CHAPTER-IV

Assessment of Neuromodulatory efficacy of Withania somnifera in STZ-induced diabetic mice model
Preface

The primary objective of this study was to understand the neuroprotective impact of a standardized extract of *Withania somnifera* (WSE) in a Streptozotocin (STZ) –induced diabetic model in mice.
1.0 INTRODUCTION

Diabetes, a rising epidemic around the world, has no signs of abatement and remains one of the supreme challenging health problems (Singh et al., 2012). Diabetes mellitus, a metabolic disorder is characterized by inadequate or complete lack of insulin essential for the metabolism of carbohydrate, proteins and lipids (American Diabetic Association, 2012). Multiple evidences demonstrate that brain disorders are leading to the unavoidable consequence of overt diabetes. Among other factors, a disproportionate elevation in the production of free radicals that deplete cellular antioxidant pool has been implicated in the development of oxidative stress mediated neuronal loss in diabetes (Shrilatha and Muralidhara, 2007; Chandrashekar and Muralidhara, 2009; Kamboj and Sandhir, 2011).

Diabetes encephalopathy is the long term complication of diabetes and is known to be associated with cognitive decline and increased risk of dementia (Biessels et al., 2005). The neurobehavioral and cognitive abnormalities in experimental diabetes are known to be associated with neuronal loss and oligodendrocytes in both gray and white matter of brain. The pathophysiology of diabetic encephalopathy appears to be multifactorial, and the underlying mechanism are poorly understood. Nevertheless a consistent feature common to all cell type loss that are damaged by hyperglycemia is elevated ROS production (Zheng et al., 2009; Kamboj and Sandhir, 2011). Numerous evidences in experimental models of diabetes suggest that oxidative stress underlies cognitive decline which contributes to hyperglycemia –induced neuronal apoptosis (Alvarez et al., 2009; Kamboj et al., 2008).

Oxidative stress is one of the important key factor in the process of neurodegeneration. Neuronal cells are particularly vulnerable to oxidative damage due to their high ATP expenditure and oxygen demand. Cellular depletion of ATP in the central nervous system (CNS) represents a pathophysiologic event leading to a series of biochemical, physiological and morphological changes, leading to reactive oxygen species (ROS) formation and oxidative stress (Dhuna et al., 2013). ROS generation is a vital mechanism accounting for cellular damage in many neurodegenerative disorders (Valko et al., 2006),
affecting the morphological and functional disability of macromolecules such as proteins, membrane lipids and nucleic acids. Although cellular defense against oxidative stress markers (ROS, HP and NO) mediated injury is provided by antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, and nonenzymatic (viz., GSH, α-tocopherol, vitamin C, urate, etc.) radical scavenging systems (Abdollahi et al., 2004), these systems are commonly susceptible of being affected and diminished under pathophysiologic conditions.

Mitochondria are the major source of ROS production in the brain and mitochondrial dysfunctions have been proposed the central mediators of neurodegeneration (DiFilippo et al., 2006). Involvement of mitochondria in hyperglycemia –induced apoptotic pathways have been demonstrated in diabetic neuropathy (Schmeichel et al., 2003; Prasad and Muralidhara, 2014). Since limited quantities of GSH is available in mitochondria, maintenance of adequate redox status is a prerequisite to protect against the deleterious effects of ROS. In view of this therapies based on enhancing GSH levels in mitochondria are speculated to be beneficial in conditions of enhanced mitochondrial ROS production. N-Acetylcystiene (NAC), a precursor of GSH has been in clinical use and is demonstrated to diminish OS in mitochondria in AD patients (Moreira et al., 2007). A recent study has shown that L-cysteine supplements significantly lower the glucose levels, markers of vascular inflammation under diabetes condition (Jain et al., 2009).

Many herbal bioactives per se or in various formulations are being extensively used in traditional medicine of both Indain and Chinese origin. A variety of compounds such as curcumin, cinnamaldehyde, berberine, withanolides, bacopasides have been demonstrated to possess significant preventive as well as curative properties in experimental animal models (Hosamani and Muralidhara, 2010; Manjunath and Muralidhara, 2013; Fenq et al, 2014; Jayaraj et al., 2014). In the past decade, there is a renewed interest toward screening of phytochemicals that can be used to improve diabetes-associated neurological dysfunctions in children and adolescents with high safety value. These phytomedicines are presumed to be safe and less toxic compared to the oral hypoglycaemic agents (such as sulfonylureas, metformin) which are demonstrated to cause various side effecs.
Withania somnifera L. (WSE, Ashwagandha) with antioxidant, antiaging, anti-inflammatory, adaptogenic and neuroprotective properties is widely used to promote health and longevity in ayurvedic formulations (Bhaskar et al., 2014). Earlier studies have shown that WSE bioactives significantly reduce blood glucose levels in mild NIDDM patients (Anadulla et al., 1995). Further, WSE was reported to increase the haemoglobin levels (Ziauddin et al., 1996) in mice model with chemically suppressed immunity. Interestingly, dried fruit extract of Withania coagulans has been shown to possess hypoglycemic potential in type I diabetic rats (Hemalatha et al., 2004).

Growing evidence demonstrates WSE to improve the health of neurons by influencing the elevation in the antioxidant enzyme levels and free radical scavenging in vivo (Manjunath and Muralidhara, 2014). In the recent past, antidiabetogenic effects of WSE is receiving wide attention, as multiple reports suggest its hypoglycemic, antiperoxidative and hypolipidemic properties, which prove to be beneficial in the management of the disease complications (Anwer et al., 2008; Udayakumar et al., 2010).

However, not many comprehensive attempts have been made to assess the modulatory effects of WSE extract in experimental diabetic models. In view of this, the neuroprotective effect of the standardized Withania somnifera extract (WSE) was examined employing a multiple low dose streptozotocin model of mice. The modulatory effects were assessed employing behavioural, haematological, histopathological and biochemical approaches.

2.0 OBJECTIVE

The primary objective of the investigation was to determine the neuroprotective efficacy of WSE in an experimentally induced diabetic mice model employing Streptozotocin (STZ) as diabetogen.
3.0 EXPERIMENTAL DESIGN

3.1 Animals

Adult (4–6 weeks old) male albino rats (CFT-swiss strain) were drawn from the stock colony of the Institute Animal Facility. Animals were provided a commercial chow diet and water ad libitum.

3.2 Diabetogen

Streptozotocin (STZ) was used to induce diabetes in male mice. A multiple low dose of STZ administration protocol was adopted to induce the hyperglycemia in male mice (AMDCC).

3.3 Experimental procedure

Mice were intraperitoneally administered with freshly dissolved STZ (50 mg/kg b.w/d for 5d) in a 0.1mol/liter citrate buffer (pH 4.5). Control mice were injected with citrate buffer. STZ-injected mice were provided with 5% glucose in drinking water for 48 hr. 24 hrs after the last STZ injection, blood glucose levels were measured with an Accuchek comfort sensor glucometer. Animals with glucose levels ≥350 mg/dL were included in the study. On day 7 mice were grouped as follows: Control mice- untreated diabetic mice were also divided into two groups: group II, diabetic untreated; group III, diabetic mice provided oral supplements of WSE 400mg/kg b.w/day, po, 4 weeks. Group I and group III mice received equivolume vehicle alone (saline).

Terminally mice were sacrificed under anesthesia, brain were excised washed several times in saline and blotted dry before sample preparation

3.4 Food intake, growth and blood glucose levels

All mice were monitored daily for feed intake and weekly for body weights throughout the experimental period of 4 weeks. Further, both control and diabetic mice were monitored for blood glucose levels biweekly
3.5 Behavioral assessments

STZ treated mice were assessed for the development of sensory and motor dysfunctions by tail immersion tests (hyperalgesia and allodynia), landing foot spread distance (LFSD), stride length (SL), elevated plus maze (EPM) and rotarod test (as described in Chapter I).

3.6 Hematological parameters

Selected haematological parameters viz., RBC and WBC counts, concentration of Hemoglobin, platelet count and haematocrit value were determined in all the experimental groups following standard procedures.

3.7 Histopathological studies

Standard protocols were followed to process the hippocampus and striatal tissue to examine histological alterations by light microscopy.

3.8 Biochemical alterations

The following biochemical markers were determined in different brain regions

*Induction of oxidative damage, GSH levels and antioxidant enzymes*

The induction of oxidative damage was assayed by measuring the levels of ROS, MDA and HP levels in the cytosol/ mitochondrial fractions and activity levels of selected antioxidant enzymes were also measured in brain regions. As a marker of redox status, GSH levels were also determined.

*Measurement of mitochondrial dysfunctions*

Selected activity levels of mitochondrial enzymes were also determined.

* Determination of neurotoxicity markers*

The modulatory effect of WSE on cholinergic functions were assessed by determining the activity level of AChE. The dopaminergic function was assessed in striatum.
4.0 RESULTS

4.1 Food intake and growth

While the food intake among mice rendered diabetic was relatively higher (15-20%), the body weight gain was not proportional (Table 4.1). Terminally the average body weights were nearly 30% lower compared to non-diabetic mice. However, diabetic mice provided with WSE supplements gained normal weight over the experimental period of 4 weeks. Likewise, the mean liver weights of diabetic mice were markedly reduced (40%) and those given WSE supplements showed improved liver weights.

4.2 Blood glucose levels

Induction of diabetes was ascertained by determining the blood glucose levels 24hrs after the last STZ injection. Initially at the end of week 1, the blood glucose levels in normal untreated mice were 131 ± 4.3mg/dL, STZ mice showed nearly 3-fold increase (385 ± 3.6mg/dL) (Table 4.2 and Figure 4.1). There was a progressive elevation in the levels of blood glucose among the diabetic mice and terminally the levels were 453+ 5.4mg/dL. Interestingly, the blood glucose levels among diabetic mice provided with WSE supplements were significantly lower (285 ± 5.9mg/dL) clearly indicating the hypoglycemic effect.

4.3 Hematological parameters

Data on the some of the hematological parameters determined terminally in all the three groups are presented in Table 4.3. Both RBC and WBC counts among diabetic mice were significantly diminished (nearly 30% decrease) when compared to the non-diabetic mice. Interestingly diabetic mice provided with WSE supplements showed normal counts. The platelets counts were elevated among diabetic mice which significantly restored with WSE supplements.

4.4 Behavioral phenotype

Diabetic mice exhibited significant alteration in the walking ability only beyond week 3. Reduction in stride length (SL) and increased LFSD was
evident among diabetic mice only at week 4. These measurements were restored among diabetic mice which received WSE supplements (Fig. 4.2).

Further, data on the exploratory activity measured terminally among the three groups is presented in Fig. 4.3. Diabetic mice spent more time (30%) in the closed arm and spent less time in the open arm. However, diabetic mice which received WSE supplements displayed normal exploratory activity as evidenced by the time spent in closed and open arm. Likewise in the rotarod test, diabetic mice spent significantly less time at both the sampling points compared to the non-diabetic mice (Fig. 4.4). However diabetic mice provided with WSE supplements showed normal behaviour on the rotarod as evident by the time spent on the rotarod.

Diabetic mice did not exhibit any signs of sensitivity towards either hot or cold stimuli during the first two weeks of STZ injection and the responses were highly comparable to those of controls (Fig. 4.5). However, during the subsequent measurement (week 3 and 4), developed sensitivity towards both hot and cold stimuli. They displayed significant reduction in the latency period for hot hyperalgesia and cold hyperalgesia (reduction was nearly 20-30%). However, diabetic mice provided with WSE supplements showed progressive improvement in both the tests. Terminally diabetic mice showed significant motor deficits, while the WSE supplemented ones were nearly normal.

4.5 WSE modulates oxidative stress in brain

Data on the status of oxidative stress in cytosol measured in terms of ROS, MDA and hydroperoxide levels is presented in Fig. 4.6, 4.7 and 4.8. While a consistent increase in the ROS levels was evident in all brain regions of diabetic mice, the levels were diminished and normalised among diabetic mice provided with WSE supplements. A similar effect of WSE was noticeable in the levels of MDA among diabetic mice. Interestingly the elevated HP levels among diabetic mice were also restored to normalcy with WSE supplements in all the brain regions clearly suggesting the antioxidant property of WSE.
4.6 Effect of WSE on redox status in brain regions

Data on the GSH levels and total thiols in different brain regions in cytosol is presented in Table 4.4. In general the GSH levels were depleted in brain regions of diabetic mice, and were significantly restored among WSE supplemented diabetic mice. Likewise the total thiols determined in different brain regions also showed a similar response. A similar trend was also evident in the mitochondrial GSH levels (Table 4.5).

4.7 WSE restores antioxidant defences in brain regions

Data on the activity levels of enzymic antioxidants, SOD and Catalase are presented in Fig. 4.9 and 4.10. While the activity levels of SOD among diabetic mice were diminished significantly in all the brain regions, the levels were restored to normalcy, in cortex, stratum among mice given WSE supplements. In both cerebellum and hippocampus the activity levels were elevated among diabetic mice which received WSE supplements suggesting differential response. Further, there was a similar effect of WSE with respect to the levels of catalase activity among diabetic mice receiving WSE.

4.8 Modulatory effect of WSE on Phase II enzyme activity

The activity levels of GST enzyme in different brain regions was significantly diminished among diabetic mice (Fig. 4.11). However, the activity levels were not only restored, but were higher than the basal levels among diabetic mice provided with WSE supplements.

4.9 WSE modulaes mitochondrial oxidative stress and function

Data obtained on the MDA, ROS generation and hydroperoxide levels in mitochondrial fractions of brain regions are presented in Fig. 4.12, 4.13 and 4.14. There was a similar effect of WSE among diabetic mice in mitochondrial milieu since the elevated levels were restored.
4.10 WSE ameliorates cholinergic function

Data on the activity levels of AChE and BChE measured in different brain regions are presented in **Fig. 4.15 A-D.** In general the activity levels of AChE among diabetic mice were significantly elevated in all the brain regions. However, WSE supplements caused varying degree of restoration in the activity levels of AChE in brain regions clearly suggesting its ability to modulate cholinergic function. Likewise the activity levels of BChE were also elevated in all regions except cortex and the levels were restored among diabetic mice given WSE supplements.

4.11 Effect of WSE on dopamine levels in straium

Among diabetic mice, a significant depletion in DA levels was evident only in the striatum (**Fig. 4.15** lower panel). While WSE supplements *per se* had no effect on DA levels in non-diabetic mice (data not shown), the levels were significantly replenished among diabetic mice.

4.12 Modualtory effect of WSE on mitochondrial function enzymes

Data on the activity levels of SDH and complex I-III and complex II-III are presented in **Fig. 4.16** and **Fig. 4.17.** In general, varying degree of diminution was evident in the brain regions of diabetic mice. Interestingly both cerebellum and straitum of diabetic mice showed relatively higher degree of reduction in the activity levels, while WSE supplemented diabetic mice exhibited significantly replenished activity levels.

4.13 Molecular markers in hippocampus: Western blots

Data analysed from western blot studies of hippocampal tissue of control, diabetic mice and WSE supplemented diabetic mice is presented in **Fig. 4.18.** While there was marginal increase, not reaching significance levels, of the pro-apoptotic protein BAD in hippocampal extracts prepared from diabetic rats, treatment of diabetic mice with WSE caused significant lowering of BAD indicating that the extract had an overall positive influence leading to lowering of apoptotic events. The observed effects could be partly explained on the basis of significant up-regulation of VEGF, a trophic factor that exerts neuroprotective
effects, in diabetic mice provided with WSE supplements. Such an enhancement of VEGF levels are likely to have profound permissive effects on survival pathway owing to its effects on PI3/akt pathway, inhibition of caspase-3 and enhancement of proliferation and migration of neuronal progenitor cells.

4.14 Histopathological alterations in hippocampus and Straitum

Histopathological analysis of hippocampi and striatal region of diabetic mice and those provided with WSE supplements is presented in plate 4.1 and 4.2 respectively. The hippocampi of the control group showed a normal architecture and damaged cell were minimal. The number of damaged neurons among diabetic mice was increased in hippocampi, compared to controls. However, significant reduction in the number of damaged neurons was evident in the hippocampi of diabetic mice provided with WSE supplements. A similar trend was also evident in the striatal region among diabetic mice provided with WSE supplements.
Table 4.1

Modulatory effect of *Withania somnifera* extract on STZ-induced alteration in the body weights of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weekly body weights (g)</th>
<th>Liver weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; week</td>
</tr>
<tr>
<td>Control</td>
<td>25±1.32</td>
<td>27±1.32</td>
</tr>
<tr>
<td>Diabetic</td>
<td>25±1.39</td>
<td>25±1.61</td>
</tr>
<tr>
<td>Diabetic + WSE</td>
<td>25±1.12</td>
<td>26±1.32</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).

**Body weights**: in grams

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

*Streptozotocin (STZ)*: 50mg/kg b.w/d, i.p, 5d
Table 4.2

Modulatory effect of *Withania somnifera* supplements on STZ –induced alteration in the level of blood glucose in male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days after administration of STZ</th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Induction of glucose</td>
<td>1(^{st}) week</td>
<td>2(^{nd}) week</td>
<td>Terminal week</td>
</tr>
<tr>
<td>Control</td>
<td>129 ± 3.32</td>
<td>131 ± 4.35</td>
<td>131 ± 3.42</td>
<td>130 ± 2.55</td>
<td>134 ± 6.62</td>
</tr>
<tr>
<td>Diabetic</td>
<td>125 ± 3.39</td>
<td>385 ± 3.65(^*)</td>
<td>402 ± 6.57(^*)</td>
<td>425 ± 4.50(^*)</td>
<td>453 ± 5.47(^*)</td>
</tr>
<tr>
<td>Diabetic + WSE</td>
<td>127 ± 4.12</td>
<td>376 ± 4.52</td>
<td>328 ± 7.51(^**)</td>
<td>302 ± 5.71(^**)</td>
<td>285 ± 5.97(^**)</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 4.1

Effect of oral supplementation of *Withania somnifera* on STZ –induced alteration in the level of blood glucose in male mice

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

**Blood glucose:** in mg/dL  
*Withania somnifera* (WSE): 400mg/kg b.w/d, oral, 4 weeks  
Streptozotocin (STZ): 50mg/kg b.w/d, i.p, 5d
Table 4.3

Modulatory effect of *Withania somnifera* supplements on STZ–induced alteration in red blood cells, white blood cells and hemoglobin count in whole blood of male mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Terminal blood analysis</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RBC</td>
<td>WBC</td>
<td>HB</td>
<td>PLT</td>
</tr>
<tr>
<td>Control</td>
<td>9.5 ± 0.18</td>
<td>9.4 ± 0.36</td>
<td>15.1 ± 0.51</td>
<td>423.7 ± 37.27</td>
<td>48.8 ± 0.77</td>
</tr>
<tr>
<td>Diabetic</td>
<td>6.7 ± 3.34*</td>
<td>-6.2 ± 0.09*</td>
<td>17.4 ± 0.25</td>
<td>756.7 ± 36.14*</td>
<td>-26.2 ± 2.07*</td>
</tr>
<tr>
<td>Diabetic + WSE</td>
<td>9.7 ± 2.20**</td>
<td>9.0 ± 0.67**</td>
<td>15.4 ± 0.31</td>
<td>518.7 ± 67.95**</td>
<td>50.4 ± 1.92**</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).

**RBC:** Red Blood Cells: Percentage (%)

**WBC:** White Blood Cells: Count (10^3)/µL of whole blood

**HB:** Hemoglobin: Grams of Hb/dL of whole blood

**PLT:** Platelet count: Platelet count/µL of whole blood

**HCT:** Hematocrit: % of RBC/dL of whole blood

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.2

Attenuation effects of oral supplementation of *Withania somnifera* extract on behavioral phenotype measured in landing foot spread distance (LFSD) and stride length (SL) among diabetic and non-diabetic mice.

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

**LFSD**: Landing Foot Spread Distance (centimeters); **SL**: Stride Length (centimeters)

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

*Streptozotocin (STZ)*: 50mg/kg b.w/d, i.p, 5d
Figure 4.3

Effect of *WSE* on STZ–induced alteration in time spent in closed (A) and open arm (B) and number of entries stored in closed (C) and open arm (D) of the elevated plus maze; behavioral alteration in the 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).

EPM: Elevated plus maze

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.4

Modulatory effect of *Withania somnifera* extract on STZ–induced alteration in rotarod test: behavioral alteration in the 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).

**RRT:** Rotarod Test

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

*Streptozotocin (STZ):* 50mg/kg b.w/ d, i.p, 5d
Figure 4.5

Modulatory effect of *Withania somnifera* extract on STZ–induced alteration in hot hyperalgicia (A) and cold hyperalgicia (B); behavioral alteration in the 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).

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

*Streptozocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.6

Modulatory effect of *Withania somnifera* on STZ–induced alteration in cytosolic reactive oxygen species levels 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).

*Ct*: Cortex; *Cb*: Cerebellum; *Hc*: Hippocampus; *St*: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
**Figure 4.7**

Modulatory effect of *Withania somnifera* on STZ–induced alteration in cytosolic lipid peroxidation levels 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).

**Ct**: Cortex; **Cb**: Cerebellum; **Hc**: Hippocampus; **St**: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.8

Modulatory effect of *Withania somnifera* on STZ –induced alteration in cytosolic hydroperoxide levels 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).

*Ct*: Cortex; *Cb*: Cerebellum; *Hc*: Hippocampus; *St*: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.9

Modulatory effect of *Withania somnifera* on STZ – induced alteration in cytosol superoxide dismutase activity levels 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).

**Ct:** Cortex; **Cb:** Cerebellum; **Hc:** Hippocampus; **St:** Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.10

Modulatory effect of *Withania somnifera* on STZ – induced alteration in cytosol catalase activity levels 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).

**Ct**: Cortex; **Cb**: Cerebellum; **Hc**: Hippocampus; **St**: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.11

Modulatory effect of *Withania somnifera* on STZ-induced alteration in cytosol glutathione-s-transferase activity levels 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).

**Ct**: Cortex; **Cb**: Cerebellum; **Hc**: Hippocampus; **St**: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.12

Modulatory effect of *Withania somnifera* on STZ-induced alteration in mitochondrial reactive oxygen species levels 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).

*Ct*: Cortex; *Cb*: Cerebellum; *Hc*: Hippocampus; *St*: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.13

Modulatory effect of *Withania somnifera* on STZ–induced alteration in mitochondrial lipid peroxidation levels 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).

**Ct**: Cortex; **Cb**: Cerebellum; **Hc**: Hippocampus; **St**: Striatum

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.14

Modulatory effect of *Withania somnifera* on STZ–induced alteration in mitochondrial hydroperoxide levels in different brain regions of male mice.

![Graphs showing hydroperoxide levels in different brain regions](image)

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

**Ct:** Cortex; **Cb:** Cerebellum; **Hc:** Hippocampus; **St:** Striatum

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

*Streptozotocin (STZ):* 50mg/kg b.w/d, i.p, 5d
Table 4.4

Modulatory effect of *Withania somnifera* oral administration on STZ – induced alteration in cytosolic reduced glutathione and total thiols in cortex and cerebellum of male mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic + WSE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>12.07 ± 1.03</td>
<td>8.23 ± 0.54*</td>
</tr>
<tr>
<td>Cortex</td>
<td>Total thiols&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10.21 ± 0.40</td>
<td>7.90 ± 0.47*</td>
</tr>
<tr>
<td></td>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>13.68 ± 1.01</td>
<td>10.65 ± 0.84*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Total thiols&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10.53 ± 0.74</td>
<td>8.87 ± 0.65*</td>
</tr>
<tr>
<td></td>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>12.45 ± 1.43</td>
<td>9.70 ± 0.74*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Total thiols&lt;sup&gt;2&lt;/sup&gt;</td>
<td>12.24 ± 1.48</td>
<td>10.37 ± 0.44*</td>
</tr>
<tr>
<td></td>
<td>GSH&lt;sup&gt;1&lt;/sup&gt;</td>
<td>14.50 ± 1.55</td>
<td>12.20 ± 0.95*</td>
</tr>
<tr>
<td>Striatum</td>
<td>Total thiols&lt;sup&gt;2&lt;/sup&gt;</td>
<td>14.33 ± 1.34</td>
<td>11.73 ± 1.05*</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<sup>1</sup>: Reduced Glutathione**: µg GSH/mg protein  
**TSH<sup>2</sup>: Total thiols**: nmol DTNB oxidized/min/mg protein

*Withania somnifera* (WSE): 400mg/kg b.w/d, oral, 4 weeks  
*Streptozotocin* (STZ): 50mg/kg b.w/ d, i.p, 5d
Table 4.5

Modulatory effect of *Withania somnifera* oral administration on STZ—induced alteration in mitochondrial reduced glutathione in different brain regions of male mice

<table>
<thead>
<tr>
<th></th>
<th>Reduced Glutathione (µg GSH/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>Control</td>
<td>9.38 ± 0.47</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8.00 ± 0.74*</td>
</tr>
<tr>
<td>Diabetic + WSE</td>
<td>8.86 ± 0.87**</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).

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

*Streptozotocin (STZ):* 50mg/kg b.w/d, i.p, 5d
Figure 4.15

Effect of *Withania somnifera* extract on STZ-induced alteration in the activity levels of AChE (A), (B); BChE (C), (D) and dopamine (Striatum) levels of cytosolic fraction of different brain regions of mice.

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

AChE: Acetylcholinesterase; BChE: Butrylcholinesterase

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.16

Effect of *Withania somnifera* extract on STZ–induced alteration in the activity levels of succinate dehydrogenase 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).

SDH: Succinate dehydrogenase;
*Withania somnifera* (WSE): 400mg/kg b.w/d, oral, 4weeks
Streptozotocin (STZ): 50mg/kg b.w/d, i.p, 5d
Modulatory effect of *Withania somnifera* on STZ–induced alteration in the levels of comp I-III (A, B) and comp II-III (C, D) of mitochondrial fraction of hippocampus 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).

**Comp I-III:** Complex I-III and **Comp I-III:** Complex II-III.

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

*Streptozotocin* (STZ): 50mg/kg b.w/d, i.p, 5d
Figure 4.18

Western blot analysis of BAD protein, and VEG factor in hippocampus of diabetic mice
Plate 4.1

Hematoxylin and eosin stained sections of hippocampi of prepubertal male mice rendered diabetic by STZ. Sampled after 4 weeks of WSE supplements. **Control** (left panel): Neurons are in their actual phase; **STZ group** (middle panel): loss in the shape and integrity of the neurons; **WSE+STZ group** (right panel): stabilized shape and integrity of the neurons.
Plate 4.2

Hematoxylin and eosin stained sections of striatum of prepubertal male mice rendered diabetic by STZ. Sampled after 4 weeks of WSE supplements. **Control** (left panel): Neurons are in their actual phase; **STZ group** (middle panel): loss in the shape and integrity of the neurons; **WSP+STZ group** (right panel): stabilized shape and integrity of the neurons.
5.0 DISCUSSION

The use of phytomedicines for the management of various diabetes-associated complications is increasing, largely due to the general notion that medicinal plants are less toxic and are free from side effects compared with regular synthetic drugs (Murthy et al., 2010; Wang et al., 2008). Among the various antidiabetic plants, WS enjoys a special status owing to its multiple pharmacological attributes.

Withania somnifera (WS) is an important medicinal plant widely used as a home remedy for several diseases in the Indian subcontinent and other parts of the world. WS is a dietary supplement composed of various nutrients, polyphenols and alkaloids that have free radical scavenging capacity, as well as other chemical constituents that possess anti-inflammatory, antitumor, anti-stress, antioxidant, immunomodulatory, and rejuvenating properties (Gohil, 2010; Murthy et al., 2010; Gokul et al., 2012; Kumar et al., 2015). The mechanism of action for these properties is not fully understood. WS also appears to influence the endocrine, cardiopulmonary and central nervous systems.

In this study, diabetic mice were provided with WSE supplements at a dosage of 400mg/kg b.w/d. Several researchers have also employed similar dosage of WSE in various experimental paradigms (Kulkarni and Dhir, 2008; Sehgal et al., 2011). At the dosage used, mice did not develop any sedation or any other noticeable symptoms clearly suggesting that the extract was well tolerated. This is consistent with previous toxicity studies which revealed that W. somnifera can be used without side effects (Alam et al., 2012).

In the present model, STZ administration to growing mice induced typical symptoms of diabetes after 5 doses, as evidenced by marked hyperglycemia, decreased body weight gain, hyperphagia, and polydipsia, which is consistent with earlier findings. Interestingly, blood glucose levels were moderately (25%), but significantly, reduced in WSE-supplemented diabetic mice. This finding corroborates with previous reports of hypoglycemic effects of WSE in experimental animals (Hemalatha et al., 2004; Anwer et al., 2008; Upadhya and Gupta, 2011; Shukla et al., 2012; Ojha et al., 2014).
In the area of diabetes management, it is well accepted, that other than strict control of blood glucose levels there are no therapies that can offset diabetic neuropathy and encephalopathy. Although the vital role of oxidative stress in the development of diabetic complications is well established, classical antioxidants such as vitamin E have failed to demonstrate the anticipated beneficial results in many clinical trials. Hence there has been a constant need to identify natural antioxidants that would be effective in attenuating oxidative stress in the brain and also prevent or delay nerve damage under hyperglycemic conditions. Accordingly the potential of herbal bioactives to modulate endogenous redox status in vivo has been considered as an effective approach to achieve neuroprotection (Dumont and Beal, 2009). Hence, it was hypothesized that WSE, which possess excellent ability to attenuate endogenous levels of oxidative markers in the brain regions (as evidenced in the chemical neurotoxin model- Chapter 3) would be an ideal candidate to ameliorate oxidative impairments and mitochondrial dysfunctions in an experimental diabetic model.

In the present study, diabetic mice displayed a time dependent progression in neuropathic signs, those provided with WSE supplements exhibited significant improvement against noxius (i.e. hyperalgesia) and non-noxius (allogdynia) stimuli clearly suggesting their efficacy to restore the sensory function under diabetic conditions. Further the locomotor phenotype also was significantly improved among WSE supplemented diabetic mice. Although the underlying mechanisms responsible for the WSE effects are not clear from this study, it is quite likely to be related to the antioxidant and anti-inflammatory property of the bioactives present in the WSE. This merits further studies in the diabetic model.

Interestingly, in the present model, brain regions of diabetic mice exhibited a marked increase in the oxidative markers. These findings are consistent with previous findings in STZ diabetic rats reported by various researchers (Tiwari and Kakkar, 2009; Kamboj and Sandhir, 2011) and our findings (Chandrasekhara et al., 2013; Prasad and Muralidhara, 2014). However, WSE supplementation among diabetic mice significantly restored the levels of
oxidative markers which indicate the potential of WSE to effectively offset the oxidative stress \textit{in vivo}.

Further, WSE supplements significantly elevated the reduced GSH levels in brain regions of diabetic mice and also among non-diabetic mice (previous Chapter data). This is also consistent with our earlier findings in the Drosophila model in which short term supplements of WSE enhanced the GSH levels. The ubiquitous thiol tripeptide, GSH is well-known to provide protection from oxidative stress-induced damage through the reduction of ROS. It acts alone or in concert with various other enzymes to scavenge superoxide, hydroxyl and peroxynitrite radicals (Dringen, 2000). Utilization/ degradation of reduced GSH in brain regions are strongly associated with oxidative stress (Cho et al., 2003; Miller et al., 2009). Thus, WSE elicited significant anti-oxidative effect as evident by the enhanced GSH levels and restoration of antioxidant defense.

Mitochondrial oxidative stress has been proposed as a major mediator of neurodegeneration in diabetic situations (Fernyhough et al., 2010; Kamboj and Snadhir, 2011). Accordingly, another salient finding evident in the present model, is the reduction in mitochondrial complex I activity in diabetic mice. This impaired activity may further predispose mitochondria to generate more ROS, thus hampering the bioenergetic stat of neurons. In this study, diabetic mice displayed diminished the membrane function, as evident by lowered activity levels of complex I, along with elevated MDA and HP levels in striatal mitochondria. The restorative effect of WSE supplements on the mitochondrial enzyme activities and membrane stability demonstrate its protective effects on mitochondria as well.

In the present investigation, the restorative effect of WSE supplements on cholinergic function among diabetic mice is consistent with the findings that WSE posses the potential to inhibit AChE activity (Choudhary et al., 2005). Interestingly we also observed a similar effect of WSE in the rotenone model of neurotoxicity in mice (Chapter 2). Post mortem studies are suggestive of the fact that elevated AChE activity levels have been observed in brain regions of PD patients with depression and mild memory deficits (Aarsland et al., 2012; Blonder et al., 2013). At present there is a poor understanding on the relative involvement of multisystem degenerative pathways leading to cognitive decline.
in PD patients (Bohnen et al., 2014). This line of thinking is consistent with a large body of evidence which suggests the therapeutic importance of various natural products possessing AChE inhibitory activity owing to their relative safety compared to regular AChE. Although speculative, this observation may be of therapeutic relevance to AD, since various acetylcholine esterase inhibitors are being employed to alleviate cognitive symptoms of AD.

In this diabetic model, elevated levels of oxidative markers were detected in cerebellum which is consistent with the thinking that oxidative stress-related neurodegeneration in cerebellum and striatum lead to severe locomotor dysfunctions. The neuromodulatory propensity of WSE is demonstrated with the reduced levels of cerebellar oxidative markers. Evidences from experimental animals suggest that phytoconstituents possess the ability to modulate cellular redox and mitochondrial function in cerebellum neurons resulting in the recovery of locomotor phenotype under a neurotoxin exposed condition (Prasad and Muralidhara, 2013; Denny and Muralidhara, 2013). In the current model, in accordance with the previous reports in diabetic mice, mitochondrial dysfunctions in cerebellum corroborated the effects in striatum along with the behavioral phenotype. The protective effects of WSE were evident with the normalization of these deleterious effects.

The underlying molecular mechanisms responsible for the protective effect of WSE are not clear from this study. However, both in the hippocampus and striatum, we measured the expression of specific protein BAD and VEGF, a trophic factor by western blotting technique. While there was marginal increase, not reaching significance levels, of the pro-apoptotic protein BAD in hippocampal extracts prepared from diabetic rats, treatment of diabetic rats with WSE caused significant lowering of BAD indicating that the extract had an overall positive influence leading to lowering of apoptotic events. The observed effects could be partly explained on the basis of significant up regulation of VEGF, a trophic factor that exerts neuroprotective effects, in diabetic rats treated with extracts. Such an enhancement of VEGF levels are likely to have profound permissive effects on survival pathway owing to its effects on PI3/akt
pathway, inhibition of caspase-3 and enhancement of proliferation and migration of neuronal progenitor cells.

In conclusion, it is proposed that WSE supplements have the potential to modulate the markers of oxidative stress, mitochondrial dysfunctions, and aberration in neurotransmission under diabetic conditions. Based on behavioral assessments and biochemical evidences, it can be reasonably proposed that WSE is a promising therapeutic adjuvant to treat or manage diabetes associated oxidative perturbations in brain regions as well as associated neuropathy. While the protective action of WSE is largely attributed to but not limited to its antioxidant and anti-inflammatory properties, further comprehensive investigations are required to delineate the underlying molecular mechanisms in experimental diabetic models.

6.0 SUMMARY

1. Mice administered with low multiple doses of STZ exhibited marked hyperglycemia (nearly 3-fold higher than controls), indicating the onset of diabetic condition.

2. Diabetic mice showed polyphagia, and the body weight gain was lower compared to non-diabetic mice. Interestingly, WSE supplements restored the body weight gain among diabetic mice.

3. While the blood glucose among the diabetic mice progressively increased, diabetic mice provided with WSE supplements exerted hypoglycemic effect.

4. Oral supplements of WSE significantly improved the stride length and LFSD measurements among the diabetic mice. Further, they exhibited normal exploratory behavioural phenotype and rotarod performance.

5. WSE supplements significantly ameliorated both sensory (hyperalgesia and allodynia) among diabetic mice
6. WSE supplements markedly attenuated the status of lipid peroxidation in brain regions as evidenced by diminished levels of ROS, MDA and hydroperoxide levels.

7. Likewise, WSE supplements were effective in restoring the oxidative stress markers in the mitochondrial milieu of brain regions examined.

8. WSE supplements restored some of the enzymic antioxidant defences in brain regions and concomitantly the redox status among the diabetic mice was replenished. The activity of GST was markedly elevated among diabetic mice provided with WSE supplements suggesting its protective role in detoxification process under such condition.

9. In the mitochondrial milieu, WSE supplements significantly attenuated diabetes associated diminution in the activity levels of ETC enzymes (SDH, Complex I-II, and Complex II-III).

10. WSE supplements among diabetic mice resulted in significant restoration of cholinergic function as evidenced by the activity levels of both AChE and BChE.

11. WSE supplements among diabetic mice resulted in significant restoration of dopaminergic function as evidenced by the restoration of DA levels in the striatum.

12. Significant up-regulation of VEGF (a trophic factor) evident in the hippocampus of Diabetic mice provided with WSE supplements suggested its neuroprotective role in alleviating diabetes-associated hippocampal dysfunctions.

13. Collectively these findings suggest that oral supplements of a standardized extract of WS roots can effectively attenuate hyperglycemia, locomotor deficits, brain oxidative stress and brain function in a an experimental diabetic model.