Assessment of Anti-Diabetic, Hypo-Lipidemic and Antioxidant Activity of *Cynodon dactylon* (L.) Pers.
CHAPTER – 3

ASSESSMENT OF ANTI-DIABETIC, HYPO-LIPIDEMIC AND ANTIOXIDANT ACTIVITY OF Cynodon dactylon (L.) Pers.

3.1. INTRODUCTION

Diabetes mellitus is a serious, complex chronic condition, which is a major health concern worldwide. This metabolic disorder is characterized by hyperglycemia and disturbances of carbohydrate, protein and fat metabolisms (Alberti and Zimmet, 1998). Numerous clinical studies have focused on the diabetes management and metabolic control to get new insights into the pathogenesis of diabetes in order to discover novel possibilities of preventive and effective treatment. Plants have an advantage in this area based on their long-term use by humans (middle paleolithic age some 60,000 years ago) (Solecki, 1975). According to the World Health Organization (WHO), 80% of the world’s people depend on traditional medicine and 25% of the medical drugs are based on plants for their primary health care needs (Chellaiah et al., 2006). The number of people affected with diabetes worldwide has increased dramatically over recent years. Indeed, by 2015 it has been estimated that the diabetic population will increase to 221 million around the world (Carter, 2004). The Diabetes Control and Complications Trial (DCCT) Research Group (1993) stated that tight control of blood glucose is an effective strategy in reducing clinical complications of diabetes mellitus significantly and even optimal control of blood glucose cannot prevent complications suggesting that an alternative approaches is needed. Ethnobotanical field studies revealed that, a number of plant remedies were used to alleviate the complications of diabetes. However, only a few have been evaluated scientifically to confirm the claimed activity (Sezik et al., 2005).

Hyperglycemia also causes oxidative stress, which in turn can result in cellular tissue damage. The uncontrolled hyperglycemia can lead to disturbances of the cell structure and functions of organs (West, 2000). Diabetes is associated with the generation of reactive oxygen species (ROS) leads to oxidative damage in vascular
system and other organs particularly to heart and kidney. The schematic representations of role of antioxidant activity are shown in figure 3.1.

Diabetes has conditions called ‘risk factors’ that contribute to atherosclerosis and its complications. These include high blood pressure, excess weight and high blood glucose levels. Dyslipidemia further raises the risk of atherosclerosis with diabetes. The most common dyslipidemia in diabetes is the combination of high triglycerides and low HDL levels. Diabetes may also have elevated LDL-cholesterol. Hyperlipidemia is implicated as the cause for coronary heart diseases (Hemalatha et al., 2006).

*Cynodon dactylon* (L.) Pers. (Fam: Poaceae) is commonly known as ‘Doob’ in India (Arugampul: Tamil). It is a weed and possesses varied medicinal properties. Leaf, root and rhizome of the plant have been used in folk medicine in different countries, as an anti-inflammatory and anticystitis (Uncini and Tomei, 1999). Antihypertensive, antiviral, hypolipidemic agent, antihysteria, antipsychotic and antigonorrheal infection (Auddy et al., 2003). In India, the plant is used in the treatment of melena, thirst, anorexia, burning sensations in the body, pruritis, miscarriage and erysipelas (Shinwari and Khan, 2000) and its leaf juice with a pinch of common salt has been used orally to treat stomachache. Decoction of whole plant is given orally to cure menstrual problem (Katewa et al., 2001).

**Figure 3.1 – Schematic representation of role of antioxidant activity.**
The aim of this study is to investigate the effect of aqueous and ethanolic extract of *C. dactylon* leaves on antidiabetic, hypolipidemic and antioxidant activity in normal and alloxan diabetic rats. In addition to study whether the extract of *C. dactylon* leaves has a protective effect on the liver and heart of alloxan diabetic rats and related toxicological study.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and Materials

The chemicals and materials required for this experimental research were obtained from Sigma–Aldrich and SD fine.

3.2.2. Plant Material and Extract Preparation

Fresh leaves of *C. dactylon* were collected from PRIST University area. The plants were taxonomically identified and authenticated by Rev Dr S John Britto SJ, Director, The Rapinat Herbarium, St Joseph’s College (Autonomous), Tiruchirappalli, Tamil Nadu, India. The voucher specimen’s number is RHCD02. Fresh leaves of *C. dactylon* were air dried in shade and powdered. Aqueous extract: the powdered plant up to 450 g was extracted with boiling water for 10 h. Ethanolic extract: the extraction was carried out by mixing the powdered (550 g) leaves with 1:2 (w/v) in 70% ethanol (v/v) for 2 days. The resulted extracts were filtered and concentrated by rotary evaporator under reduced pressure and low temperature separately (Chopra et al., 1992).

3.2.3. Experimental Animals

Eight-week-old male adult albino rats (Rat: *Rattus norvegicus*) of 130–150 g body weight were used for all experiments. They were housed in separate cages under 12 hr light/dark cycles and kept at constant room temperature (22–25°C). Rats have free access to standard food and water *ad libitum*. Diabetes was induced by a single
intraperitoneal injection of an alloxan with 0.9% saline solution at a dosage of 150 mg/kg body weight. Control animals received a vehicle injection (Uma et al., 2006). All procedures were performed with the guidelines of Institutional Animal Ethics Committee (IAEC No.: 743/03/abc/CPCSEA dt 3.3.03-approval no.: PhD2/2009-2010). Animals with plasma glucose > 200 mg/dl were classified as diabetic. They were a total of four groups (n=6 for each group): group I: control ad libitum; group II: alloxan diabetic, untreated; group III: alloxan diabetic treated with aqueous extract of C. dactylon leaves (450 mg/kg/bw/oral) at 24 h intervals for 15 days; and group IV: alloxan diabetic treated with ethanolic extract of C. dactylon leaves (450 mg/kg/bw/oral) at 24 h intervals for 15 days. After 15 days of treatment, the animals were euthanized and collected plasma [Collect the blood with heparin (10 IU)] and organs for this study.

3.2.4. Estimation of Glucose (AGAPPE Diagnostics Kit)

10 µl of plasma sample mixed with 1000 µl of enzyme reagent and incubated for 10 minutes at 37 °C. At the same time blank and standard solution was prepared. The absorbance of sample against reagent blank was read at 505 nm. The activity was calculated by using the formula. Glucose (mg/ml) = Abs. sample/Abs. standard × conc. of standard. The values are expressed in mg/dl.

3.2.5. Estimation of Plasma and Organs Protein Profile

3.2.5.1. Total Protein (Autozyme kit)

To 0.01 ml of plasma/tissues extract and 1 ml of working solution was added and incubated the assay mixture for 5 minutes at 37 °C. After completion of incubation period the absorbance was measured at 546nm. The activity was calculated by using the formula. Total protein in gm% = Absorbance of sample/Absorbance of standard x 5
3.2.5.2. Total Albumin (Autozyme Kit)

To 0.01 ml of plasma and 1 ml of working solution was added and incubated the assay mixture for 1 minute at 37 °C. After completion of incubation period the absorbance was measured at 600 nm. The activity was calculated by using the formula.

\[
\text{Total albumin in gm\%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 5.
\]

3.2.5.3. Estimation of Globulin (Autozyme Kit)

To 0.01 ml of plasma and 1 ml of working solution was added and incubated the assay mixture for 1 minute at 37 °C. After completion of incubation period the absorbance was measured at 600 nm.

The activity was calculated by using the formula.

\[
\text{Total albumin in gm\%} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 3.
\]

3.2.6. Plasma Lipid Profile (Cholesterol, Triglyceride, HDL, LDL and VLDL)

3.2.6.1. Estimation of Cholesterol (ENSURE Kit)

To 1 ml of enzyme reagent and 10 µl of plasma was mixed well and kept at 37 °C for 5 minutes at room temperature and the absorbance was measured by using spectrophotometer at 505 nm. The activity was calculated by using the formula.

\[
\text{Cholesterol conc. mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. of Std (200)}.
\]

3.2.6.2. Estimation of Triglycerides

10 µl of plasma sample mixed with 1000 µl of enzyme reagent and incubated for 5 minutes at 37 °C. At the same time blank and standard solution was prepared. The absorbance of sample against reagent blank was read at 540 nm. The activity was calculated by using the formula.

\[
\text{Triglycerides conc. (mg/dl)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times 200
\]
3.2.6.3. HDL-Cholesterol

To 200 µl of plasma sample 300 µl of HDL ppt reagent was added and mixed well and kept at room temperature for 10 minutes then centrifuged at 3000 rpm for 10 minutes. Then the pellet was discarded and 1ml of enzyme reagent was added to 100 µl of supernatant, then incubated for 5 minutes at 37 °C and the absorbance was read at 505 nm. The activity was calculated by using the formula. **HDL Cholesterol conc.mg/dl:** \( \frac{\text{Abs of Test}}{\text{Abs of standard}} \times \text{Conc of Std (50)} \).

3.2.6.4. LDL-Cholesterol

The reason for choosing LDL cholesterol as a target for lipid profile is that it represents the fraction of cholesterol, which is most deleterious and has been mostly directly correlated with clinical studies. The LDL was calculated by using the formula.

\[
\text{LDL} = (\text{Total cholesterol}) - (\text{HDL Cholesterol}) - (\text{Triglyceride}/5)
\]

LDL cholesterol levels were expressed as mg/dl plasma.

3.2.6.5. VLDL-Cholesterol

The fraction of VLDL is to transport endogenously synthesized triglyceride and cholesterol into the peripheral tissue. VLDL cholesterol values were calculated using the following formula; the values are expressed in mg/dl.

\[
\text{VLDL} = \text{Triglyceride}/5
\]

3.2.6.6. Atherogenic Index (AI) was calculated by using the following formula;

\[
\text{Atherogenic index} = \frac{\text{Total cholesterol} - \text{HDL-cholesterol}}{\text{HDL-cholesterol}}
\]
3.2.7. Plasma Enzymes Profile (AST, ALT, ALP, AP LDH and CPK)

3.2.7.1. Assay of Aspartate Transaminase (AGAPPE Diagnostics Kit)

To 1000 µl of working reagent and 100 µl of sample was added, mixed and incubate for 1 minute at 37 °C and the absorbance was measured. The activity was calculated by using the formula.

\[
\text{ALT activity in (U/L)} = (\Delta \text{OD/min}) \times 1745
\]

3.2.7.2. Assay of Alanine Transaminase (AGAPPE Diagnostics Kit)

To 1000 µl of working reagent and 100 µl of plasma was added, mixed and incubate for 1 minute at 37 °C and the absorbance was measured at 340 nm. By using the following formula the AST activity was measured.

\[
\text{AST activity (U/L)} = (\Delta \text{OD/min}) \times 1745
\]

3.2.7.3. Assay of Alkaline Phosphatase (ALP)

To 1000 µl of working reagent and 20 µl of plasma was added, mixed and incubate for 1 minute at 37 °C and the absorbance was measured at 405nm. The activity was calculated by using the formula.

\[
\text{ALP Activity (U/L)} = (\Delta \text{OD/min}) \times 2750
\]

3.2.7.4. Assay of Acid Phosphatase (AP)

To 1000 µl of working reagent and 20 µl of plasma was added, mixed ad incubate for 1 minute at 37 °C and the absorbance was measured at 405nm. The activity was calculated by using the formula.

\[
\text{AP Activity (U/L)} = 750 \times (\Delta \text{OD/min})
\]
3.2.7.5. Lactate Dehydrogenase

To 1000µl of working reagent and 10µl of serum/organ extract was added, mixed and incubated for 1 minute at 37°C and the absorbance was measured at 340 nm. The activity was calculated by using the formula.

\[
LDH -P \text{ activity (U/L)} = (\Delta \text{ OD/min}) \times 16030
\]

3.2.7.6. Creatinine Phosphatase (CPK)

To 1000µl of working reagent and 10 µl of plasma was added, mixed and incubated for 1 minute at 37 °C and the absorbance was measured at 340 nm. The activity was calculated by using the formula.

\[
\text{OD}_{40\text{min}} - \text{OD}_{20\text{min}} \times 150
\]

\[
\text{CK (U/L)} = \frac{\text{OD}_{\text{CALIBRATOR}} - \text{OD}_{\text{H2O}}}{\text{OD}_{\text{CALIBRATOR}} - \text{OD}_{\text{H2O}}}
\]

3.2.8. Estimation of Antioxidant Profile

3.2.8.1. Assay of Superoxide Dismutase (Das et al., 2001)

Pipette 1.4 ml aliquot of the reaction mixture in a test tube containing 1.1 ml phosphate buffer, 75 µl methionine, 40 µl triton X-100, 75 µl hydroxylamine hydrochloride and 100 µl EDTA. 100 µl of the sample was added followed by preincubation at 37 °C for 5 min. 80 µl of riboflavin was added and the tubes were exposed for 10 min to 200 W philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 1.0 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50 % of nitrite formation under assay condition.
3.2.8.2. Assay of Catalase (Sinha, 1972)

To 0.9 ml of phosphate buffer, 0.1 ml of plasma and 0.4 ml of hydrogen peroxide was added. At 0 seconds and after 60 seconds 2.0 ml of dichromate-acetic acid mixture was added. The tubes were kept in boiling water bath for 10 minutes and the colour developed was read at 620 nm. Standards in the range of 1.2-6.0 µmol were taken and processed as test and blank containing reagent alone. The activity of catalase was expressed as µmole of H$_2$O$_2$ decomposed/min/mg protein or ml of plasma.

3.2.8.3. Assay of Glutathione Peroxidase (Ellman, 1959)

To 0.4 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione and 0.1 ml of H$_2$O$_2$ were added to two test tubes labeled as test (T) and control (C). To the test added, 0.1 ml of sample and to the control added 0.2 ml of water was added. The contents were mixed well and incubated at 37 °C for 10 minutes. The reaction was arrested with the addition of 0.5 ml of 10% TCA. To determine the glutathione content, 1.0 ml of supernatant was removed by centrifugation. To that added, 3.0 ml of buffer and 0.5 ml of Ellman’s reagent were added. The colour developed was read at 412 nm. Standards in the range of 40-200 µg of reduced glutathione was taken and treated in the similar manner. The activity was expressed in term of µg of glutathione consumed/min/mg protein or ml of plasma.

3.2.8.4. Estimation of Reduced Glutathione (Moron et al., 1979)

Pipette out 0.2 to 1.0 ml standard Glutathion solution corresponding to 40-200 µg concentration. The volume in all the tubes was made up to 1.0 ml with distilled water. 0.1 ml of plasma was pipetted out and precipitated with 2.0 ml of 5% TCA. 1.0 ml of supernatant was taken after centrifugation. To all the tubes added 0.5 ml of Ellman’s reagent and 3.0 ml of phosphate buffer were added. The absorbance was read at 412 nm within 2 min against the reagent blank. The amount of glutathione was expressed as µg/g tissue or ml of plasma.
3.2.8.5. Estimation of LPO (Niehius and Samuelsson, 1968)

Pipette out 0.1 ml of plasma into a test tube, labeled as test and 1.0 ml of water in another tube as blank. Added 2.0 ml of the TBA-TCA-HCl reagent mixture was added to all test tubes. Standards in the range of 20 to 100 µg were treated similarly. Mixed well and placed in boiling water bath for 5 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm.

3.2.9. Histological Studies (Kleiner et al., 2005)

The classic paraffin sectioning and haematoxylin eosin staining techniques were used for the histological studies. The various steps involved in the preparation of tissues for histological studies are as follows:

3.2.9.1. Fixation

In order to avoid tissue by the lysosomal enzymes and to preserve its physical land chemical structure, a bit of tissue from each organ was cut and fixed in bouin’s fluid immediately after removal from the animal body. The tissues were fixed in bouin’s fluid for about 24 hours. The tissues were then taken and washed in glass distilled water for a day to remove excess of picric acid.

3.2.9.2. Dehydration

The term dehydration means the removal of water from the tissues by alcohol of varying grades. For dehydration ethanol was used. The tissues were kept in the following solutions for an hour each: 30%, 50%, 70% and 100% alcohol. Inadequately dehydrated tissues cannot be satisfactorily infiltrated with paraffin. At the same time over dehydration results in making the tissues brittle, which would be difficult for sectioning. So, the tissues were carefully dehydrated.
3.2.9.3. Clearing

Dealcoholization or replacement of alcohol from the tissues with a clearing agent is called as clearing. Xylene was used as the clearing agent for one or two hours, two or three times. Since, the clearing agent is miscible with both dehydration and embedding agents, it permits paraffin to infiltrate the tissues. So, the clearing was carried out as the next step after dehydration to permit tissue spaces to be filled with paraffin. The tissues were kept in the clearing agent till they become transparent and impregnated with xylene.

3.2.9.4. Impregnation

In this process the clearing agent xylene was placed by paraffin wax. The tissues were taken out of xylene and were kept in molten paraffin embedding bath, which consists of metal pots filled with molten wax maintained at about 50 °C. The tissues were given three changes in the molten wax at half an hour intervals.

3.2.9.5. Embedding

The paraffin wax used for embedding should be fresh and heated upto the optimum melting point at about 56–58 °C. A clear glass plate was smeared with glycerine. L-shaped mould was placed on it to from a rectangular cavity. The molten paraffin wax was poured and air bubbles were removed by using a hot needle. The tissue was placed in the paraffin and oriented with the surface to be sectioned. Then the tissue was pressed gently towards the glass plate to make settle uniformly with a metal pressing rod and allowed the wax to settle and solidity room temperature. The paraffin block was kept in cold water for cooling.

3.2.9.6. Section Cutting

Section cutting was done with a rotatory microtome. The excess of paraffin around the tissue was removed by trimming, leaving ½ cm around the tissue. Then the
block was attached to the gently heated holder. Additional support was given by some extra wax, which was applied along the sides of the block. Before sectioning, all set screws holding the object holder and knife were hand tightened to avoid vibration. To produce uniform sections, the microtome knife was adjusted to the proper angle in the knife holder with only the cutting edge coming in contact with the paraffin block. The tissue was cut in 7 µ thickness.

### 3.2.9.7. Flattening and Mounting of Sections

This was carried out in tissue flotation warm water bath. The sections were spread on a warm water bath after they were detached from the knife with the help of hair brush. Dust free clean slides were coated with egg albumin (not for histochemistry) over the whole surface. Required sections were spread on clean slide and kept at room temperature.

### 3.2.9.8. Staining

The sections were stained as follows; deparaffinization with xylene two times each for five minutes

- Dehydration through descending grades of ethyl alcohol
  - 100% alcohol (absolute) - 2 minute
  - 90% alcohol - 1 minute
  - 50% alcohol - 1 minute

- Staining with Ehrlich’s haematoxylin for 15-20 minutes. Thoroughly washed in tap water for 10 minutes. Rinsed with distilled water and stained with eosin. Dehydration again with ascending grades of alcohol.
  - 70% alcohol - 2 minute
  - 90% alcohol - 2 minute
  - 100% alcohol - 1 minute

- Clearing with xylene two times, each for about 3 minutes interval.
3.2.9.9. Mounting

On the stained slide, DPX mountant was applied uniformly and microglass cover slides were spread. The slides were observed in Nikon microscope and microphotographs were taken.

3.2.10. Statistical Analysis

The data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed by one-way analysis of variance (ANOVA). The results were considered statistically significant, if the $P$ values were 0.05 or less.

3.3. RESULTS

3.3.1. Body Weight and Organs Weight

The body weight and various organs weight of the experimental rats i.e. alloxan treated diabetic rats and control are Tabulated as 3.1 and 3.2. The total body weight decreased during diabetes, but the individual organ weight like heart, liver, spleen and kidney increased in diabetic rats, whereas weight of brain decreased ($P < 0.05$). These changes in organ weight were alleviated to different extents by feeding $C$. dactylon leaves extract. It has been reported that, the weight of the liver increases during diabetes and our results are also in agreement with the same.

Table 3.1 – Effect of aqueous and ethanolic extract of $C$. dactylon on body weight in control and experimental animals. Values are expressed mean ± SEM of six animals. $^aP < 0.05$, as compared to diabetic induced.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
</tr>
<tr>
<td>Group I</td>
<td>134.8 ± 8.24</td>
</tr>
<tr>
<td>Group II</td>
<td>135.1 ± 10.2</td>
</tr>
<tr>
<td>Group III</td>
<td>137.8 ± 9.31</td>
</tr>
<tr>
<td>Group IV</td>
<td>137.0 ± 9.28</td>
</tr>
</tbody>
</table>
Table 3.2 – Effect of aqueous and ethanolic extract of C. dactylon on organs weight in control and experimental animals. Values are expressed mean ± SEM of six animals. *P < 0.05, as compared to diabetic induced.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Organs Weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>Group I</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>Group II</td>
<td>0.56 ± 0.12</td>
</tr>
<tr>
<td>Group III</td>
<td>0.59 ± 0.19</td>
</tr>
<tr>
<td>Group IV</td>
<td>0.42 ± 0.19</td>
</tr>
</tbody>
</table>

3.3.2. Blood Glucose Level

The effect of aqueous and ethanolic extract of C. dactylon leaves on blood glucose levels is shown in Figure 3.2. A significant difference in the blood glucose levels in all groups were observed at the end of the 15-day of treatment. The blood glucose levels were increased significantly in alloxan diabetic rats as compared with the control rats (*P < 0.05). In diabetic rats, significant decrease in blood glucose levels were observed after the oral administration of aqueous and ethanolic extract of C. dactylon leaves. Blood glucose levels of normal, diabetic rats and treated animals were 101.0 ± 5.2, 210.0 ± 6.9, 125 ± 3.15 and 110.5 ± 8.92 mg/dl, respectively (*P < 0.05).
3.3.3. Plasma and Organs Protein Profile

Total protein content was significantly reduced, whereas albumin and globulin increased in diabetic rats as compared with control rats. In diabetic rats, increase in the total protein and decrease in the albumin and globulin content were observed due to the *C. dactylon* treatment and values are indicated in Figure 3.3 ($P < 0.05$). The different organ protein content of control, diabetic and treated groups are presented in Table 3.3 and 3.4. A decrease in protein content of diabetic rat liver, spleen, kidney, brain, pancreases, muscle and adipose tissue but, an increase in heart protein content was observed when compare with control rats ($P < 0.05$). The organ protein contents were restored to normal level after the treatment with aqueous and ethanolic extract of *C. dactylon* leaves.

Figure 3.3 – Effect of aqueous and ethanolic extract of *C. dactylon* on plasma protein profile in control and experimental animals. Values are expressed mean ± SEM of six animals.
Table 3.3 – Effect of aqueous and ethanolic extract of C. dactylon on organs protein profile in control and experimental animals. Values are expressed mean ± SEM of six animals. *P < 0.05, as compared to diabetic induced.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein Concentration (mg of protein/gm of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>Group I</td>
<td>41.14 ± 5.21</td>
</tr>
<tr>
<td>Group II</td>
<td>43.51 ± 6.32</td>
</tr>
<tr>
<td>Group III</td>
<td>42.13 ± 3.25</td>
</tr>
<tr>
<td>Group IV</td>
<td>33.60 ± 7.230</td>
</tr>
</tbody>
</table>

Table 3.4 – Effect of aqueous and ethanolic extract of C. dactylon on organs protein profile in control and experimental animals. Values are expressed mean ± SEM of six animals. *P < 0.05, as compared to diabetic induced.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein Concentration (mg of protein/gm of tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
</tr>
<tr>
<td>Group I</td>
<td>20.24 ± 2.31</td>
</tr>
<tr>
<td>Group II</td>
<td>17.60 ± 3.89</td>
</tr>
<tr>
<td>Group III</td>
<td>20.10 ± 1.75</td>
</tr>
<tr>
<td>Group IV</td>
<td>29.20 ± 4.580</td>
</tr>
</tbody>
</table>

3.3.4. Plasma Lipid Profile (Cholesterol, Triglyceride, HDL, LDL and VLDL)

Differences in the plasma cholesterol levels of all groups were observed at the end of the 15-day of treatment (P < 0.05) as indicated in Table 3.5. Plasma cholesterol levels were significantly increased in diabetic rats when compared with control group. Oral administration of aqueous and ethanolic extract of C. dactylon leaves had significantly reduced the plasma cholesterol levels in diabetic treated rats 27.8 % and 32.7% respectively. In diabetic rats, a significant elevation of plasma LDL-cholesterol
and VLDL-cholesterol was observed as compared with control rats. No change in HDL-cholesterol level was observed. After administration of aqueous and ethanolic extract of *C. dactylon* leaves, it was found to reduce LDL-cholesterol (43.6 % and 36.6 %) \((P < 0.05)\) and VLDL-cholesterol (50.0 % and 39.5 %) and increase HDL-cholesterol (24.2 % and 17.1 %) in diabetic treated rats \((P < 0.05)\). Triglyceride and atherogenic index in plasma were increased significantly in diabetic rats as compared with normal rats. Administration of leaf extract reversed these effects \((P < 0.05)\).

**Table 3.5 – Effect of aqueous and ethanolic extract of *C. dactylon* on plasma lipid profile in control and experimental animals. Values are expressed mean ± SEM of six animals. \(^aP < 0.05, \text{ as compared to diabetic induced.}\)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Lipid Profile (mg/dl)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>TG</td>
</tr>
<tr>
<td>Group I</td>
<td>31.9 ± 3.21</td>
<td>64.9 ± 4.24</td>
</tr>
<tr>
<td>Group II</td>
<td>42.8 ± 2.75</td>
<td>105.6 ± 6.84</td>
</tr>
<tr>
<td>Group III</td>
<td>30.9 ± 4.25</td>
<td>48.6 ± 5.96</td>
</tr>
<tr>
<td>Group IV*</td>
<td>28.8 ± 4.23</td>
<td>55.6 ± 5.38</td>
</tr>
</tbody>
</table>

**3.3.5. Plasma Enzymes Profile (AST, ALT, ALP, AP LDH and CPK)**

A significant elevation of plasma AST and ALT activity \((P < 0.05)\) were observed in diabetic rats as compared with control. Treatment of diabetic rats with aqueous and ethanolic extract of *C. dactylon* leaves resulted in a significant decrease in the levels of AST (30.5 % and 28 %) and ALT (21.5 % and 32.2 %), when compared with diabetic control. A significant increase in the levels of ALP, AP, LDH and CPK \((P < 0.05)\) were observed in diabetic control when compared with control group. A significant level of reduction in plasma-marker enzymes [ALP: (21.5 % and 27.2 %); AP: (15 % and 22.5 %); LDH: (20.1 % and 25.5 %) and CPK: (7 % and 9.5 %)] were noticed due to the effect of aqueous and ethanolic extract of *C. dactylon* leaves as indicated in Table 3.6.
Table 3.6 – Effect of aqueous and ethanolic extract of C. dactylon leaves on plasma marker enzyme profile in control and experimental animals. Values are expressed mean ± SEM of six animals. *P < 0.05, as compared to diabetic induced.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Enzyme Profile (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AST</td>
</tr>
<tr>
<td>Group I</td>
<td>50.3 ± 5.21</td>
</tr>
<tr>
<td>Group II</td>
<td>88.6 ± 7.52</td>
</tr>
<tr>
<td>Group III</td>
<td>69.5 ± 6.35</td>
</tr>
<tr>
<td>Group IV</td>
<td>60.0 ± 6.35</td>
</tr>
</tbody>
</table>

Table 3.7 – Effect of aqueous and ethanolic extract of C. dactylon on antioxidant activity in control and experimental animals. Values are expressed mean ± SEM of six animals. *P < 0.05, as compared to diabetic induced. U₁/one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min; U₂/mmol of hydrogen peroxide consumed/minute; U₃/mg of glutathione consumed/minute.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antioxidant Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD (U₁/mg Protein)</td>
</tr>
<tr>
<td>Group I</td>
<td>8.90 ± 1.25</td>
</tr>
<tr>
<td>Group II</td>
<td>6.32 ± 0.96</td>
</tr>
<tr>
<td>Group III</td>
<td>14.7 ± 2.35</td>
</tr>
<tr>
<td>Group IV</td>
<td>12.8 ± 2.35</td>
</tr>
</tbody>
</table>

Identification and Characterization of Novel Proteins in Diabetic and Diabetic Treated [Cynodon dactylon (L.) Pers] Albino Rats
3.3.6. Antioxidant Level

Table 3.7 illustrates the activities of SOD, CAT, GPx, GSH and LPO in plasma of control and experimental groups. There were a significant reduction in the activities of SOD, CAT GPx and GSH during diabetes. Administration of aqueous and ethanolic extract of *C. dactylon* leaves tends to bring the values to near control [SOD (133 % and 103.1%), CAT (6.3 % and 9.4 %), GPx (29.8 % and 27.2 %) and GSH (17.1 % and 7.1 %)]. There was a significant elevation in plasma LPO during diabetes when compared to the corresponding control group. Administration of aqueous and ethanolic extract tends to bring the values to near control 35.5 % and 21% respectively.

3.3.7. Histological Studies for Pancreatic Tissue

Figure 3.4 (A–D) represents the photomicrographs of hematoxylin– eosin staining of pancreatic tissues section of control and experimental groups of rat. Figure 3.4A shows the pancreatic tissue of control rats exhibiting a concentric arrangement of the pancreatic cell (alpha and beta) around the central vein and arteries. Figure 3.4B portrays the section of pancreatic tissues of alloxan-induced group of rat exhibiting distortion in the arrangement of beta cell around pancreas. Figure 3.4C and D demonstrates the section of pancreatic tissues of alloxan-induced group of rats treated with aqueous and ethanolic extract of *C. dactylon* leaves presenting the normal pancreatic arrangement around the vein and arteries.
3.4. DISCUSSION

Diabetes mellitus is a pathologic condition, resulting in severe metabolic imbalances and non-physiologic changes in many tissues, where oxidative stress plays an important role in the etiology (Baynes and Thorpe, 1996). Alloxan causes diabetes by rapid depletion of β-cells which leads to reduction in insulin release (Gupta et al., 2004). Hyperglycemia causes oxidative damage by generation of ROS
Identification and Characterization of Novel Proteins in Diabetic and Diabetic Treated [Cynodon dactylon (L.) Pers] Albino Rats

Chapter–3

(Mohamed et al., 1999) and development of diabetic complications (Baynes and Thorpe, 1999).

The administration of _C. dactylon_ leaves extract to alloxan diabetic rats reduced blood glucose levels, is in accordance with our previous (Ravi et al., 2004). These effects may be attributed to the insulin mimicking effect of plant extract (Bolkent et al., 2005). Lowering the plasma lipid levels through dietary or drug therapy appears to be associated with a decrease in the risk of vascular disease (Grudy et al., 1999). The increase in the plasma lipids on the diabetic subject is mainly due to the increased mobilization of free fatty acids from peripheral deposits, since insulin inhibits the hormone sensitive lipase (Al-Shamaony et al., 1994). On the other hand, glucagon, catecholamines and other hormones enhance lipolysis. Liver, an insulin dependent tissue that plays a pivotal role in glucose and lipid homeostasis and it is severely affected during diabetes (Stone and Van Thiel, 1985). Diabetes results in decrease in glucose utilization and an increase in glucose production in insulin-dependent tissues, such as liver. A marked increase in hepatic lipid concentration has been earlier observed during diabetes (Gupta et al., 1999).

Alloxan is a chemical that induces loss of insulin-producing islet β-cells and causes a hypoinsulinemic condition and resultant diabetes mellitus in animals. This effect is thought to be mediated by a sequence of redox reactions involving the production of superoxide anion radicals in or near the β-cells (Heikkila and Cabbat 1978; Jorns et al., 1999). It has been reported that damage to beta cells by alloxan profoundly decreases the blood glucose uptake for the peripheral cell. These changes can be restored to a normal level by insulin treatment (Henderson et al., 1981). The administration of the aqueous and ethanolic extract of _C. dactylon_ leaves to diabetic rats changes were restored to a normal level quite similar to that of control group of rats. The results of the histopathological studies supported and well interrelated with data obtained from biochemical analysis.

Cholesterol is a powerful risk factor for many coronary heart diseases. The degree of hypercholesterolemia is directly proportional to severity in diabetes. In the present study, we have observed higher levels of cholesterol in plasma of diabetic rats.
The increased level of cholesterol in plasma is due to the decreased level of HDL-cholesterol. This in turn results in decreased removal of cholesterol from extrahepatic tissues by the HDL-cholesterol (Pari and Satheesh, 2004). Administration of *C. dactylon* leaves extract to diabetic rats normalizes plasma cholesterol levels. The underlying mechanism by which plant extract exerts its cholesterol lowering effect seems to be a decrease in cholesterol absorption from the intestine, by binding with bile acids within the intestine and increasing bile acids excretion (Eddouks *et al.*, 2005). Plant extract may acts by decreasing the cholesterol biosynthesis especially by decreasing the HMG-CoA reductase activity, a key enzyme of cholesterol biosynthesis and/or by reducing the NADPH required for fatty acids and cholesterol biosynthesis (Sharma *et al.*, 2003). In addition, plant extract may improve hypercholesterolemia by modifying lipoprotein metabolism: enhanced uptake of LDL by increasing LDL receptors (Slater *et al.*, 1980) and/or by increasing the lecithin:cholesterol acyl transferase activity (Khanna *et al.*, 2002) which may contribute to the regulation of blood lipids. HDL-cholesterol is recognized as a factor that protects against development of atherosclerotic disease and low HDL-cholesterol is associated with an increased risk of CHD in individuals both with and without diabetes. Total cholesterol/HDL and LDL/HDL cholesterol ratio are also predictor of coronary risk (Laakso *et al.*, 1993). In the present study, rats treated with *C. dactylon* leaves extract had markedly reduced ratios. These results indicated that *C. dactylon* leaves extract might have some protective effects against hypercholesterolemia risks in diabetes. In our study, diabetic rats exhibited clear-cut abnormalities in lipid metabolism as evidenced from the significant elevation of VLDL-cholesterol level and atherogenic index. Treatment with *C. dactylon* leaves extract for 15 days significantly and greatly reduced VLDL-cholesterol level and decreased in atherogenic index in diabetic rats indicating its potent antihyperlipidemic and antiatherogenic activities of plant extract represent the reduction of CV complication.

The marked increase in plasma triglyceride observed in diabetic rats is in agreement with the finding of the some researcher (Nikkila and Kekki, 1973). The increased level of triglycerides in alloxan diabetes observed in our study may be due to lack of insulin, which normally activates the enzyme lipoprotein lipase. Lopez-Virella
et al. reported that treatment with insulin served to lower plasma triglyceride levels, by returning lipoprotein lipase levels to normal (Lopez-Virella et al., 1983). Oral administration of *C. dactylon* leaves extract to diabetic rats reduced plasma triglycerides levels. Our finding is a very desirable biochemical state for prevention of atherosclerosis.

Oxidative stress in diabetes coexists with a reduction in the antioxidant capacity, which can increase the deleterious effects of free radicals. Increased oxidative stress is believed to play an important role in the aetiology and pathogenesis of chronic complications of diabetes. This study analyzed the effect of an aqueous and ethanolic extract from *C. dactylon* leaves on biomarkers of oxidative stress in plasma antioxidant of diabetic rats. In our study, the activity of SOD was found to be lower in diabetic rats. This decrease in SOD activity could result from inactivation by \( \text{H}_2\text{O}_2 \) or by glycation of the enzyme, which are known to occur during diabetes due to hyperglycemia (McCord et al., 1976). Reduced activities of CAT in plasma have been observed during diabetes. CAT is a heme protein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals (Chance et al., 1952). Therefore, reduced in the activity of this enzymes may result in a number of deleterious effects due to the accumulation of superoxide anion radicals and hydrogen peroxide. Administration of *C. dactylon* leaves extract resulted in the elevation of the SOD and CAT level, which protects oxidative damage in tissues.

Under *in vivo* conditions, GSH acts as an antioxidant and its level was reduced in diabetes mellitus (Baynes and Thorpe, 1999). During diabetes, we also observed a significant decreased in GSH levels in plasma. In a similar manner, Matkovics et al. showed decreased amounts of GSH in the skeletal muscle, liver and lung homogenates of mice with diabetes (Matkovics et al., 1997). The decrease in GSH levels represents increased utilization due to oxidative stress (Anuradha and Selvam, 1993). The depletion of GSH content also may lower glutathione-S-transferase (GST) activity, because GSH is required as a substrate for glutathione-S-transferase activity (Rathore et al., 2000). Reduction in glutathione peroxidase (GPx) activity was also observed in plasma during diabetes. GPx has been shown to be an important adaptive response to conditions of increased peroxidative stress (Matcovic et al., 1982). Administration of
C. dactylon leaves extract resulted in the elevation of the GSH level, which protects the cell membrane against oxidative damage by regulating the redox status of protein in the cell membrane. The increase in the GSH content may protect the tissues against diabetes-associated tissue injury by reducing the susceptibility to toxic radicals.

Lipid peroxidation is a marker of cellular oxidative damage initiated by reactive oxygen species (Farber et al., 1990). The increase in oxygen free radicals in diabetes could be due to an increase in levels of blood glucose, which upon autooxidation generates free radicals. Insulin secretion is also closely associated with lipoxygenase-derived peroxides (Walsh and Pek, 1984). Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled lipid peroxidation leading to cellular infiltration and islet cell damage in type 1 diabetes (Metz, 1984). Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. Its products (lipid radical and lipid peroxide) are harmful to the cells in the body and associated with atherosclerosis, brain, kidney and other tissue damage (Orhan et al., 2005). In the present study, the concentrations of lipid peroxidation, indicating an increased in the generation of free radicals were increased in the stomach and spleen tissues of diabetic rats. After administration of C. dactylon leaves extract, the levels of lipid peroxidation declined significantly and thus prevent tissue damage.

Some transition metals, such as nickel, chromium and vanadium might act as catalysts of the oxidative deterioration of biological molecules (Wronska-Nofer et al., 1999). This might occur via formation of reactive oxygen species and enhanced lipid peroxidation, depletion of sulfhydryls and oxidative tissue injury (Stohs and Bagchi, 1995). Vanadium compounds may behave as antioxidants and pro-oxidants, depending on experimental conditions and the dose of vanadium. In our results, induced diabetes by alloxan caused a significant decrease in plasma GSH and increase in plasma LPO. The administration of C. dactylon leaves extract to diabetic rats reduced LPO and turned GSH toward its normal values. These results suggest that the administration of C. dactylon leaves extract may have a protective effect against tissues damage in pro-oxidant conditions during diabetes.
Administration of aqueous and ethanolic extract of *C. dactylon* leaves improved the liver function by decreasing the plasma AST, ALT and ALP levels in diabetic treated rats. The increase of AST and ALT will increase the incidence of heart and liver diseases. AST is an enzyme found primarily in the cells of the liver, heart, skeletal muscles, kidneys, pancreas and to a lesser extent, in red blood cells. Its plasma concentration is in proportion to the amount of cellular leakage or damage. It is released into plasma in larger quantities when any one of these tissues is damaged. Its increased levels are usually associated with heart attacks or liver disease (Maiti *et al*., 2004). The aqueous and ethanolic extract of *C. dactylon* leaves were decreased the AST level, which is an indication of the protective effect on liver and heart.

ALT an enzyme found primarily in the liver is far greater. Its enhanced release into the bloodstream is the result of liver abnormality. It therefore serves as a specific indicator of liver status and its elevated levels in plasma indicate liver damage. Increased levels of ALP indicate bone disease, liver disease or bile tract blockage (Kesari *et al*., 2007). The aqueous and ethanolic extract of *C. dactylon* leaves were reduced the ALT and ALP levels too indicating its protective effect over liver and improvement in liver functional efficiency. Measurement of enzymic activities of acid phosphatases (AP) is of clinical and toxocological importance as changes in their activities are indicative of tissue damage by toxicants or in disease conditions. Acid phosphatase activity of plasma of diabetic rats was found to be increased as compared with control. At the dose of 450 mg/kg for 15 days, aqueous and ethanolic extract is reported to inhibit acid phosphatase activity and reduced the AP level in plasma (Chauhan *et al*., 1979).

The significant increases of LDH are mainly due to leakage of these enzymes in to the blood because of alloxan toxicity in liver. Higher activity of glucose-6-phosphatase provides H⁺ which binds with NADP⁺ to form NADPH which is helpful in the synthesis of fats from carbohydrates. When glycolysis slows down because of cellular activity, pentose phosphate pathway that is still active in liver provides NADPH, which converts acetyl radicals in to long chain fatty acids during diabetes mellitus. Similar results were reported by other researchers in experimental
diabetes (Grover et al., 2000). However, treatment of alloxan diabetic rats with aqueous and ethanolic extract of *C. dactylon* leaves for 15 consecutive days could restore the normal metabolism by shifting the balance from lipids metabolism to carbohydrate metabolism (Rajagopal and Sasikala, 2008).

It is well known that diagnosis of cardiac enzymes is very important. Plasma CPK activity is a more sensitive indicator in early stage of myocardial ischemia, while peak rises in myocardial tissue damage (Chatterjea and Shinde, 2002). The results in diabetic treated animals in this experiment shows a protective effect of aqueous and ethanolic extract of *C. dactylon* leaves on the heart of experimental animals. Moreover, the significantly lowered activities of CPK, scientifically suggest that the leaf extract of *C. dactylon* have the potential of reducing the factors that produce infarction in the myocardium. This is so because the metabolism of alloxan-induced infarct myocardium may be studied by assessing the level of marker enzyme in the plasma (Edet et al., 2009). The present study shows that improved diabetic condition by administration of *C. dactylon*. The GC-MS analysis of plant extract revealed the presence of 7 major compounds in aqueous extract and 6 major compounds in ethanolic extract. 2-Propenoic acid and tetra methyl-2-hexadecen-1-ol are present in both aqueous and ethanolic extract of *C. dactylon*. Based on our GC-MS result, we could predict that, 2-propenoic acid and tetra methyl-2-hexadecen-1-ol could be having anti-diabetic activity (Refer chapter-2). In this study, the aqueous extract demonstrated higher activity than the ethanolic extract. This suggests that the active principle(s) were more soluble in ethanol than in the aqueous medium.

In conclusion, the administration of *C. dactylon* leaves extract may be able to reduce hyperglycemia and hyperlipidemia related to the risk of diabetes. This has clinical implications, that the relatively nontoxic *C. dactylon* extract, if used as a hypoglycemic agent, may also reverse dyslipidemia associated with diabetes and prevent the CV complications, which are very prevalent in diabetic patients. GC-MS analyses of the extracts of chemical constituents are active antidiabetic and antioxidant as well as hypolipidemic principles in *C. dactylon*. The present investigation has also opened avenues for further research especially with reference to the development of potent phytomedicine for diabetes mellitus from *C. dactylon* leaves.