CHAPTER – IV

ANTIDIABETIC ACTIVITY OF *Pimenta dioica*

METHANOLIC LEAF EXTRACT IN STZ – INDUCED DIABETIC RATS

4.1 INTRODUCTION

Diabetes mellitus is a metabolic disorder of the endocrine system. It is considered to be one of the most serious endocrine syndromes. The disease occurs worldwide and its incidence is increasing rapidly in most part of the world. People suffering from diabetes are not able to produce or properly use insulin in the body and hence they have a high level of blood glucose. Diabetes is becoming the third killer disease of mankind, after cancer and cardiovascular diseases, because of its high prevalence, morbidity and mortality. Approximately 4% of the population worldwide suffering from diabetes is expected to increase by 5.4% in 2025 (Kim, 2006). The management of diabetes is considered a global problem and a cure has yet to be discovered. Despite various studies have been carried out in understanding the management of diabetes and also the presence of known antidiabetic medications available in the market, diabetes as well as its related metabolic complications have continued to be a challenging medical problem. This is imputable to the increasing side
effects of the antidiabetic medications and also their inadequacy in treating various related ailments associated with diabetes.

The searching for new antidiabetic drugs from natural plants is growing attention as they contain phytochemical compounds which can be used as an alternative and may also prove helpful in subsiding the vast abnormalities associated with diabetes mellitus. Concurrently, Phytochemicals identified from traditional medicinal plants are presenting exciting opportunities for the development of new drug therapies. The ethnobotanical information reports about 800 plant species that may possess the antidiabetic activity. Among that maximum species have possessed antioxidant property and multi therapeutic properties. Many indigenous Indian medicinal plants have been found to be useful for managing diabetes. After recommendations made by the World Health Organization on medicinal plants for antidiabetic drugs, various researchers concentrated on traditional medicinal plants for more effective as well as safer hypoglycemic drug candidates. Many useful plants as well as herbs which have been introduced in pharmacological and clinical trials confirmed to reduce blood sugar levels (Thirumalai 2012; Arumugam , 2013). So it is important to pharmacologically evaluate various plants used in the
traditional system of medicine for larger scope of isolating novel antidiabetic drugs (Gupta, 2007).

The number of people diagnosed with type 2 diabetes is estimated to be at 2% - 3% of the world population and is rising at a rate of 4% - 5% per year (Patel, 2003). Currently available oral hypoglycemic drugs for the treatment of DM have the characteristic profile of adverse effects. Hence, research is focused to screen the medicinal plants that are used traditionally for the treatment of DM to find a newer lead drug molecule from phyto constituents with more potential and lesser side effects than the existing hypoglycemic agents (Chandramohan, 2008). World Health Organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective, non-toxic with less or no side effects and are considered to be excellent candidates for oral therapy (Patel, 1997). Many review articles and research papers appeared in the journals and books showed that most of the plants used in the traditional system of medicine for the treatment of DM proved to be scientifically effective (Grover, 2002; Ray, 2010). Hence, the antidiabetic drug discovery has diverted their focus to natural plant derived drugs which also minimized the side effects associated with conventional diabetes treatments.
Plants have always played a very important role in contributing new therapeutic agents for treatments of various diseases. Metformin is a very good example and was discovered from a medicinal plant, *Galega officinalis* and later synthesized as a commercial drug for treating diabetes (Aiman, 1970). The increased blood glucose leads to increased levels of superoxide anions in diabetes, which causes the generation of hydroxyl radicals via Haber-Weiss reaction and result in per-oxidation of membrane lipids as well as protein glycation. This results in oxidative damage to membranes of cells. These radicals can further impose its damage effects on other major biomolecules such carbohydrates, proteins and can also lead to DNA damage (Sato, 1970). Streptozotocin (STZ) can selectively destroy the β-cells of the pancreas by inducing increased ROS generation and carbonium ion (CH3+) which lead to DNA breaks via alkylating DNA bases. The N-nitrous-N methyl urea section of the molecule imposes diabetogenic activity. Glucose may play an important role as carriers for this cytotoxic group (Verspohl, 2002). Disturbance in lipid metabolism is so common in diabetes that diabetes is sometimes called as “more a disease of lipid than carbohydrate metabolism” (Rawi et al., 1998).

*Pimenta dioica* (L.) Merril (Fam: Myrtaceae) is usually known as Allspice in culinary uses. It gets its name from the berries aroma,
that smells like the blend of spices, such as cinnamon, cloves, ginger and nutmeg. Allspice gets its characteristic odour because of the presence of essential oil in the pericarp of its seeds. The plant Allspice is mentioned in the Wealth of India (Wealth of India, 1969). Pimenta berry oil is used mostly as a carminative and stimulant. It shows bactericidal, fungicidal as well as antioxidant properties. Allspice is used as a paste and relieve tooth ache and also as a mouth wash to freshen the breath. Pimenta is an aromatic stimulant and carminative to the gastro-intestinal tract, which resemble cloves in its activities. Antioxidants are helpful in preventing disease such as cardiovascular, diabetes, cancer, rheumatoid arthritis, inflammatory bowel disease, pancreatitis, hematological and neurodegenerative diseases (Irshed, 2002; Craig, 1997). In India, the leaves of Pimenta are used to flavor rice which gives adds a typical aroma. Allspice is considered as a very important spice in the meat industry which use the berry powder for meat tenderizing (Seidemann, 2005; Sharma, 2003). In this context, the present study was aimed to investigate the antidiabetic activity of the methanolic leaf extracts of Pimenta dioica in streptozotocin induced diabetic rats.
4.2 MATERIALS AND METHODS

4.2.1 Estimation of blood glucose

Blood glucose was estimated by the method O-toluidine using the modified reagent of Sasaki et al. (1972).

Reagents

1. Trichloro acetic acid- 10%

2. O-toluidine reagent- 12.5g of thiourea and 12.0 g of boric acid were dissolved in 50.0 ml of distilled water by heating over a mild flame. 75.0 ml of redistilled O-toluidine and 375 ml of acetic acid were mixed and the total volume was made upto 500 ml with distilled water. The reagent was left in a refrigerator overnight and filtered.

3. Stock standard – 1 mg/ml :100 mg of anhydrous D-glucose was dissolved in 100 ml of water containing 0.01% benzoic acid.

4. Working standard- 1μg/ml: 10.0 ml of stock glucose solution was diluted to 100 ml distilled water.

Procedure

0.1ml of freshly drawn blood was immediately mixed with 1.9 ml of 10% TCA to precipitate the proteins and then centrifuged. 1.0 ml of the supernatant was mixed with 4.0 ml of O-toluidine reagent and was kept in a boiling water bath for 15 minutes. The green color
developed was read colorimetrically at 620nm. A set of standard glucose (20 -100μg) was treated simultaneously using reagent blank. Glucose concentration was expressed as mg/dl of blood.

4.2.2 Estimation of haemoglobin

Haemoglobin in the blood was estimated by the method of Drabkin and Austin (1932). The dilution of blood in an alkaline solution containing potassium cyanide and potassium ferricyanide form the basis of this method. Haemoglobin gets oxidized forming cyanmethaemoglobin whose absorbance was then measured at 540 nm.

Reagents

1. Drabkin’s reagent: 200 mg of potassium ferricyanide, 50 mg of potassium cyanide and 1.0 g of Na₂CO₃ were dissolved in distilled water and made up to one liter. The reagent had a pale yellow color of pH 9.6 and was stored in brown bottle.

2. Cyanmethaemoglobin standard solution: 16g/dl.

Procedure

0.02 ml of blood and 5.0 ml of Drabkin’s reagent were added, mixed well and allowed to stain for 10 minutes. The solution was read at 540 nm together with the standard solution against a reagent blank. Values were expressed as g/dl of blood.
4.2.3 Estimation of glycosylated haemoglobin (HbA\textsubscript{1}C)

Glycosylated haemoglobin in the blood was estimated by the method of Sudhakar and Pattabiraman (1981).

Reagents

1. 1 m potassium oxalate in 2 m hydrochloric acid (Oxalate hydrochloric acid).
2. Phenol: 80%
3. Concentrated H\textsubscript{2}S\textsubscript{0}\textsubscript{4}
4. TCA: 40%
5. Saline
6. Stock solution: Stock solution was prepared by dissolving 100 mg of fructose in 100 ml of distilled water.
7. Working standard: Stock standard was diluted to get a concentration of 100\textmu g/ml

Procedure

0.5 ml of saline washed erythrocytes were lysed with 5.5 ml of water, mixed and incubated at 37\textdegree C for 15 minutes. The contents were centrifuged and the supernatant was discarded, then 0.5 ml of saline water was added, mixed and processed for estimation. About 0.2 ml of aliquot and 4 ml of oxalate hydrochloric solution were added and mixed. The contents were mixed at 100 °C for 4 h, cooled and
precipitated with 2 ml of 40% TCA. The mixture was centrifuged and
to 0.5 ml of supernatant, 0.5 ml of 80% phenol and 3.0 ml of
concentrated sulphuric acid were added. The colour developed was
read at 480 nm after 30 minutes. The concentration of glycosylated Hb
was expressed as mg/g of haemoglobin.

4.2.4 Quantitative determination of plasma insulin

The plasma insulin was assayed by ELISA method (Enzyme
Linked Immunosorbant Assay) using Boehringer Manheim Kit
(Boehringer analyzer ES 300).

Reagents


2. Phosphate buffer 40mM/l, pH-7.0.

3. Anti-insulin POD conjugates (POD-peroxidase).


5. Substrate: Phosphate/ citrate 100mM/l, H₂O₂ Ph-4.4/ (as
perborate) 3.2 mM/l

6. Chromogen: Di-ammonium 2, 2-azino-bis (3-ethyl benzothia-
zoline-6-sulphonate).
**Procedure**

0.1 ml of plasma was injected into the plastic tubes coated with monoclonal anti-insulin antibodies. Phosphate buffer and anti-insulin POD conjugate were added to form anti-insulin antibody-POD conjugate. Substrate-chromogen solution was then added to form indicator reaction. A set of standards were also treated in similar manner. After the development of colour, the absorbance was read at 420 nm. The values were expressed as µU/ml of plasma.

**4.2.5 Estimation of urea**

Urea in the plasma was estimated by using the diagnostic kit based on the method of Fawcett and Scott (1960).

Urea hydrolyse in the presence of water and urease to produce ammonia and carbon dioxide. Under alkaline conditions, the ammonia so formed reacts with hypochlorite and sodium salicylate in the presence of sodium nitroprusside to form a green colored chromophore. The intensity of the colour produced is proportional to the concentration of urea in the sample.

**Reagents**

1. Buffered enzyme: Phosphate buffer, urease, sodium nitroprusside and ethylenediamine tetra acetic acid.
2. Colour developing reagent: Buffer, sodium hypochlorite, sodium salicylate and sodium hydroxide.

3. Urea standard: 40 mg/dl.

Procedure

To 1.0 ml of buffered enzyme, 10µl of sample was added, mixed well and kept at 37°C for 5 minutes. 10 µl of standard and 10 µl of distilled water (blank) were also processed simultaneously. To all the tubes, 1.0 ml of colour developing reagent was added and mixed well. Exactly after 5 minutes of incubation at 37°C, 1.0 ml of distilled water was added and the colour developed was read at 600 nm. The value was expressed as mg/dl of plasma.

4.2.6 Estimation of uric acid

Uric acid in the plasma was estimated by using the diagnostic kit based on the enzymatic method described by Caraway (1955)

Uric acid in the sample is oxidized by uricase to allantoin. In this reaction for every mole of uric acid oxidized, hydrogen peroxide react with 3,5-dichloro-2-hydroxybenzene sulthonic acid and 4 - aminoantipyrine to give quioneimine dye. Intensity of the color of this dye was proportional to the concentration of uric acid in the sample.
Reagents

1. Enzyme reagent: 4-aminoantipyrine (4mM) 3,5-dichloro 2-hydroxybenezensulfonate (2.0mM) microbial uricase 150, u/L), horseradish peroxidase (10,000 U/L)

Procedure

To 1 ml of the enzyme reagent 25 μL of sample was added and mixed by inversion. 25 μL of standard and 25 μL of distilled water (blank) were also processed simultaneously. The tubes were incubated at 37°C for 5 min. and the color developed was read at 510 nm.

The values were expressed as mg/dl of plasma.

4.2.7 Estimation of creatinine

Creatinine in the plasma was estimated by using the diagnostic kit based on the method of Tietz, (1987) using Jaffé’s (1886) colour reaction. The assay of creatinine has been measured based on the reaction of creatinine with alkaline picrotate as described by Jaffé. Most of the contaminants reacting with the Jaffé reagent produce a colour at a lower rate. The colour formation are proportional to the concentration of creatinine in the sample.

Reagents

1. Saturated picric acid
2. Sodium hydroxide: 0.75 N
3. Creatinine standard: 2.0 mg/dl

Procedure

0.1ml of sample was added to a reagent mixture containing 0.5 ml of picric acid solution and 0.5 ml of sodium hydroxide. The tubes were mixed well and incubated for 20 seconds. With the spectrophotometer adjusted to zero absorbance with distilled water, reading was taken at 510 nm for 20 seconds ($A_1$) and exactly after 45 seconds ($A_2$). Changes in absorbance ($A_2 - A_1$) were measured for test and standard solutions which was used to determine the creatinine concentration in the test sample. The values were expressed as mg/dl of plasma.

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-value of less than 0.05 was considered to be significant.

4.3 RESULTS

The present study brought out the changes in the body weight, blood glucose and plasma insulin in control and experimental animal groups (Table-1). Body weight was significantly (P<0.05) decreased in diabetic rats when compared with that of the normal control rats. In
diabetic rats there was a significant increase in the level of plasma glucose and significant decrease in plasma insulin. Oral administration of *Pimenta dioica* (75 mg and 150 mg/kg bw) and glibenclamide to diabetic rats significantly (P<0.05) increased the body weight and plasma insulin and markedly decreased the plasma glucose level when compared with that of the diabetic control rats.

**Table 1**: Effect of methanolic leaf extracts of *Pimenta dioica* on body weight, plasma glucose and plasma insulin in the normal control, diabetic induced and treated rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th></th>
<th>Plasma glucose (mg/dl)</th>
<th>Plasma Insulin (μU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (0 day)</td>
<td>Final (45days)</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>182.89±4.10a</td>
<td>210.03±3.81a</td>
<td>86.71±4.50a</td>
<td>15.80±1.08a</td>
</tr>
<tr>
<td>Diabetic</td>
<td>184.0±4.20b</td>
<td>140.45±4.66c</td>
<td>287.78±11.45a</td>
<td>7.40±0.31c</td>
</tr>
<tr>
<td>Diabetic+<em>P.dioica</em>@75mg/kg</td>
<td>178.51±3.30d</td>
<td>190.09±4.40c</td>
<td>135.24±5.84b</td>
<td>14.21±0.70d</td>
</tr>
<tr>
<td>Diabetic+<em>P.dioica</em>@150mg/kg</td>
<td>172.25±4.21e</td>
<td>187.51±2.30d</td>
<td>105.23±8.81c</td>
<td>15.31±1.20b</td>
</tr>
<tr>
<td>Glibenclamide (0.6μg)</td>
<td>185.48±4.50a</td>
<td>206.00±3.80b</td>
<td>97.54±6.80d</td>
<td>15.08±1.15c</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (n=6 rats) significantly different at P<0.05 when compared with control group

The levels of haemoglobin, glycosylated haemoglobin, urea, uric acid and creatinine in normal control and experimental animals are shown in Table - 2. The diabetic rats revealed significant decrease in
the level of haemoglobin and significant (P<0.05) increase in the level of HbA1c. The level of blood urea, uric acid and creatinine significantly increased in the diabetic control group when compared with that of the normal control group. The level of haemoglobin after the administration of *Pimenta dioica* (75 mg and 150 mg/kg bw) and glibenclamide significantly increased in the diabetic rats. The level of blood urea, uric acid and creatinine after orally administering the leaf extract of *Pimenta dioica* (75 mg/150 mg/kg bw) significantly decreased in the diabetic control group.

**Table 2: Effect of methanolic leaf extracts of *Pimenta dioica* on haemoglobin, glycosylated haemoglobin, urea, uric acid and creatinine in the normal control, diabetic induced and treated rats.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Haemoglobin (g/dl)</th>
<th>Glycosylated haemoglobin (HbA1C) %</th>
<th>Urea (mg/dl)</th>
<th>Uric acid (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.06±1.02^a</td>
<td>0.42±0.01^c</td>
<td>24.90±1.80^c</td>
<td>1.29±0.08^c</td>
<td>0.43±0.030^c</td>
</tr>
<tr>
<td>Diabetic</td>
<td>9.08±0.46^c</td>
<td>1.38±0.10^a</td>
<td>44.41±3.40^a</td>
<td>2.29±0.19^a</td>
<td>0.82±0.04^a</td>
</tr>
<tr>
<td>Diabetic + <em>P. dioica</em> (75mg/kg)</td>
<td>11.01±0.84^d</td>
<td>0.49±0.03^b</td>
<td>31.71±2.91^b</td>
<td>1.79±0.13^b</td>
<td>0.52±0.08^b</td>
</tr>
<tr>
<td>Diabetic + <em>P. dioica</em> (150mg/kg)</td>
<td>12.04±1.06^c</td>
<td>0.47±0.05^a</td>
<td>30.33±2.35^a</td>
<td>1.71±0.14^a</td>
<td>0.49±0.11^d</td>
</tr>
<tr>
<td>Glibenclamide (0.6µg)</td>
<td>12.75±1.13^b</td>
<td>0.45±0.03^d</td>
<td>29.17±2.21^d</td>
<td>1.64±0.01^d</td>
<td>0.45±0.12^c</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (n=6 rats), significantly different at P<0.05 when compared with control group.
Fig. 4. Effect of *P. dioica* leaf extract on the body weight in the normal, diabetic induced and treated rats.

Fig. 5. Effect of *P. dioica* leaf extract on the plasma glucose level in the normal, diabetic induced and treated rats.
Fig. 6. Effect of *P. dioica* leaf extract on the plasma insulin level in the normal, diabetic induced and treated rats.

Fig. 7. Effect of *P. dioica* leaf extract on the haemoglobin level in the normal, diabetic induced and treated rats.
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