62 ± 0.72</td>
<td>20.69 ± 0.62</td>
<td>22.07 ± 0.83*</td>
</tr>
<tr>
<td>CAT²</td>
<td>Cb</td>
<td>3.21 ± 0.16</td>
<td>3.38 ± 0.12</td>
<td>3.56 ± 0.11*</td>
<td>3.62 ± 0.14*</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>3.51 ± 0.14</td>
<td>3.71 ± 0.11</td>
<td>3.99 ± 0.10</td>
<td>4.06 ± 0.16</td>
</tr>
<tr>
<td>GPx³</td>
<td>Cb</td>
<td>12.42 ± 0.63</td>
<td>14.40 ± 0.69</td>
<td>15.10 ± 0.59*</td>
<td>17.07 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>14.22 ± 0.54</td>
<td>15.60 ± 0.67</td>
<td>16.60 ± 0.71</td>
<td>20.89 ± 0.75*</td>
</tr>
<tr>
<td>GST⁴</td>
<td>Cb</td>
<td>31.32 ± 1.56</td>
<td>27.60 ± 1.82</td>
<td>32.90 ± 1.78</td>
<td>34.25 ± 1.54</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>40.10 ± 1.91</td>
<td>42.05 ± 1.78</td>
<td>42.60 ± 1.69</td>
<td>43.29 ± 1.76</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6);
Data was analyzed by one way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Cb: Cerebellum; St: Striatum

SOD¹: Superoxide dismutase- Units/mg protein.
CAT²: Catalase- nmol hydroperoxides/min/mg protein
GPx³: Glutathione peroxidase- µmol NADH oxidized/min/mg protein
GST⁴: Glutathione-s-transferase- µmol GS-DNB oxidized/min/mg protein
Figure 3.7

Modulatory effect of *Withania somnifera* supplements on Rotenone-induced oxidative stress in cerebellum and striatum of male mice: ROS (A); MDA (B) and HP (C)

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc 'Tukey's test' (*a,b* p<0.05).

Rotenone (ROT): 0.5mg/kg b.w/d, i.p, 7d

*Withania somnifera* (WSE): 400mg/kg b.w/d, oral, 4 wks.
Figure 3.8

Modulatory effect of *Withania somnifera* on Rotenone -induced oxidative stress in mitochondrial fractions of cerebellum and striatum of male mice: ROS (A); MDA (B) and HP (C).

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (a,b,p<0.05).

**ROS**: Reactive oxygen species; **MDA**: Malondialdehyde; **HP**: Hydroperoxides

**Rotenone (ROT)**: 0.5mg/kg b.w/d, i.p, 7d;

**Withania somnifera (WSE)**: 400mg/kg b.w/d, oral, 4 wks.
Figure 3.9

Modulatory effect of *Withania somnifera* prophylaxis on Rotenone – induced nitric oxide levels in cytosol (A) and mitochondria (B) of cerebellum and striatum of male mice

![Graph A](image1)

**A**

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (a,b,p<0.05).

**Rotenone (ROT):** 0.5mg/kg b.w/d, i.p, 7d

**Withania somnifera (WSE):** 400mg/kg b.w/d, oral, 4 wks.
Figure 3.10

Modulatory effect of *Withania somnifera* on Rotenone-induced perturbations in cytosolic GSH (A), TSH (B) and mitochondrial GSH (C), TSH (D) of cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (a, b \( p<0.05 \)).

**Rotenone (ROT):** 0.5mg/kg b.w/d, i.p, 7d  
*Withania somnifera (WSE):* 400mg/kg b.w/d, oral, 4 wks.
Table 3.5

Modulatory effect of *Withania somnifera* prophylaxis on Rotenone-induced alteration in the levels of antioxidant enzymes in cerebellum and striatum of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTR</th>
<th>ROT</th>
<th>ROT + WSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD(^1) Cyto</td>
<td>Cb</td>
<td>16.66 ± 0.65</td>
<td>21.34 ± 0.77*</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>10.92 ± 0.48</td>
<td>14.20 ± 0.45*</td>
</tr>
<tr>
<td></td>
<td>Mito</td>
<td>Cb</td>
<td>22.00 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>14.90 ± 0.49</td>
<td>12.30 ± 0.55</td>
</tr>
<tr>
<td>CAT(^2) Cyto</td>
<td>Cb</td>
<td>2.30 ± 0.11</td>
<td>3.70 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>0.90 ± 0.04</td>
<td>1.60 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>Mito</td>
<td>Cb</td>
<td>2.80 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>4.10 ± 0.12</td>
<td>1.70 ± 0.05*</td>
</tr>
<tr>
<td>GPx(^3) Cyto</td>
<td>Cb</td>
<td>34.70 ± 1.10</td>
<td>38.30 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>25.00 ± 1.11</td>
<td>30.00 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>Mito</td>
<td>Cb</td>
<td>37.70 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>27.40 ± 1.41</td>
<td>24.60 ± 1.12</td>
</tr>
<tr>
<td>GST(^4) Cyto</td>
<td>Cb</td>
<td>34.70 ± 1.09</td>
<td>38.30 ± 1.13</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>25.00 ± 1.61</td>
<td>30.00 ± 1.12*</td>
</tr>
<tr>
<td></td>
<td>Mito</td>
<td>Cb</td>
<td>26.70 ± 1.24</td>
</tr>
<tr>
<td></td>
<td>St</td>
<td>37.30 ± 1.22</td>
<td>40.30 ± 1.83</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s test’ (*p<0.05).

**Cyto**: Cytosol; **Mito**: Mitochondria; **Cb**: Cerebellum; **St**: Striatum

**SOD\(^1\)**: Superoxide dismutase- Units/mg protein.
**CAT\(^2\)**: Catalase- nmol Hydroperoxides/min/mg protein
**GPx\(^3\)**: Glutathione peroxidase- µmol NADH oxidized/min/mg protein
**GST\(^4\)**: Glutathione-s-transferase- µmol GS-DNB oxidized/min/mg protein
Table 3.6

Effect of Rotenone (ROT) administration on the activity levels of Acetylcholinesterase in cerebellum and striatum of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>AChE$^1$ Cerebellum</th>
<th>AChE$^1$ Striatum</th>
<th>DA$^2$ Cerebellum</th>
<th>DA$^2$ Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.81 ± 0.80</td>
<td>10.11 ± 0.92</td>
<td>13.5 ± 0.57</td>
<td>15.5 ± 0.27</td>
</tr>
<tr>
<td>ROT</td>
<td>16.17 ± 0.98*</td>
<td>12.87 ± 1.04*</td>
<td>11.6 ± 0.43</td>
<td>11.2 ± 0.21*</td>
</tr>
<tr>
<td>ROT + WSE</td>
<td>12.44 ± 1.02</td>
<td>13.91 ± 1.01**</td>
<td>13.1 ± 0.25</td>
<td>15.3 ± 0.19**</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (*p<0.05).

AChE$^1$- Acetylcholinesterase- pmol DTNB hydrolyzed/min/mg protein
DA$^2$ – Dopamine- µg DA/mg protein
Figure 3.11

Modulatory effect of *Withania somnifera* prophylaxis on Rotenone-induced alterations in the activity levels of Comp-I-III (A), Comp-II-III (B), SDH (C) and MTT reduction (D) in cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (*a,b,p<0.05).

**Comp-I-III:** Complex I-III; **Comp-II-III:** Complex II-III; **SDH:** Succinate dehydrogenase; **MTT:** MTT reduction

**Rotenone (ROT):** 0.5mg/kg b.w/d, i.p, 7d
**Withania somnifera (WSE):** 400mg/kg b.w/d, oral, 4 wks.
Table 3.7
Effect of Ferulic acid supplements on endogenous oxidative stress markers in cytosol of different brain regions of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTR</th>
<th>FA1</th>
<th>FA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS(^1)</td>
<td>12.05±1.05</td>
<td>10.79±0.67</td>
<td>10.11±0.55*</td>
</tr>
<tr>
<td>MDA(^2)</td>
<td>17.27±1.00</td>
<td>14.94±0.75</td>
<td>14.50±0.30*</td>
</tr>
<tr>
<td>HP(^3)</td>
<td>5.57±0.35</td>
<td>14.97±0.47</td>
<td>4.91±0.22</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS(^1)</td>
<td>12.71±0.95</td>
<td>10.80±0.61*</td>
<td>11.08±0.59</td>
</tr>
<tr>
<td>MDA(^2)</td>
<td>16.65±0.55</td>
<td>14.28±0.44</td>
<td>14.67±0.53</td>
</tr>
<tr>
<td>HP(^3)</td>
<td>6.50±0.45</td>
<td>5.26±0.27*</td>
<td>5.90±0.32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTR</th>
<th>FA1</th>
<th>FA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS(^1)</td>
<td>7.37±0.45</td>
<td>6.56±0.37</td>
<td>7.05±0.45</td>
</tr>
<tr>
<td>MDA(^2)</td>
<td>12.36±0.41</td>
<td>10.97±0.75</td>
<td>11.35±0.50</td>
</tr>
<tr>
<td>HP(^3)</td>
<td>6.45±0.25</td>
<td>5.72±0.67</td>
<td>5.45±0.15</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS(^1)</td>
<td>11.46±0.75</td>
<td>10.08±0.37</td>
<td>9.69±0.60*</td>
</tr>
<tr>
<td>MDA(^2)</td>
<td>15.96±0.25</td>
<td>13.37±0.75</td>
<td>13.80±0.45</td>
</tr>
<tr>
<td>HP(^3)</td>
<td>5.10±0.45</td>
<td>4.31±0.34</td>
<td>4.22±0.23</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Ferulic acid (FA): FA1: 25 mg/kg b.w/d; FA2: 50mg/kg b.w/d, Oral, 15d
ROS\(^1\): Reactive oxygen species- pmol DCF/min/mg protein
MDA\(^2\): Malondialdehyde- nmol MDA/mg protein
HP\(^3\): Hydroperoxides- nmol hydroperoxides/mg protein
Table 3.9

Effect of Ferulic acid supplements on reduced glutathione levels in different brain regions of male mice

<table>
<thead>
<tr>
<th>Groups/ Brain regions</th>
<th>CTR ± SE</th>
<th>FA1 ± SE</th>
<th>FA2 ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>12.0 ± 0.34</td>
<td>13.5 ± 0.35</td>
<td>13.9 ± 0.87</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>13.6 ± 0.43</td>
<td>17.1 ± 0.32*</td>
<td>18.2 ± 1.30*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>12.2 ± 0.67</td>
<td>14.4 ± 0.98*</td>
<td>15.7 ± 0.85*</td>
</tr>
<tr>
<td>Striatum</td>
<td>14.8 ± 0.45</td>
<td>18.1 ± 0.97*</td>
<td>18.4 ± 1.05*</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

**Ferulic acid (FA): FA1**: 25 mg/kg b.w/d; **FA2**: 50mg/kg b.w/d, oral, 15d
Figure 3.12

Effect of Ferulic acid supplements on endogenous levels of antioxidant enzymes and glutathione-s-transferase in different brain regions of adult male mice: SOD (A); CAT (B) and GST (C)

Values are mean ± SE (n=6); Data was analyzed by One way ANOVA followed by post hoc ‘Tukey’s test’ (‘p<0.05).

Ferulic acid (FA): FA1: 25 mg/kg b.w/d; FA2: 50mg/kg b.w/d, oral, 15d
Figure 3.13

Modulatory effect of Ferulic acid supplements on Rotenone -induced impairments in cytosolic oxidative markers in different brain regions of male mice: ROS (A, D); MDA (B, E) and HP (C, F)

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Ferulic acid (FA): 25mg/kg b.w/d, 15days; Rotenone (ROT): 0.5mg/kg b.w/d, 7d
Figure 3.14

Modulatory effect of Ferulic acid supplements on Rotenone -induced impairments in mitochondrial oxidative markers in different brain regions of male mice: ROS (A, D); MDA (B, E) and HP (C, F)

![Graphs showing modulatory effect of Ferulic acid supplements on Rotenone-induced impairments in mitochondrial oxidative markers in different brain regions of male mice: ROS (A, D); MDA (B, E) and HP (C, F).]

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

**Ferulic acid (FA):** 25mg/kg b.w/d, 15days; **Rotenone (ROT):** 0.5mg/kg b.w/d, 7d
Table 3.10

Modulatory effect of Ferulic acid prophylaxis on ROT- induced alteration in reduced glutathione and activities of antioxidant enzyme levels in cytosolic fractions of different brain regions of male mice

<table>
<thead>
<tr>
<th>Parameters/Groups</th>
<th>Brain regions</th>
<th>Cortex</th>
<th>Cerebellum</th>
<th>Hippocampus</th>
<th>Striatum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH</strong>&lt;sup&gt;a&lt;/sup&gt; CTR</td>
<td>12.04 ± 1.2</td>
<td>13.58 ± 1.6</td>
<td>12.24 ± 2.3</td>
<td>14.77 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>ROT</td>
<td>10.04 ± 1.8</td>
<td>2.28 ± 0.9</td>
<td>14.96 ± 3.0</td>
<td>12.31 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>ROT+FA</td>
<td>10.87 ± 2.2</td>
<td>7.62 ± 1.5*</td>
<td>15.89 ± 0.03</td>
<td>15.89 ± 2.8**</td>
<td></td>
</tr>
<tr>
<td><strong>SOD</strong>&lt;sup&gt;b&lt;/sup&gt; CTR</td>
<td>13.08 ± 1.01</td>
<td>16.66 ± 0.69</td>
<td>13.15 ± 1.87</td>
<td>10.92 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>ROT</td>
<td>11.52 ± 2.10</td>
<td>21.34 ± 0.73</td>
<td>10.49 ± 1.68</td>
<td>14.23 ± 0.67</td>
<td></td>
</tr>
<tr>
<td>ROT+FA</td>
<td>14.49 ± 1.41**</td>
<td>20.08 ± 2.95</td>
<td>18.23 ± 2.63**</td>
<td>15.15 ± 1.19</td>
<td></td>
</tr>
<tr>
<td><strong>CAT</strong>&lt;sup&gt;c&lt;/sup&gt; CTR</td>
<td>1.87 ± 0.091</td>
<td>2.32 ± 0.011</td>
<td>2.87 ± 0.028</td>
<td>0.90 ± 0.040</td>
<td></td>
</tr>
<tr>
<td>ROT</td>
<td>2.00 ± 0.082</td>
<td>3.71 ± 0.012*</td>
<td>3.72 ± 0.037</td>
<td>1.72 ± 0.011*</td>
<td></td>
</tr>
<tr>
<td>ROT+FA</td>
<td>1.77 ± 0.022</td>
<td>2.86 ± 0.032**</td>
<td>3.40 ± 0.026</td>
<td>1.61 ± 0.029**</td>
<td></td>
</tr>
<tr>
<td><strong>GST</strong>&lt;sup&gt;d&lt;/sup&gt; CTR</td>
<td>28.03 ± 1.04</td>
<td>31.32 ± 1.56</td>
<td>19.06 ± 1.26</td>
<td>40.10 ± 1.91</td>
<td></td>
</tr>
<tr>
<td>ROT</td>
<td>31.32 ± 1.21</td>
<td>38.23 ± 1.71</td>
<td>22.89 ± 1.17</td>
<td>47.99 ± 1.51*</td>
<td></td>
</tr>
<tr>
<td>ROT+FA</td>
<td>29.94 ± 1.01</td>
<td>37.14 ± 1.51</td>
<td>19.41 ± 1.41</td>
<td>30.40 ± 1.72**</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Dunnett’s test’ (*p<0.05).

Ferulic acid (FA): 25mg/kg b.w/d, 15days; Rotenone (ROT): 0.5mg/kg b.w/d, 7d

GSH<sup>a</sup>: µg GSH/mg protein  
SOD<sup>b</sup>: Units/mg protein  
CAT<sup>c</sup>: nmol hydroperoxides/min/mg protein  
GST<sup>d</sup>: µmol GS-DNB/min/mg protein
Figure 3.15

Modulatory effect of Ferulic acid supplements on ROT–induced elevation in cytosolic protein carbonyls (A, B) and nitric oxide (C, D) in different brain regions of male mice

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Ferulic acid (FA): 25mg/kg b.w, 15days; Rotenone (ROT): 0.5mg/kg b.w, 7d
Table 3.11

Modulatory effect of FA supplements on ROT –induced alteration in AChE and Striatal Dopamine levels in different brain regions of male mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTR</td>
</tr>
<tr>
<td>AChE</td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>7.72 ± 0.44</td>
</tr>
<tr>
<td>Striatum</td>
<td>7.21 ± 0.34</td>
</tr>
<tr>
<td>Dopamine</td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>3.14 ± 0.14</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

** Acetylcholinesterase**: pmol substrate hydrolyzed/min/mg protein
** Dopamine**: µg dopamine/mg protein

**Ferulic acid (FA)**: 25mg/kg b.w/d, 15days; **Rotenone (ROT)**: 0.5mg/kg b.w/d, 7d
Figure 3.16

Modulatory effect of Ferulic acid prophylaxis on ROT –induced alteration in mitochondrial SDH (A), MTT (B) levels in different brain regions of adult male mice

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

SDH: Succinate dehydrogenase; MTT: MTT reduction assay

**Ferulic acid (FA):** 25mg/kg b.w/d, 15days; **Rotenone (ROT):** 0.5mg/kg b.w/d, 7d
Figure 3.17

Modulatory effect of FA prophylaxis on ROT –induced alteration in mitochondrial ETC enzymes; Comp I-III (A, B), Comp II-III (C, D) in different brain regions of adult male mice

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

**Comp I-III**: Complex I-III; **Comp II-III**: Complex II-III

**Ferulic acid (FA)**: 25mg/kg b.w/d, 15days; **Rotenone (ROT)**: 0.5mg/kg b.w/d, 7d
Figure 3.18

Combined effect of WSE+FA on ROT –induced impairments in endogenous oxidative markers in different brain regions of male mice: ROS (A); MDA (B) and HP (C)

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Rotenone (ROT) - i.p, 0.5mg/kg b.w, 7days; Ferulic acid (FA): Oral, 25mg/kg b.w, 15 days; Withania somnifera (WSE): 400mg/kg b.w 4weeks
Table 3.12

Combined effect of WSE+FA oral supplementation on levels of nitric oxide and total thiols in different brain regions of male mice administered with ROT

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTR</th>
<th>ROT</th>
<th>ROT+WSE</th>
<th>ROT+WSE+FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>5.51 ± 0.32</td>
<td>6.39 ± 0.19</td>
<td>5.09 ± 0.23**</td>
<td>4.13 ± 0.23**</td>
</tr>
<tr>
<td>St</td>
<td>4.96 ± 0.35</td>
<td>6.31 ± 0.37*</td>
<td>4.50 ± 0.36**</td>
<td>3.24 ± 0.21**</td>
</tr>
<tr>
<td>Total thiols&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>10.53 ± 0.53</td>
<td>12.18 ± 0.57</td>
<td>13.92 ± 0.62</td>
<td>14.66 ± 0.65**</td>
</tr>
<tr>
<td>St</td>
<td>14.33 ± 0.65</td>
<td>17.54 ± 0.59*</td>
<td>21.47 ± 0.97**</td>
<td>22.24 ± 0.89**</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s test’ (*p<0.05)

<sup>1</sup>Nitric oxide – Absorbance/mg protein
<sup>2</sup>Total thiols – nmol DTNB oxidized/mg protein

Rotenone (ROT) – 0.5mg/kg b.w, i.p, 7days;
Ferulic acid (FA): 25 mg/kg b.w, oral, 15 days;
Withania somnifera (WSE): 400mg/kg b.w, oral, 4weeks
Table 3.13

Combined effect of WSE+FA supplementation against ROT – induced alteration in the level of reduced GSH and antioxidant activity levels in cytosolic fraction of different brain regions of male mice

<table>
<thead>
<tr>
<th>Brain regions</th>
<th>Groups</th>
<th>Control</th>
<th>ROT</th>
<th>ROT+WSE</th>
<th>ROT+WSE+FA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GSH$^1$</td>
<td>SOD$^2$</td>
<td>CAT$^3$</td>
<td>GST$^4$</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td>13.58 ± 0.94</td>
<td>16.66 ± 1.04</td>
<td>2.3 ± 0.04</td>
<td>34.3 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.27 ± 0.12*</td>
<td>21.34 ± 1.75*</td>
<td>3.7 ± 0.01*</td>
<td>38.3 ± 1.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.63 ± 0.11**</td>
<td>25.43 ± 1.32**</td>
<td>2.8 ± 0.02**</td>
<td>32.1 ± 1.77**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.13 ± 0.10**</td>
<td>27.23 ± 1.23**</td>
<td>2.48 ± 0.03**</td>
<td>26.43 ± 1.24**</td>
</tr>
<tr>
<td>Striatum</td>
<td></td>
<td>14.77 ± 0.95</td>
<td>10.92 ± 0.90</td>
<td>0.9 ± 0.002</td>
<td>25.0 ± 1.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.90 ± 0.89*</td>
<td>14.20 ± 0.84*</td>
<td>1.6 ± 0.01*</td>
<td>30.0 ± 2.04*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.19 ± 0.86**</td>
<td>18.94 ± 1.34**</td>
<td>1.2 ± 0.01**</td>
<td>22.4 ± 1.63**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.12 ± 1.34**</td>
<td>20.24 ± 1.22**</td>
<td>1.03 ± 0.01**</td>
<td>17.34 ± 1.01**</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

GSH$^1$: Reduced glutathione: $\mu$g GSH/mg protein
SOD$^2$: Superoxide dismutase: Units/mg protein.
CAT$^3$: Catalase: nmol Hydroperoxides/min/mg protein
GST$^4$: Glutathione peroxidase: $\mu$mol NADH oxidized/min/mg protein
GPx$^5$: Glutathione-s-transferase: $\mu$mol GS-DNB oxidized/min/mg protein

Rotenone (ROT) - i.p. 0.5mg/kg b.w, 7days; Ferulic acid (FA): oral, 25 mg/kg b.w, 15 days; Withania somnifera (WSE): 400mg/kg b.w 4weeks
Table 3.14

Combined effect of WSE+FA supplementation on ROT–induced acetylcholinesterase activity levels in cytosolic fraction of cerebellum and striatum of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>AChE (pmol DTNB hydrolyzed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cerebellum</td>
</tr>
<tr>
<td>Control</td>
<td>10.81 ± 0.45</td>
</tr>
<tr>
<td>ROT</td>
<td>16.17 ± 0.56</td>
</tr>
<tr>
<td>ROT+WSE</td>
<td>12.44 ± 0.77</td>
</tr>
<tr>
<td>ROT+WSE+FA</td>
<td>10.23 ± 0.86</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Figure 3.19

Combined effect of WSE+FA oral supplementation on ROT–induced acetylcholinesterase activity levels in cytosolic fraction of cerebellum and striatum of male mice

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Rotenone (ROT) - i.p, 0.5mg/kg b.w, 7days; Ferulic acid (FA): oral, 25 mg/kg b.w, 15 days; Withania somnifera (WSE): 400mg/kg b.w 4weeks
Figure 3.20

Synergistic modulatory effect of WSE+FA oral supplementation on ROT altered impairments in the activity levels of Complex I-III (A) and Complex II-III (B) in mitochondrial fraction of different brain regions of male mice.

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s’ test (*p<0.05).

Rotenone (ROT) - i.p, 0.5mg/kg b.w, 7days; Ferulic acid (FA): oral, 25 mg/kg b.w, 15 days; Withania somnifera (WSE): 400mg/kg b.w 4weeks
Table 3.15

Synergistic modulatory effect of WSE+FA oral supplementation on levels of succinate dehydrogenase and MTT in different brain regions of male mice administered with ROT

<table>
<thead>
<tr>
<th>Groups</th>
<th>CTR</th>
<th>ROT</th>
<th>ROT+WSE</th>
<th>ROT+WSE+FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDH¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>7.2 ± 0.43</td>
<td>5.6 ± 0.39*</td>
<td>6.7 ± 0.54**</td>
<td>8.2 ± 0.69**</td>
</tr>
<tr>
<td>St</td>
<td>7.4 ± 0.54</td>
<td>5.1 ± 0.52*</td>
<td>6.4 ± 0.55**</td>
<td>7.2 ± 0.57**</td>
</tr>
<tr>
<td>MTT²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb</td>
<td>10.4 ± 0.53</td>
<td>9.5 ± 0.57</td>
<td>10.6 ± 0.62</td>
<td>11.9 ± 0.65**</td>
</tr>
<tr>
<td>St</td>
<td>9.6 ± 0.65</td>
<td>8.7 ± 0.59</td>
<td>11.5 ± 0.97**</td>
<td>12.4 ± 0.89**</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n=6); Data was analyzed by one way ANOVA followed by post hoc ‘Tukey’s test’ (*p<0.05)

SDH¹ – Succinate dehydrogenase: ng INT reduced/min/mg protein

MTT² – MTT reductase: Absorbance/mg protein

Rotenone (ROT) – 0.5mg/kg b.w, i.p, 7days;
Ferulic acid (FA): 25 mg/kg b.w, oral, 15 days;
Withania somnifera (WSE): 400mg/kg b.w, oral, 4weeks
5.0 DISCUSSION

Neuromodulatory efficacy of WSE

Due to the fact that WS root extracts are extensively used in ayurvedic system of medicine, they are generally considered as safe. However, their large scale applications and the availability of various forms formulations over the counter with specific health benefits, a comprehensive understanding of its possible mechanism/s of action in vivo is relevant (Gokul, manjunath and Muralidahra, 2012; Alam et al., 2012). Although earlier studies have documented the antioxidant and immune-modulatory activity of WS, it is only recently that researchers are examining its neuromodulatory potential employing various animal models (Sankar et al., 2007; Ahmad et al., 2005; Kumar and Kumar, 2009; Kumar et al., 2010; Sehgal et al., 2012). Accordingly in the present study, the potential of WSE to modulate levels of endogenous oxidative markers in specific brain regions of mice was investigated. Since this model aimed to understand the neurotoxic susceptibility that could be extrapolated to children and preadolescents, it was logical to examine its prophylactic efficacy in young mice employing a well-known neurotoxin such as rotenone. Further, the objective of selecting young mice was based on the following criteria: i) the brain of a 4 week old mice is still in the process of developing new inter neuronal connections and will continue during the postnatal development (Spear, 2007) ii) lack of data on the neuromodulatory potential of WSE prophylaxis and ii) the requirement of enhancement of cognitive abilities during the growing stages.

The therapeutic doses of WSE employed in the present study have been reported to be generally not associated with any known side effects (Alam et al., 2012). In the preliminary dose-determinative study, no symptoms or mortality was evident among mice orally administered with WSE even at a dosage 400mg/kg b.w/d for 4 weeks. This is consistent with an earlier report in which the acute toxicity of ashwagandholine (total alkaloids from WS roots) in mice was reported to be 465mg/kg b.w/d (Malhotra et al., 1965). Further, oral supplements of WSE significantly reduced the levels of endogenous oxidative markers and nitric oxide levels in brain regions viz., St and Cb clearly
suggesting its potential to attenuate neurotoxic insult/s. Enhanced levels of GSH and thiols in these brain regions with concomitant elevation in the activity levels of antioxidant enzymes (such as SOD, GPx) may further render the brain regions less susceptible to neurotoxic -mediated oxidative dysfunctions. Based on this property, it can be presumed that WSE serve as a good candidate for neuroprotection (Dumont and Beal, 2011; Girish and Muralidhara, 2012). These findings are consistent with the antioxidant activity of WS reported previously in vitro and in vivo. Withanolides of WS administered (i.p) to rats at low doses (10-20 mg/kg b.w/d, 21d) were previously shown to enhance the activity levels of various antioxidant enzymes in frontal cortex and striatum (Bhattacharya et al., 1997b; Bhattacharya et al., 2001). Studies in vitro employing chemical systems also demonstrated the ability of WSE to scavenge various radicals such as DPPH, superoxide, nitric oxide radicals and inhibition of deoxyribose oxidation (as presented in Chapter 2). These data are in line with the recent findings in which an aqueous extract of WS exhibited significant neuroprotective effect against hydrogen peroxide and Aβ-induced cytotoxicity in PC 12 cells in vitro (Jayaprakasham et al., 2010; Kumar et al., 2010).

Chemical models of PD that have been primarily developed to understand the pathophysiology of the disease are often useful in exploring the protective efficacy of various putative phytochemicals for their possible therapeutic use (Betarbet et al., 2000; Sherer et al., 2007; Swarnkar et al., 2010; Santiago et al., 2010; Shinomol et al., 2012; Coulom and Birman, 2004; Girish and Muralidhara, 2012). Although the effects of ROT in adult rodents are well known, the susceptibility of young mice to short-term ROT exposure have been less investigated. Consistent with previous findings from our laboratory (Girish and Muralidhara, 2012), in the present study ROT caused significant oxidative stress in St and Cb. Interestingly, St showed robust increase in the levels of ROS, hydroperoxides, and MDA. Besides, St displayed maximum increase in total protein carbonyls and depletion of GSH thus indicating some mode of selectivity by ROT. Data obtained in behavioural studies indicated that ROT – induced attenuation of motor function in mice was significantly alleviated by WSE prophylaxis. This data corroborates with one finding in Drosophila system,
wherein WSE offered robust protection against ROT–induced motor deficits in a co-exposure paradigm (Manjunath and Muralidhara, 2015, Chapter 2).

Following the recapitulation of ROT–induced oxidative stress and neurotoxic effects in prepubertal male mice, the efficacy of WSE to offset these events was investigated employing a prophylactic paradigm. In the present model, mice were provided with WSE prophylaxis for 4 weeks. As anticipated, ROT–induced significant oxidative stress in both cytosolic and mitochondrial fractions of St and Cb (Manjunath and Muralidhara, 2013). The major findings in the present model such as marked elevation in ROS and MDA levels, alterations in the activities of antioxidant enzymes, diminished GSH levels in St and Cb clearly suggested that the brain regions are indeed subjected to significant oxidative stress (Aoyama et al., 2008).

Interestingly, WSE prophylaxis completely attenuated ROT–induced oxidative stress in cytosol in St and Cb suggesting its ability to render protection against neurotoxicant insult. However, the differential effect observed in brain regions evidenced with WSE per se (absence of neurotoxin) and in the prophylactic study (under conditions of neurotoxin exposure) may be attributed to the altered dynamics of the prevailing oxidative status in vivo. Interestingly this data is consistent with our recent findings in Drosophila system wherein WS-enriched diet not only diminished the basal levels of oxidative markers, but also offered marked protection against ROT–induced mortality oxidative stress and neurotoxicity (Manjunath and Muralidhara, 2015). In the present study, WSE prophylaxis significantly attenuated ROT–induced motor dysfunction as evidenced by restoration of LFSD measurements. We have also obtained similar neuroprotective effect of WSE against 3-NPA–induced oxidative stress and mitochondrial dysfunctions in St suggesting its broad neuromodulatory activity (Manjunath and Muralidhara, unpublished).

GSH-associated metabolism is a major mechanism for cellular protection against toxicants that generate oxidative stress (Denny and Muralidhara, 2012; Bharath and Andersen, 2005). GSH participates in detoxification at several levels, and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, GSH provides the cell with multiple defenses not only against ROS, but also against their toxic products (Bharath and
Andersen, 2005). In this study, WSE supplements besides enhancing the basal levels of GSH, total/ non-protein thiols in cytosol of brain regions in mice, also offered significant protection against ROT –induced GSH depletion as demonstrated by the restoration of GSH levels in these brain regions. Interestingly, one of the most important events which occur during the pathogenesis of PD is depletion of cytoplasmic GSH within the substantia nigra dopaminergic neurons and one of the approaches to alleviate the problem is to replenish the GSH pool either by increasing the synthesis or by slowing its metabolism (Bharath and Andersen, 2005). Accordingly, we speculate that GSH-related mechanism/s may play a vital role in the observed protective effect of WSE against ROT –induced oxidative stress. Studies in cell models would provide better insight on the potential of WSE to modulate GSH metabolism. In addition, the observed enhanced levels of enzymatic antioxidant defenses in St and Cb could also have played a significant role in ameliorating the effects of ROT.

In the present study, the activities of enzymes such as SDH and Complex I-III, II-III were measured in brain regions of ROT administered mice as a marker of altered physiology and membrane damage of mitochondrial function (Rustin et al., 2002). The decrease in the activity levels of SDH among the mice treated with ROT suggests its specific effect on the mitochondrial enzymes. Further, the MTT reduction has been used as a marker for mitochondrial function in view of the fact that, the presence of succinate increases the reduction of MTT to formazan via the system called succinate tetrazolium reductase. As a result, treatment with ROT markedly decreases the MTT levels. Further, ROT administration significantly decreased the NADH depended cytochrome C reductase in both Cb and St. The ETC enzymes play a central role in energy metabolism and also the site for cellular ROS generation (Murphy, 2009). One of the main mechanisms of generation of free radicals following ROT exposure is due to its interference in normal physiology of ETC enzymes. The alterations in SDH and MTT of mitochondria were restored to normalcy among mice provided with WSE prophylaxis. The protective effect of WSE was further discernable in terms of restoration of the Complex I-III and
Complex II-III enzymes, the central enzymes, which play an important role in oxidative stress.

It is well known that inhibition of AChE is an important therapeutic target for the treatment of neurodegenerative diseases (Meshorer et al., 2002). Significant decrease in the activity of AChE indicates an increase in the acetylcholine levels in the synaptic cleft and is known to enable an improvement in cognitive functions. In this study, mice provided with WSE prophylaxis exhibited consistent reduction in the activity of AChE in brain regions clearly suggesting alterations in cholinergic functions in vivo. Consistent with this, earlier studies have demonstrated the AChE inhibitory potential of Withanolides in vitro (Choudhary et al., 2005). Withania somnifera was also found to improve the cognitive capabilities of the brain by increasing the cortical muscarinic acetylcholine capacity in lateral septum and frontal cortex, which suggest their capacity to affect events in the cortical cholinergic-signal transduction cascade (Schliebs et al., 1997). In another study, chronic administration of WS significantly restored the activity levels of AChE in brain regions of rats administered with 3-nitropropionic acid and improved the cognitive functions as well (Kumar and Kumar, 2009). The propensity of WSE to inhibit cholinesterase and its calcium antagonistic ability are speculated to be chiefly responsible for its clinical use in AD and other associated disorders.

In conclusion, our findings clearly suggest that oral supplements of standard WSE possess the potential to modulate endogenous levels of oxidative markers in the brain regions of mice. Further, data obtained in the ROT model clearly demonstrated that compounds present in the WSE provide significant neuroprotection against ROT –induced oxidative stress and motor deficits. This is the first evidence of the potential of WSE against Parkinsonian motor symptoms in mice model and it is largely attributable to its ability to attenuate oxidative stress and mitochondrial dysfunction in striatum and cerebellum resulting in neuroprotection.
Neuromodulatory efficacy of FA and FA enriched WSE

Numerous evidences suggest that phytochemicals might have an impact on brain pathology, aging and neurodegenerative diseases. Epidemiological studies have suggested the potent interaction of dietary flavonoids in the neurodegenerative cascade (Kanski et al., 2002; Ogiwara et al., 2002; Srinivasan et al., 2007). However, neither their mechanisms of action nor their cell targets are completely known. Numerous evidences have demonstrated the beneficial effects of various flavonoids in many models of neurodegenerative diseases viz., Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and Amyotropic lateral sclerosis. Most of the biological roles of flavonoids are related to their antioxidant properties. Dietary intervention studies in several mammalian species, including humans, using flavonoid-rich plant or food extracts have indicated the ability of flavonoids to improve memory and learning by protecting vulnerable cells, enhancing existing neuronal function, or by stimulating neuronal regeneration (Castelluccio et al., 1996; Trombino et al., 2004). It has been speculated that neuroprotective actions of flavonoids are likely to be due to (a) modulation of intracellular signalling cascades which control neuronal survival, death and differentiation, (b) effect on gene expression and c) interactions with mitochondria.

Oxidative stress has been proposed to play a vital role in the pathological development of various neurodegenerative disorders, including PD. Hence after demonstrating the neuroprotective effect of FA in the ROT fly model, we sought to validate its protective efficacy employing a prophylactic paradigm in a mice model of neurotoxicity in order to draw a comparison. Dosage and duration of FA was selected based on our preliminary studies and earlier studies (Bolling et al., 2011; Sultana, 2012). Consistent with our earlier observations, ROT administration caused a significant induction of oxidative stress in brain regions of mice (Shinomol et al., 2012; Manjunath and Muralidhara, 2013). Our salient findings such as marked elevation in ROS, MDA and HP levels, diminished GSH levels and perturbations in the activities of several antioxidant enzymes were suggestive of the oxidative stress induction in brain regions, although not uniform in different regions. Interestingly, FA prophylaxis completely attenuated ROT –induced oxidative stress in cytosol of St, Cb and Ct. However; we did
observe a differential protective effect of FA under ROT exposure with respect to MDA and HP levels, which could be related to the altered dynamics of the oxidative status of the specific brain region. The higher degree of neuroprotection evident in the Drosophila system may be explained due to the less complex system which would allow a higher bioavailability of FA compared to the higher complexity of the organ systems in the rodent model. It is likely that free FA may be better utilized in the fly model due to simple pharmacokinetic distribution.

The mechanism/s underlying or contributing to the neurodegeneration in PD seem to be multifactorial, mitochondrial dysfunctions is widely taken as a central cause for the many forms of the disease. Whether oxidative stress is a cause or a consequence of dopaminergic death, there is substantial evidence for oxidative stress both in human PD patients and in animal models of PD, especially using rotenone, a complex-I inhibitor (Mythri and Bharat, 2011). The modulatory effects of FA in flies were discernible in the mitochondrial milieu as evidenced by significant restoration in the levels of oxidative markers in the mitochondria of head region (unpublished data). Defects in electron transport chain and ROS generation are known to play a significant role in the etiology of various neurodegenerative disorders. In the present model, FA not only diminished the degree of oxidative stress, but also attenuated the activity levels of mitochondrial complex enzymes.

GSH mediated metabolism of neurotoxicants is a major mechanism for cellular protection, since it is not only involved in the detoxification of ROS, but also detoxifies the toxic end products (BalzFerei, 1994). In the present model, ROT administration significantly diminished the GSH levels and the data is consistent with our earlier observation in N27 and mice models (Shinomol et al., 2012). Hence we believe that, the protective effect of FA may be related at least in part to its ability to restore the GSH levels. It may be pertinent here to mention that one of the important events which occur during the pathogenesis of PD in humans is depletion of GSH levels in the substantianigra dopaminergic neurons and accordingly replenishment of the GSH pool either by accelerating its synthesis or slowing down its metabolism have often been used as strategies. Hence we speculate that GSH mediated protective effects may be considered
as important in the observed neuroprotective effects of FA in the ROT model. We have also observed similar protective effects previously with several other phytoconstituents such as *Bacopa monneri* and *Withania somnifera* in the ROT mice model (Shinomol et al., 2012; Manjunath and Muralidhara, 2013). Further, the enhanced activities of several other important enzymatic defenses could also have contributed to the alleviation of ROT –induced oxidative stress. Our data is consistent with the neuroprotective effect of FA measured in terms of cognitive function in mice treated with buthionine sulfoximine (Mamiya et al., 2008). Previously several studies have reported the differential tissue-specific effects of FA and its ethyl ester in terms of induction of enzymic antioxidant defenses (phase II enzymes such as glutathione transferase, Thioredoxin reductase). The protective effect of dietary FA against CCl₄ –induced renal damage through elevated antioxidant enzymes such as SOD and glutathione peroxidase in rats (Srinivasan et al., 2005).

It is well established that one of principal mechanism of generation of free radical under ROT exposure is due to its specific interference in the normal functioning of ETC enzymes. In the resent model, mitochondrial dysfunctions in the brain regions of ROT administered mice were evident as measured by the significant perturbations in the activity levels of SDH and complex I-III and II-III. Further, ROT administration also caused significant decrease in MTT reduction and vital alterations viz., reduced SDH activity and MTT reduction were restored to normalcy among mice given FA prophylaxis. The neuroprotective action of FA was also evident in terms of the restorative effect on the activity levels of ETC enzymes viz., complex I-III and complex II-III clearly suggesting that FA is likely to be acting on several pathways in the mice model.

Previous studies suggest that sodium ferulate protects mice against learning and memory deficits by centrally administered β-amyloid (Yan et al., 2001). Further it is speculated that the primary site of action of FA could be microglia and astrocytes (Kim et al., 2004; Cho et al., 2005). In the present study, another important effect of FA in mice model was on the activity of AChE, which is primarily involved in cholinergic function. As previously observed ROT administration resulted in significant increase in the AChE activity (Shinomol et al., 2013). Interestingly FA prophylaxis restored the AChE activity in striatum,
hippocampus, while the activity levels were above normal in cerebellum and cortex. While the relevance of AChE restorative effect of FA in the ROT model is not clear from this study, it suggests a specific action of FA which can be exploited in therapy of dxneurodegenerative disorders such as PD. Further, ROT administration caused a significant reduction in the striatal DA levels and FA significantly restored the levels in striatum.

6.0 SUMMARY

1. Oral supplements of WSE in mice significantly modulated the endogenous oxidative markers as evidenced with reduced levels of MDA, HP, PC and ROS in brain regions striatum (St) and cerebellum (Cb).

2. WSE significantly elevated the GSH levels in striatal regions, while the effect was marked in Cb.

3. Intraperitonial injection of ROT (1.0 mg/ kg b.w/ d, 7d) causes progressive locomotor deficits in terms of reduced stride length, increased LFSD, increased narrow beam latency. These findings are in lines with the previous reports.

4. The protective effects of WSE (400mg/ kg b.w/d) against ROT –induced neurotoxicity was evident with improved motor function of mice.

5. Interestingly ROT –induced oxidative markers in both cytosol and mitochondria of striatal region were also diminished among mice given WSE prophylaxis.

6. WSE markedly modulated the ROT –induced changes in the antioxidant enzyme activities in striatal tissue.

7. WSE significantly modulated the ROT –induced reduction in the activity levels of mitochondrial marker enzymes in striatum.

8. ROT –induced elevation in the AChE activity was normalized with WSE prophylaxis.

9. In conclusion, these in vivo findings in the ROT model of neurotoxicity clearly demonstrated that WSE supplements provide robust neuroprotective against ROT –induced behavioral phenotype, oxidative stressed neurotoxicity.
11. FA prophylaxis in mice significantly diminished endogenous levels of oxidative markers, enhanced the GSH levels, and antioxidant defense in cerebellum and striatum.

12. In the ROT model, FA prophylaxis offset oxidative stress and protein oxidation.

13. FA markedly restored the activity levels of AChE, replenished the DA levels in striatum and mitochondrial fractions.

14. In general, a combination of FA and WSE offered higher level of protection against ROT–induced oxidative stress and neurotoxicity.

15. Taken together, these findings provide significant evidence on the neuroprotective efficacy of WSE in an experimental paradigm and the possible utilization as a therapeutic adjuvant under conditions of oxidative stress-mediated NDD.

16. Collectively, our findings provide reasonable evidence on the therapeutic potential FA enrichment approach to achieve enhanced neuroprotection. Since FA is ubiquitously present in a variety of commonly consumed foods, increased consumption of FA-rich foods may provide enhanced neuroprotection under specific situations of oxidative stress mediated neurodegenerative disorders such as PD.