CHAPTER – VI

ANTIOXIDANT ACTIVITY OF *Pimenta dioica*

METHANOLIC LEAF EXTRACT IN STZ – INDUCED DIABETIC RATS

6.1 INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and insufficiency in the secretion of endogenous insulin. Although the etiology of this disease is not well defined, viral infection, autoimmune disease and environmental factors have been implicated (Like, 1979; Kataoka, 1983; Sandler, 2000; Shewade, 2001). Oxygen free radicals are formed disproportionately in diabetes mellitus by glucose oxidation, non-enzymatic glycation of proteins and the subsequent degradation of glycosylated proteins. Diabetic complications are also associated with overproduction of free radicals and accumulation of lipid peroxidation by-products. Enhanced oxidative stress has been well documented in both experimental and human diabetes mellitus (Baynes, 1991).

Hyperglycemia-induced over production of free radicals is widely recognized as the link between diabetes and diabetic complications. Oxidative stress is a constant feature of uncontrolled diabetes in humans and animals (Low, 1991; Jennings, 1987; Horie,
Antioxidants, which are produced either endogenously or derived from dietary sources, are categorized into two groups: enzymatic and non-enzymatic. Catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, tyrodoxin reductase, ariel esterase and paraoxonase are included in the enzymatic group, while the non-enzymatic group includes vitamins A, C, and E, carotenoids, glutathione, flavonoids, other compounds such as a-lipoic acid and co-enzyme Q10, and copper, zinc, magnesium, and selenium (Esteghamati, 2008).

Hyperglycemia and intake of free fatty acid are the main causes of oxidative stress induced (Evans, 2002). Thus, diabetic patients tend to have a more oxidative environment in the organs of healthy people, which is influenced by the increased ROS generation (Ceriello, 2001; Mahadev, 2004; Rains, 2011). Moreover, diabetic patients show a reduction in the levels of antioxidants, which further influences the increase in the oxidative stress (Maritim, 2003; Rains, 2011). Oxidative stress in diabetes leads to tissue damage, with lipid peroxidation, inactivation of proteins and protein glycation as intermediate mechanisms (Wolff et al., 1991). The polyol pathway comprising of enzymes aldose reductase and sorbitol dehydrogenase catalyzing the conversion of excessive glucose to sorbitol and sorbitol
to fructose respectively is activated in diabetic subjects (Lee and Chung, 1999). Tissue antioxidant status is also altered in diabetes resulting in increased oxidative damage and tissue injury which is stimulated by the generation of free radicals (Saxena et al., 1993; Genet et al., 2002; Lou, 2003). Activity of important biological antioxidant enzymes, glutathione peroxidase and glutathione reductase, that directly scavenge free radicals or prevent their conversion to toxic products (Freeman and Crapo, 1982), is also altered in diabetic condition (Wohaieb and Godin, 1987; Genet et al., 2002).

Aerobic organisms have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants that are usually effective in blocking harmful effects of ROS. Oxidative stress may be either contribute to or be produced due to tissue damage leading to both a primary and secondary source of diabetic pathology (Baynes, 1996). Oxidative stress can not only damage receptors, enzymes, signal transduction pathways and transport proteins but also lead to secondary damage to other biomolecules in the system. The oxidatively damaged proteins may be recognized as ‘foreign’ by the immune system, which can trigger immune response leading to antibody production. Small deviations from the physiological activity of antioxidant enzymes may
have a dramatic effect on the resistance of cells to oxidant-induced
damage to the genome and cell killing (Amstad, 1994; Limoli, 1998).

Animal catalases (CAT) are heme-containing enzymes that
convert hydrogen peroxide (H₂O₂) to water and O₂, and they are
largely localized in subcellular organelles such as peroxisomes.
Mitochondria and the endoplasmic reticulum contain little CAT. Thus,
intracellular H₂O₂ cannot be eliminated unless it diffuses to the
peroxisomes. The glutathione peroxidase (GSH-Px) removes H₂O₂ by
coupling its reduction with the oxidation of GSH-Px that can also
reduce other peroxides, such as fatty acid hydro peroxides. These
enzymes are present in the cytoplasm at (Roberto, 2000) milli molar
concentrations and are also present in the mitochondrial matrix. Most
animal tissues contain both CAT and GSH-Px activity.

The superoxide anion radicals undergo dismutation to hydrogen
peroxide, which if not degraded by catalase or glutathione peroxidase
and in the presence of transition metals, can lead to the production of
Superoxide anion radicals can also react with nitric oxide to form
reactive proxy nitrite radicals (Halliwell, 1990; Hogg, 1999). The rate
of production and elimination of free radicals is always balanced by the
antioxidant defense mechanism and any shift in this delicate balance
will lead to cellular damage. Thus antioxidants have gained importance in recent years due to their ability to neutralize free radicals and their actions (Tiwari, 2004). Increased levels of oxygen free radical activity, coupled with reduced protection against oxidative stress, could play a major role in the neurovascular abnormalities in experimental diabetes mellitus (Reckelhoff, 2000). Chemicals with antioxidant properties and free radical scavengers may help in the regeneration of β - cells and protect pancreatic islets against the cytotoxic effects of STZ (Tarique, 2007). The super oxide dismutase (SOD) and CAT play a prominent role in scavenging free radicals and restoring antioxidant activities in the tissues of diabetic animals (Tuzun, 1999; Rahimi, 2005).

Plants have been an important source of medicine and has helped human in the maintenance of health for thousands of years (Cowan, 1999). According to the World Health Organization, up to 80 percent of people still rely mainly on traditional remedies such as herbs for their health needs due to better cultural acceptability, fewer side effects and better compatibility with the human body (Thevasundari, 2011). Its civilization is very ancient and the country as a whole has long been known for its rich resources of medical plants (Arunkumar, 2009). About four thousand years ago, the medical intelligence of the Indian subcontinent was called as Ayurveda. The exact meaning of
Ayurveda is “Science of life,” which still remains a crucial system of medicines as well as drug therapy in India. The disease evolved in the body due to exogenous factors has also been reported in Ayurveda medicine. It also covered all aspects of diseases, pharmacy and therapeutics in Sanskrit literature (Ramar, 2008).

*Pimenta dioica*, commonly known as allspice was used by early Central American civilizations as a flavoring for chocolate. The Spanish explorers of the 17th century gave Allspice the name *Pimenta*, owing to its peppery flavor. As a medicine, allspice has similar use, as cloves and their oils. Antioxidant and hepatoprotective activity of *Pimenta dioica* leaves extract have been described earlier (Nayak, 2008). In such a context, the present study was aimed to investigate the antioxidant activity of the methanolic leaf extracts of *Pimenta dioica* in streptozotocin induced diabetic rats.

**6.2 MATERIALS AND METHODS**

**6.2.1 Estimation of reduced glutathione (GSH) (Ellman, 1959)**

This method was based on the development of yellow colour when dithio-dinitrobis benzoic acid (DTNB) was added to compounds containing sulfhydryl groups.
Reagents

1. Phosphate buffer: 0.1 M, pH 7.0.
2. 5% TCA.
3. Ellman’s reagent: 34 mg dithio-dinitrobis benzoic acid was dissolved in 10 ml 0.1% sodium citrate.
4. 0.3 M disodium hydrogen phosphate.
5. Stock standard glutathione: 1000 µg/ml in distilled water.
6. Working standard: 100 µg/ml in distilled water.

Procedure

A known weight of the liver and kidney tissues were homogenized in phosphate buffer. 0.5 ml of the tissue homogenized mixture was treated with 2.0 ml of 5% TCA, mixed and centrifuged. 2.0 ml of the supernatant was then treated with 1.0 ml of Ellman’s reagent and 4.0 ml of 0.3 M disodium hydrogen phosphate. The absorbance of the yellow colour developed was read in a UV Spectronic Spectrophotometer at 412 nm. A series of standards (20-100 µg) were treated in a similar manner along with a blank containing 1.0 ml buffer and was expressed as µmoles/mg of protein in tissue.
6.2.2 Estimation of glutathione peroxidase (GPx) (Rotruck et al., 1973)

A known amount of the enzyme preparation was allowed to react with H₂O₂ and GSH for a specific time period. Then the GSH content remaining after the reaction was measured.

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2 \]

Reagents

a. Tris-HCl buffer-0.4 m, pH 7.0
b. Sodium azide solution-10mM
c. 10% TCA
d. EDTA- 0.4mM
e. Hydrogen peroxide-1.0mM
f. Glutathione solution (GSH)-2mM

Procedure

To 0.2 ml Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.2 ml enzyme preparation of the liver and kidney tissue homogenate was added and mixed well. To this 0.2ml of GSH followed by 0.1ml of H₂O₂ were added. The contents were mixed and incubated at 37 °C for 10 minutes. The reaction was arrested by the addition of 0.5 ml 10% TCA. The tubes were centrifuged and the remaining was determined as in the GSH procedure. The activities were expressed as μg of GSH consumed/min/mg protein in tissues.
6.2.3 Estimation of superoxide dismutase (SOD) (Kakkar et al., 1984)

The assay was based on the inhibition of the formation of NADH-phenazine-methosulphate and nitroblue tetrazolium formation. The reaction was initiated by the addition of NADH. After incubation for 90 seconds the reaction was stopped by adding glacial acetic acid. The colour developed at the end of the reaction was extracted into n-butanol layer and measured at 520 nm.

Reagents

1. Sodium pyrophosphate buffer -0.025 m, pH 8.3.
2. Absolute ethanol.
3. Chloroform.
4. n-butanol.
5. Phenazine methosulphate (PMS)-186 µ mol.
6. Nitroblue tetrazolium (NBT)-300 µ mol.
7. NADH-780 µ mol.

Procedure

Liver and kidney tissues (from acute phase animal) were homogenized in sodium pyrophosphate buffer (0.025 M, pH 8.3). 0.5 ml of the tissue homogenate was diluted to 1.0 ml with water followed by the addition of 2.5 ml of ethanol and 1.5 ml of chloroform (chilled reagents were added). This mixture was shaken for 90 minutes at 4 °C and then centrifuged.
The enzyme activity in the supernatant was determined as follows. The assay mixture containing 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate and 0.3 ml of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 ml were prepared and the reaction was started by the addition of 0.2 ml NADH. After incubation at 30 °C for 90 seconds, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was separated. The colour intensity of the homogenate was measured in a colorimeter at 560 nm. A system devoid of enzyme served as the control. The enzyme concentration required to produce 50% inhibition of chromogen formation in one minute under standard conditions was taken as one unit. The specific activity of the enzyme was expressed as U/mg protein in tissues.

6.2.4 Estimation of catalase (CAT) (Sinha, 1972)

The catalase preparation was allowed to split H₂O₂ for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H₂O₂ was treated with dichromate in acetic acid in hot condition. The H₂O₂-chromic acetate formed was determined colorimetrically.
Reagents

1. Phosphate buffer-0.01 M, pH 7.0.
2. Hydrogen peroxide-0.2 M.
3. 5% potassium dichromate.
4. Dichromate-acetic acid reagent- 1: 3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1 ml was diluted again with 4 ml of acetic acid.
5. Standard hydrogen peroxide-0.2 M.

Procedure

The liver and kidney tissue homogenates were prepared by using phosphate buffer (0.01 M, pH 7.0). To 0.9 ml phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of hydrogen peroxide were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 ml of dichromate-acetic acid mixture. The tube was kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 620 nm. Standards in the concentration range of 20-100 μ moles were taken and processed for the test. The specific activity of the enzymes was expressed as U/mg protein in tissues.

6.2.5 Estimation of thiobarbituric acid reactive substances (TBARS) (Niehuas and Samuelson, 1968)

Malondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with thiobarbituric acid
(TBA) in acidic condition. The reaction generates a pink colored chromophore which was read at 535nm.

**Reagents**

1. 5 % TCA
2. HCL − 0.025N.
3. TBA − 0.375 % in hot distilled water.
4. TCA − TBA − HCL reagents. Solution 1, 2 and 3 were mixed freshly in the ratio of 1:1:1.
5. Stock standard − 4.4 mol/l solution of stock was prepared from 1, 1-3, 3 - tetra methoxy propane in distilled water.
6. Working standard − Stock solution was diluted to get concentration of 50 m.mol / ml.

**Procedure**

Liver and kidney tissue homogenates were prepared in Tris − HCL buffer (0.025 M, pH 7.8). To 1.0 ml of the tissue homogenate, 2.0ml of TCA − TBA − HCl reagent was added and mixed thoroughly. The mixture was kept in a boiling water bath for 15 minutes. After cooling, the tubes were centrifuged at 1000 rpm for 10 minutes and the pink colour developed in the supernatant was measured in a systronic Spectrometer at 535 nm against a reagent blank. A series of standard solutions in the concentrations of 2.5-10 moles were treated in a similar manner and the values were expressed as nMol/mg protein in tissue.
Statistical analysis

All data are expressed as mean ± S.E. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple tests using SPSS (version 18) computer software. In all cases, P-values of less than 0.05 was considered to be significant.

6.3 RESULTS

The Table shows the effect of treatment of STZ-induced diabetic rats with different dosages of the methanolic leaf extract of *P. dioica* as well as the standard drug, glibenclamide on the liver and kidney enzymes like thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GPx). In comparison to the normal control group, diabetic control group had shown a significant increase in the level of TBARS in both the liver and kidney tissues. The increase was found to be nearly two-fold and the percentage increase was observed to be 58% in the liver and 43% in the kidney tissues respectively. Treatment with the two dosages of the methanolic leaf extract of *P. dioica* (75 mg/kg b.w and 150 mg/kg b.w) exhibited a significant decrease in TBARS at all doses. *P. dioica* leaf extract at the dose of 75 mg/Kg b.w. induced a marked reduction of TBARS and at the dose of 150 mg/kg b.w. the decrease was more pronounced. On treatment with glibenclamide, the obtained reduction in TBARS level was still notable in both the liver and kidney tissues respectively.
Table 4. Effect of methanolic leaf extract of *P. dioica* on the liver and kidney antioxidant enzymes in the normal, STZ – induced diabetic and treated rats.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group</th>
<th>Treatment</th>
<th>TBARS U/ml</th>
<th>SOD U/ml</th>
<th>CAT U/ml</th>
<th>GSH U/ml</th>
<th>GPx U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>I</td>
<td>Normal control</td>
<td>3.90±0.10a</td>
<td>112.90±2.28a</td>
<td>32.55±0.90a</td>
<td>241.28±3.40a</td>
<td>34.50±0.81a</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Diabetic control</td>
<td>8.41±0.16a</td>
<td>81.01±1.48a</td>
<td>21.60±0.31a</td>
<td>220.35±1.32a</td>
<td>22.10±0.39c</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Diabetic + <em>P. dioica</em> extract (75 mg/kg b.w.)</td>
<td>4.60±0.11b</td>
<td>96.39±2.19d</td>
<td>28.92±0.51d</td>
<td>235.89±3.10d</td>
<td>29.45±0.89d</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Diabetic + <em>P. dioica</em> extract (150 mg/kg b.w.)</td>
<td>4.54±0.15c</td>
<td>102.48±3.12c</td>
<td>28.21±0.51c</td>
<td>237.41±0.51c</td>
<td>32.04±0.44c</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Diabetic + glibenclamide (0.6 mg/kg b.w.)</td>
<td>4.15±0.09d</td>
<td>109.69±3.64a</td>
<td>31.47±0.51b</td>
<td>240.33±2.31b</td>
<td>33.01±0.55b</td>
</tr>
<tr>
<td>Kidney</td>
<td>I</td>
<td>Normal control</td>
<td>5.13±0.10f</td>
<td>146.69±2.01a</td>
<td>28.81±0.55a</td>
<td>334.98±5.71a</td>
<td>32.60±0.89a</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Diabetic control</td>
<td>7.35±0.15f</td>
<td>115.59±2.05c</td>
<td>20.01±0.84c</td>
<td>302.18±3.13c</td>
<td>23.80±0.68c</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Diabetic + <em>P. dioica</em> extract (75 mg/kg b.w.)</td>
<td>5.93±0.10b</td>
<td>131.12±3.41d</td>
<td>27.60±0.50c</td>
<td>326.80±4.98d</td>
<td>27.02±0.59d</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>Diabetic + <em>P. dioica</em> extract (150 mg/kg b.w.)</td>
<td>5.39±0.20c</td>
<td>133.10±3.65c</td>
<td>28.16±0.55d</td>
<td>329.41±6.41c</td>
<td>30.12±1.02c</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Diabetic + glibenclamide (0.6 mg/kg b.w.)</td>
<td>5.27±0.15d</td>
<td>144.81±2.90b</td>
<td>28.61±0.51b</td>
<td>331.65±3.41b</td>
<td>31.42±1.15b</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E (n=6) and are significantly different at p < 0.005 when compared with control groups.
Fig. 17. Effect of *P. dioica* leaf extract on TBARS in the liver tissues of normal, diabetic induced and treated rats

Fig. 18. Effect of *P. dioica* leaf extract on TBARS in the kidney tissues of normal, diabetic induced and treated rats
The diabetic control group revealed a drastic decline in the level of superoxide dismutase enzyme (81.01±1.48U/ml) when compared with that of the normal control group (112.90±2.28 U/ml) in the liver tissues. A marked reduction in superoxide dismutase level was observed among the normal control and diabetic control groups in the kidney tissues as well, i.e. 146.69±2.01U/ml and 115.59±2.05U/ml respectively. The percentage reduction was found to be 32% in the liver tissues and 33% in the kidney tissues. Administration of the methanolic leaf extract of *P.dioica* at the dose of 75mg/kg b.w. induced a significant increase of superoxide dismutase in the liver tissues (96.39 ±2.19 U/ml) and kidney tissues (132.12±3.41 U/ml) respectively. Administration of the methanolic leaf extract of *P.dioica* at the dose of 150 mg/kg b.w. restored the superoxide dismutase level to near normalcy i.e 102.48±3.12 U/ml in the liver tissues and 133.10±3.65 U/ml in the kidney tissues respectively. The standard drug, glibenclamide also led to a prominent decrease in superoxide dismutase almost on par with the normal control group.
Fig. 19. Effect of *P. dioica* leaf extract on SOD in the liver tissues of normal, diabetic induced and treated rats

Fig. 20. Effect of *P. dioica* leaf extract on SOD in the kidney tissues of normal, diabetic induced and treated rats
The normal control group exhibited the catalase enzyme level at (32.55±0.90 U/ml) in the liver tissues and (28.81±0.51 U/ml) in the kidney tissues respectively, whereas a categorical decline in the level of catalase was obtained in the STZ – induced diabetic group as (21.60±0.51 U/ml) in the liver tissues and (20.01±0.84 U/ml) kidney tissues. The percentage reduction was observed to be 32% in the liver tissues and 29% in the kidney tissues respectively. Treatment with the methanolic leaf extract of *P. dioica* at the dose of 75 mg/kg b.w. yielded a significant elevation in the level of catalase in both the tissues studied, ie. 26.20±0.51 U/ml and 27.60±0.50 U/ml in the liver and kidney tissues respectively. Near normal level in the catalase enzyme was achieved in both the tissues on treatment with the methanolic leaf extract of *P. dioica* at the dose of 150 mg/kg b.w. This reveals the significant effects of the plant leaf extract on elevating the enzyme level in the diabetic control group. The percentage increase was shown to be 88% in the liver tissues and 97% in the kidney tissues. The standard drug, glibenglamide as well brought back the catalase level to near normalcy in both the tissues undertaken for the study.
Fig. 21. Effect of *P. dioica* leaf extract on CAT in the liver tissues of normal, diabetic induced and treated rats

Fig. 22. Effect of *P. dioica* leaf extract on CAT in the kidney tissues of normal, diabetic induced and treated rats
A significant decrease in the level of reduced glutathione was obtained in the diabetic control group (220.35±1.32 U/ml) when compared with that of the normal control group (241.28±3.40 U/ml) in the liver tissues. The kidney tissues also exhibited a marked decline in the reduced glutathione enzyme level in the liver tissues of diabetic control group (302.18±3.13 U/ml) on comparison with that of the normal control group (334.98±5.71 U/ml). The percentage decrease was found to be 17% in the liver tissues and 19% in the kidney tissues respectively. Administration of the methanolic leaf extract of *P. dioica* at the dose of 75 mg/kg b.w. elevated the level of reduced glutathione significantly in the liver tissues of STZ-induced diabetic control group (235.89±3.10 U/ml) as well as in the kidney tissues 326.80±4.98 U/ml. On treatment with the *P. dioica* leaf extract at the dose of 150 mg/kg b.w. the reduced glutathione enzyme level increased categorically to near normal level in both the tissues studied, i.e. 237.41±0.51 U/ml in the liver tissues and 329.41±6.41 U/ml in the kidney tissues respectively. The percentage increase was shown to be 96% in the liver tissues and 95% in the kidney tissues which is on par with the normal control group. Treatment with the standard diabetic drug, glibenclamide also led to a marked elevation in the level of reduced glutathione on both the liver and kidney tissues respectively.
Fig. 23. Effect of *P. dioica* leaf extract on GSH in the liver tissues of normal, diabetic induced and treated rats.

Fig. 24. Effect of *P. dioica* leaf extract on GSH in the kidney tissues of normal, diabetic induced and treated rats.
At the end of the respective plant extracts and diabetic drug treatments, the level of glutathione peroxidase enzyme was analyzed. In comparison to the normal control group, the diabetic control group had shown a significant decrease in the glutathione peroxidase level in both the liver and kidney tissues i.e. (34.50±0.81 U/ml) and (22.10±0.39 U/ml) in the liver tissues and (32.60±0.89 U/ml) and (23.80±0.68 U/ml) in the kidney tissues respectively. The percentage decrease was observed to be 69% in the liver tissues and 65% in the kidney tissues. Administration of the methanolic extract of P.dioica leaves at the dose of 75 mg/kg b.w. induced a significant increase in the glutathione peroxidase level in the liver (29.45±0.89 U/ml) and kidney tissues (27.02±0.59 U/ml) respectively. Whereas, administration of the plant leaf extract at the dose of 150 mg/kg b.w led to a marked elevation in the level of glutathione peroxidase enzymes in the liver 32.04±0.44 U/ml and kidney tissues 30.12±1.02 U/ml. The average percentage increase on treatment with both the doses of the P.dioica plant extract was found to be 95% in both the liver and kidney tissues respectively. Similarly, treatment with the standard diabetic drug, glibenclamide as well, markedly increased the level of glutathione peroxidase on par with the normal control group, in both the liver and kidney tissues studied.
Fig. 25. Effect of *P. dioica* leaf extract on Gpx in the liver tissues of normal, diabetic induced and treated rats

Fig. 26. Effect of *P. dioica* leaf extract on Gpx in the kidney tissues of normal, diabetic induced and treated rats
6.4 DISCUSSION

The antioxidant studies are normally conducted in animal models to find out the mechanism of action of the specific medicinal plant or any other synthetic drug. In the present study, the methanolic leaf extract of the plant *Pimenta dioica* was administered in the STZ-induced diabetic rats in two different dosages. Antioxidants are the substances or nutrients which can prevent or slow down the oxidative damage to the body. When the body cells use oxygen, they naturally produce free radicals which can cause damage (Khalil et al., 2012). Chronic hyperglycemia in diabetes leads to auto-oxidation of glucose, non-enzymatic protein glycolysation, impaired glutathione metabolism, alteration in antioxidant enzymes and formation of lipid peroxides. These events accelerate the production of free radicals and weaken the antioxidant defense system.

Hence attention has been given to naturally occurring antioxidants to counteract the deleterious effects of reactive antioxidants (Elumalai et al., 2013). Numerous plant constituents have shown free radical scavenging or antioxidant activity. In diabetic condition, increased production of reactive oxygen species (ROS) play a major role in the destruction of the pancreas and the progression of the β – cell dysfunction (Kanjimoto and Kaneto, 2004). Therefore,
oxidative stress is believed to play a pivotal role in the etiology and pathogenesis of diabetes mellitus and its long-term complications (Paolisso et al., 1993).

Antioxidants are the first line defense system against free radical damage and act to maintain optimum health and well being. In the present study, chosen antioxidant assays were performed to evaluate the antioxidant activity of Pimenta dioica in STZ – induced diabetic rats.

Robertson et al., (2005) demonstrated that antioxidant have been shown to break the worsening effects of diabetes by improving the functioning of the β- cells of pancreas in animal models and also suggested that enhancing the antioxidant defense mechanisms in pancreatic islets may be a valuable pharmacologic approach in managing diabetes.

Lipid peroxidation, a free radical mediated propagation of oxidative insult to polyunsaturated fatty acids, is a characteristic feature of chronic diabetes. It impairs cell membrane fluidity and alters the activity of membrane bound enzymes and receptors, resulting in membrane malfunctioning (Halliwell, 2000). Malondialdehyde (MDA), a secondary product of lipid peroxidation, is used as an indicator of tissue damage. Most of the tissue damage is considered to
be mediated by these free radicals by attacking membranes through the peroxidation of polyunsaturated fatty acids. The increase in oxygen free radicals in diabetes could be primarily due to an increase in blood glucose levels, which upon auto oxidation generate free radicals (Malini et al., 2011). The increased susceptibility of the tissues of the diabetic animals may be due to the activation of the lipid peroxidation system.

Lipid peroxidation is one of the characteristic features of chronic diabetes. The increased free radicals produced may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation. Lipid peroxidation will in turn result in the elevated production of free radicals (Diao et al., 2014).

In the present study, it was observed that TBARS level in the liver and pancreas of STZ-induced diabetes was significantly increased when compared to that of the control. The decreased activity of antioxidant molecules along with elevated TBARS level in diabetic rats could probably be associated with oxidative stress and decreased antioxidant defense potential (Yang et al., 2011). Diabetic rats treated with *P. dioica* leaf extract in treated groups showed markedly decreased level of TBARS.
Hyperglycemia is associated with the formation of reactive oxygen species (ROS) which cause damage particularly to liver, kidney and also pancreas. The level of lipid peroxidation (TBARS) and reactive oxygen species (Superoxide radical, hydrogen peroxide and hydroxyl radicals) are common markers of oxidative stress in diabetic rats (Mohamed et al., 1999). Lipid peroxidation refers to the oxidative degradation of lipids that impairs cell membrane functions resulting in cell damage and leading to several pathologies and cytotoxicity (Halliwell and Chirico, 1993). Malondialdehyde (MDA) is one of the end products of lipid peroxidation which is usually measured as a marker for oxidative stress (Krishnaraju et al., 2009).

It is well established that the free radicals derived from oxygen have been implicated in the pathophysiology of diabetes mellitus and other diseases (Kakker et al., 1998). A number of studies revealed that oxidative stress plays a major role in the development, progression of diabetes and its related complications (Oyedenic and Afolayan, 2011; Sajeesh et al., 2011; Poongothai et al., 2011; Mishra et al., 2011; Kapoor et al., 2011; Kumar et al., 2011). In diabetic state, free radical generation may occur via increased glycolysis, intercellular activation of polyol pathway, auto-oxidation of glucose and non-enzymatic protein glycation (Sharma et al., 2010). Moreover drastic reduction of
in vivo antioxidant enzymes level in various tissues was reported in diabetic condition (Ahmed, 2005).

Oxidative stress is suggested as the mechanism underlying diabetes and diabetic complications, that results from an imbalance between radical generating and radical scavenging systems (Neethu et al., 2014). Antioxidant enzymes as well as non-enzymatic antioxidants are the first line of defense against ROS induced oxidative damage in any living organism (Sellamuthu et al., 2013). SOD, CAT and GSH – Px are the three major scavenging enzymes that remove the toxic free radicals by catalyzing the removal of superoxide radical, converting it into \( \text{H}_2\text{O}_2 \) and molecular oxygen, which both damage the cell membrane and other biological structures.

Reduced glutathione (GSH) is a major non-protein thiol in living organisms. Thiol groups are supposed to play an important role in maintaining the intracellular and membrane redox state of the secretory function of \( \beta \)-pancreatic cells (Paolisso et al., 1993). In the present study, the empirical lower mean plasma levels of GSH in untreated diabetic rats were possibly due to increased utilization of GSH as an antioxidant defense against reactive oxygen species, as suggested by others (Gregus et al., 1996). However, \( P.\text{dioica} \) leaf extract treated diabetic rats exhibited significantly higher mean levels of GSH in plasma than that of the untreated diabetic rats. In this study, the treatment with the leaf extract of \( P.\text{dioica} \) restored the GSH
content to near normal levels in diabetic rats and recommended that this was due to a reduction in oxidative stress.

Reduced glutathione is an important biomolecule responsible for the elimination of reactive intermediates by the reduction of hydroperoxidase in the presence of glutathione peroxidase. GSH also functions as a free radical scavenger and helps to repair free radical mediated biological damage (Dubey and Batra, 2008). Glutathione peroxidase, catalyses the reaction of hydroperoxidess with reduced glutathion to form glutathione disulphide and the reduction products of the hydroperoxide (Sabu and Kuttan, 2004). In the present study, GSH and GPx levels were decreased in the liver and kidney tissues of diabetic rats than that of the normal control rats and it clearly indicates the defective functions of GSH and GPx in diabetic rats. But, increased levels of GSH and GPx were noticed in the above tissues of diabetic rats after the administration of *P.dioica* which shows the free radical scavenging ability of *P.dioica* in diabetic condition.

Superoxide dismutase, the metallo protein is primarily involved in the antioxidant defense by scavenging the superoxide radicals. In hyperglycemia, glucose undergoes auto-oxidation and produces superoxide radicals which lead to lipid peroxidation (Saumya and Basha, 2011). Superoxide dismutase (SOD) is a natural cellular
antioxidant enzyme which catalyse the dismutation of superoxide radicals into hydrogen peroxide ($\text{H}_2\text{O}_2$). Catalase, the heme protein, localized in the peroxisomes or microperoxisomes catalyses the decomposition of $\text{H}_2\text{O}_2$ and protects the cell from oxidative damage produced by $\text{H}_2\text{O}_2$ (Chance and Greenstain, 1992). Catalase is responsible for the detoxification of significant amounts of hydrogen peroxide ($\text{H}_2\text{O}_2$) (Al-Sheikh et al., 2014).

Glutathione peroxidase (GPx), being a selenium containing enzyme, aids the removal of $\text{H}_2\text{O}_2$, thereby preventing the function of hydroxyl radical. In the present study, the mean activities of SOD and CAT in liver and kidney samples were found to be significantly lower in diabetic untreated rats than in normal rats. This may be due to the inadequacy of the antioxidant defense in combating ROS mediated damage (Pari and Latha, 2004). The decrease in mean GPx activity observed in the liver and kidney tissues of diabetic untreated rats possibly represented an important adaptive response to increased peroxidative stress as hypothesized by Kinalsiki et al., (2000).

Glutathione peroxidase plays a central role in the catabolism of $\text{H}_2\text{O}_2$ and the detoxification of endogenous metabolic peroxides and hydro peroxides which catalyzes the reduced glutathione (Saravanan and Pommurugan, 2013). Glutathione functions as a free radical
scavenger and is an essential co-substrate for glutathione peroxidase (Lorenzi, 2007).

In diabetic rats, decreased level of the antioxidant enzymes was reported and it indicates the development of an imbalance between ROS production and antioxidant scavenging systems. In the present study, SOD and CAT levels were significantly decreased in the liver and kidney tissues of diabetic rats which shows the disturbance in the antioxidant status. But after the administration of *P. dioica* leaf extract, significantly increased levels of SOD and CAT were noticed in diabetic rats when compared to the diabetic control rats and this phenomenon supports the protective effect of *P. dioica* on the above organs against free radical mediated damage.

SOD, CAT and GPx, the enzymatic antioxidants play a vital role in preventing cells from being exposed to oxidative damage. SOD reduces the superoxide radical into hydrogen peroxide. CAT catalysis the reduction of hydrogen peroxide and protects the tissues against hydroxyl radicals (Eliza et al., 2010). When cells have increased levels of SOD without a proportional increase in glutathione peroxidase, cells face a peroxide overload challenge. Peroxidase can react with transitional metals and generate the radical hydroxyl, which is the most harmful radical (Halliwell and Gutteridge, 1999). In diabetes, high
glucose can inactivate antioxidant enzymes such as SOD, CAT and GPx by gylcations of these proteins thus producing induced oxidative stress, which in turn, cause lipid peroxidation (Kennedy and Lyons, 1997). CAT, SOD and GPx activities were brought to near normal indicating the efficacy of *P. dioica* in attenuating the oxidative stress in diabetic tissues.

Recent studies have suggested that the lowering of SOD, CAT and GPx activity in diabetic rats could be prevented by treatment with antioxidants. Eventually *P. dioica* leaves reduced oxidative stress in the liver and kidney tissues under diabetic condition compared to the synthetic drug, glibenclamide. In the present investigation, significantly lower mean activites of SOD, CAT and GPx were noted in the liver and kidney tissues of *P.dioica* treated diabetic rats than in diabetic untreated rats, suggesting that *P.dioica* possibly protects liver and kidney cells against oxidative insult.

In the present study, decreased levels of the liver and kidney SOD, CAT, GPx, GSH and increased level of TBARS were observed in STZ-induced diabetic rats when compared to that of the normal control rats. The decrease in the level of the above enzymes directly reflects the oxidative stress in diabetic rats and these enzyme level changes may be attributed to the generation of free radicals by polyol
pathway, auto-oxidation of glucose, glycosylation in hyperglycemic condition and STZ mediated production of ROS by its donor property to the intracellular molecules. In the present study, increased SOD, CAT, GSH and GPx levels as well as reduced TBARS level after the administration of P. dioica leaf extract represents the antioxidant property of P. dioica in diabetic condition and hence P. dioica possesses a potential to reduce or prevent the diabetic micro and macrovascular complications.

The decreased activity of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase, along with decreased glutathione level was found in the liver and pancreatic tissues of diabetic rats. The above results are in agreement with the findings of Cheng et al., (2013). It was suggested that the decreased antioxidant enzyme activity in the diabetic control group could be due to glycation of these enzymes, which occurred at persistently elevated blood glucose levels (Almedia et al., 2012). However, the administration of P. dioica leaf extract in the treated groups resulted in enhanced GSH-Px, SOD and CAT activities and GSH level in the liver and pancreas of diabetic rats.