CHAPTER VI
SUMMARY AND CONCLUSION

The thesis entitled “Antidiabetic activities of \textit{A.\textit{monilifer}} in streptozotocin-induced diabetic rats.” submitted by the author is divided into 5 chapters.

Chapter 1 deals with introduction where in details of the diabetic diseases have been highlighted.

Diabetes mellitus is one of the major metabolic disorders, afflicting a large proportion of the population all over the world. According to recent estimates, the human population worldwide appears to be in the midst of a diabetes epidemic. The WHO predicts that the number of cases worldwide for diabetes, now is 171 million will increase to 366 million or more by the year 2030. In the absence of effective and affordable interventions for diabetes, the frequency will escalate worldwide, with a major impact on the population of developing nations. Although biomedical science has unrevealed substantially the pathobiological processes involved in causing diabetes, and has designed therapeutic agents with a range of action to fight hyperglycaemia, the efficacy of these therapeutic agents is compromised in several ways. However, the practical applicability of the majority of these synthetic agents remained restricted owing to their limited action, pharmacokinetic properties, secondary failure rates and accompanying side effects. The current treatment although provides good glycaemic control but it performs little in preventing or delaying the course of long-term complications. Moreover, providing modern medical healthcare across the world, especially in developing countries, is still a far-reaching goal due to economic constraints. This partly contributes to the high prevalence of non-compliance observed in minority, disadvantages communities in developed countries, and rural folks in developing countries.
Chapter 2 The details about the literature review of herbal remedies for diabetic diseases and the profiles of plants are also presented.

Chapter 3 deals with scope and objectives of the study and the aim to discover the antidiabetic activity of medicinal plant along with methodology have been detailed out. The work plan included:

1. To select and identify the medicinal plant *A. monilifer*
2. To collect and extract the whole plant of *A. monilifer* for the study.
3. To find out the phytoconstituents present in the various extracts of *A. monilifer*
4. To analyze the chemical constituents of the various extracts of *A. monilifer* using GC-MS
5. To evaluate the antioxidant activity of different extracts of whole plant of *A. monilifer* using different *in vitro* models such as DPPH assay, nitric oxide radical scavenging activity, iron chelating activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, total antioxidant activity, total phenol and flavonoid content.
6. To evaluate the effect of extracts on various pharmacological parameters in wistar albino rats such as:
   - Estimation of the fasting blood glucose level
   - Estimation of body weight, plasma glucose and Urine sugar.
   - Estimation of plasma insulin.
   - Estimation of Oral glucose tolerance test
   - Hematological studies for the different extracts of *A. monilifer* in streptozotocin-induced diabetic rats will be assessed by the following parameters.
     - Haemoglobin (Hb) in blood
     - Glycosylated Haemoglobin (HbA1C)
     - Total white blood cell (WBC) count
   - Liver function test for the different extracts of *A. monilifer* in streptozotocin-induced diabetic rats will be assessed by the following parameters.
     - Serum alkaline phosphatase (ALP) activity
     - Serum bilirubin
     - Serum glutamate pyruvate transaminase (SGPT) activity
     - Serum glutamate oxaloacetic transaminase (SGOT) activity
Kidney function test for the different extracts of *A. monilifer* in streptozotocin-induced diabetic rats will be assessed by the following parameters.
- Serum creatinine
- Serum urea
- Uric acid

Streptozotocin-induced diabetic rats will be assessed by the following parameters.
- Serum α-Amylase activity
- Liver glycogen

Changes in the level of lipid peroxidative markers (thiobarbituric acid reactive substances, lipid hydroperoxides and conjugated dienes) in liver, kidney and pancreas.

*Invivo* enzymatic antioxidant potential of different extracts of *A. monilifer* in streptozotocin-induced diabetic rats will be assessed by the following parameters.
- Superoxide dismutase (SOD) activity in blood and tissues
- Catalyse (CAT) activity in blood and tissues
- Glutathione peroxidase (GPx) activity in blood and tissues
- Glutathione reductase (GSHRx) activity in tissues
- Glutathione S-transferase (GST) in tissues.

*Invivo* non-enzymatic antioxidants potential of different extracts of *A. monilifer* in streptozotocin-induced diabetic rats will be assessed by the following parameters.
- Vitamin C in plasma, liver and kidney
- Vitamin E in plasma, liver and kidney
- Reduced glutathione in plasma, liver and kidney.

Changes in the levels of membrane bound ATPase such as Na\(^+\) ATPase, Mg\(^{2+}\) ATPase and Ca\(^{2+}\) ATPase in erythrocytes and tissues (liver and kidney).

7. Histopathological changes in liver, kidney and pancreas
8. Isolation of the active ingredients from methanolic extract of whole plant of *A. monilifer* by column chromatographic method.
- Characterization of isolated compounds using FTIR, \(^{13}\)C NMR, \(^1\)H NMR and Mass Spectrometry.
To elucidate the structure of the isolated compounds.

Chapter 4 deals with materials and methods. The complete list of materials, chemicals, equipments and instruments used for the present studies has been listed. The methodology of research has been described:

Selection of Plant Material and Extraction

The air dried and powdered materials of *A. monilifer* subjected to Soxhlet extraction individually using different solvents with increasing order of polarity of the solvent. The solvents included petroleum ether, ethyl acetate and methanol. About 500gm of the dry powdered plant material were packed in a muslin cloth pouch and placed in a thimble of the Soxhlet extraction apparatus attached to the mouth of a round bottomed flask containing extracting solvent. Some boiling chips were added into the flask to avoid bumping during heating. The each solvent extraction was carried out at 40-60°C for 72 h continuously. Each time before extracting with the next solvent of higher polarity the powdered drug (Marc) was dried in a hot air oven below 50°C for 10min and again repacked in the thimble.

Each solvent extract was collected by distilling off the solvent, which was recovered subsequently. Then each solvent extract was concentrated by evaporation to dryness under reduced pressure using a rotary vacuum evaporator. Then each solvent was removed to obtain dried extracts and weighed separately. The percentage yield of each solvent extraction was calculated in terms of initial air dried plant material packed in the thimble. Then each solvent dried extract such as petroleum ether, ethyl acetate, methanol on plant *viz.*, The plant extracts were stored in airtight bottles and refrigerated until use. Different phytochemical tests were conducted for the plant extract of *A. monilifer* to determine the presence and absence of various phytochemical constituents.
Experimental design

Totally 36 number of rats were utilized for this experiment. The rats were divided into six groups of six each.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>1.</td>
<td>Group I</td>
<td>Normal saline (Control).</td>
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<tr>
<td>2.</td>
<td>Group II</td>
<td>Streptozotocin (50 mg/kg, i.p.)</td>
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<td>3.</td>
<td>Group III</td>
<td>STZ + Glibenclamide (600 μg (or) 0.6 mg/kg, i.p.)</td>
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<td>4.</td>
<td>Group IV</td>
<td>STZ + Pet. Ether extract of <em>A. monilifer</em> (250 mg/kg, i.p.)</td>
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<td>5.</td>
<td>Group V</td>
<td>STZ + Ethyl acetate extract of <em>A. monilifer</em> (250 mg/kg, i.p.)</td>
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<tr>
<td>6.</td>
<td>Group VI</td>
<td>STZ + Methanolic extract of <em>A. monilifer</em> (250 mg/kg, i.p.)</td>
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Rats of group II were administered intraperitoneally with diabetic induced drug streptozotocin, rats of group III were administered intraperitoneally with STZ and standard drug Glibenclamide, groups IV, V and VI were administered intraperitoneally with Pet. ether, ethyl acetate and methanolic extract of *A. monilifer* respectively. Animals were sacrificed toward the end of 70 days (10 weeks) every one by cervical dislocation after during the night fasting. Blood was gathered in heparinised tubes and plasma was isolated. Liver, kidney and pancreas were cleaned up adhering fat, measured precisely and utilized for the preparation of homogenate. Animals were sufficiently given consideration according to the Animal Ethical Committee's suggestions.

Type 2 diabetes mellitus was induced in overnight fasted rats will be administered by a single dose of STZ (50 mg/kg, ip). STZ will be dissolved in citrate buffer pH (4.5). Group III rats were administered with Glibenclamide (600 μg (or) 0.6 mg/kg, ip/day.
Group IV, V and VI were administered with petroleum ether, ethyl acetate and methanolic extracts (250 mg/kg ip) of *A. monilifer*. The elevated glucose levels in plasma will be determined at 72 hrs and then on day 7 after injection, to confirm hyperglycemia. At the end of 7, 15, 30, 45, 60 and 70 days the fasting blood samples were drawn and added with anticoagulant potassium oxalate and sodium fluoride for the estimation of blood glucose and plasma insulin.

After 70 days the blood sample will be collected to perform Hematological study, Lipidemic activity, liver function test, kidney function test, pancreatic function test, histopathological studies and *in vivo* antioxidant activity.

At the end of 70th day of experimental regimen the animals in different groups will be sacrificed by cervical dislocation, Blood will be collected in two different tubes (i.e., one with anti-coagulant i.e., for plasma separation and another without anti-coagulant for serum separation).

Plasma and serum will be separated by centrifugation and will be used for the various biochemical estimations. Liver, kidney and pancreas was dissected out, washed in ice-cold saline and kept in ice-cold container for various marker enzyme parameters and histopathological study will be carried out.

**Chapter 5** deals with a detailed account of results with corresponding interpretation and discussion on the outcome of each phase of experimentation:

**Phytochemical investigation of various extracts of *A. monilifer***

The percentage of petroleum ether, ethyl acetate and methanol extracts of *A. monilifer* was 5.38%w/w, 7.16%w/w and 14.52%w/w respectively. The phytochemical screening of whole plant of *A. monilifer* studies showed that the various extracts contains flavonoids, alkaloids, carbohydrates, free sugars, glycosides, phytosterols, tannins, proteins, amino acid compounds, saponins and phenolic compounds.
Effect of various extracts of *A. monilifer* on *in vitro* antioxidant activity

The pet. ether extract, ethyl acetate and methanolic extarct of *A. monilifer* on *in-vitro* antioxidant activities were estimated by DPPH, superoxide anion scavenging activity, Iron chelating method, hydroxy radical activity, nitric oxide scavenging activity, total antioxidant activity, FRAP method. The IC\textsubscript{50} values of pet. ether extract such as 1120\(\mu\)g/ml, 450\(\mu\)g/ml, 490\(\mu\)g/ml, 1070\(\mu\)g/ml, 970\(\mu\)g/ml, 1025\(\mu\)g/ml and 1110\(\mu\)g/ml, respectively. IC\textsubscript{50} values of ethyl acetate extract such as 960\(\mu\)g/ml, 410\(\mu\)g/ml, 360\(\mu\)g/ml, 515\(\mu\)g/ml, 735\(\mu\)g/ml, 570\(\mu\)g/ml and 425\(\mu\)g/ml, respectively. IC\textsubscript{50} values of methanolic extract such as 490\(\mu\)g/ml, 175\(\mu\)g/ml, 78\(\mu\)g/ml, 420\(\mu\)g/ml, 455\(\mu\)g/ml, 430\(\mu\)g/ml and 220\(\mu\)g/ml, respectively. These values were comparable with standard antioxidant agents such as EDTA, Quercetin, rutin and ascorbate IC\textsubscript{50} values. Among the three extracts methanolic extracts shows the more *in-vitro* anti oxidant activity when compared with standards.

Estimation of fasting blood glucose levels

The effect of treatment of the extract on fasting blood glucose levels on day 1,7,15,30,45,60 and 70 respectively in comparison to diabetic control group II. This indicated that the GBC treatment successfully reduced the blood glucose levels in the diabetic rats towards the normal level in 70 days. Similarly, methanolic extract of *A. monilifer* treated diabetic group VI showed, significant reduction in blood glucose values on day 1,7,15,30,45,60 and 70 respectively as compared with diabetic control group II. This indicated that the methanolic extract of *A. monilifer* treatment could reduce the blood glucose levels in the diabetic rats towards the normal level in the 70 days of study.
**Estimation of body weight, blood glucose and urine sugar**

The effect of various extracts of *A. monilifer* on body weight, blood glucose and urine sugar in normal and experimental rats. The blood glucose and urine sugar elevated and body weight decreased significantly in diabetic rats as compared to normal control rats. In diabetic rats, treatment with various extracts of *A. monilifer* lowered plasma and urine glucose and elevated body weight significantly as compared to diabetic control rats. Glibenclamide and methanolic extract of *A. monilifer* at a dose of 250 mg kg-1 b.wt. restored the plasma glucose and urine sugar to near normal levels and elevated body weight significantly. Since 250 mg/kg b.wt. of methanolic extract of *A. monilifer* showed highest plasma glucose lowering effect.

The urine sugar levels in normal control rats showed absence of sugar in urine. The urine sugar levels of the differentiate groups of diabetic animals treated with standard drug (Glibenclamide) and methanolic extract of *A. monilifer* for 70 days decreased towards the normal level.
**Estimation of plasma insulin, Hb and HbA1c**

The levels of plasma insulin, Hb and HbA1c. Plasma insulin and Hb decreased and HbA1c increased significantly in diabetic control rats and these values were reversed by treatment with methanolic extract of *A. monilifer* and glibenclamide. A significant elevation in plasma insulin and Hb and reduction in HbA1c were also observed in normal rats treated with methanolic extract of *A. monilifer* as compared to normal control rats.

**Estimation of Oral Glucose Tolerance Test (OGTT)**

Treatment with GBC significantly improved the glucose tolerance at normal fasting levels at 0, 30, 60 and 120 min, respectively. Further, treatment with methanolic extract of *A. monilifer* significantly reduced sugar glucose level at 120min compared to normal control. These data suggested that treatment with methanolic extract of *A. monilifer* showed tolerance to glucose administration.

**Effect of various extracts of *A. monilifer* on WBC, Bilirubin, ALP, Liver glycogen and Alpha amylase**

There was significant increase in liver glycogen level to 50.72±1.76 (p<0.01) in glibenclamide treated diabetic control group III. Similarly methanolic extract of *A. monilifer* treatment significantly (p<0.01) increased the glycogen content to 47.64±1.55 (p<0.01) in STZ-induced diabetic group VI.

The white blood counts were significantly restored to near normal after methanolic extract of *A. monilifer* administration. The presence of some phytochemicals with ability to stimulate the production of white blood count in the extract could be responsible for the observed result in the treated rats.
The activities of plasma hepatic marker enzyme ALP in control and diabetic rats are given in the table 5.33. Increased activities of hepatic marker enzymes were observed in diabetic rats. Treatment with methanolic extract of *A. monilifer* and glibenclamide normalized hepatic marker enzyme.

Bilirubin level was found to be decreased from 3.45±1.56 mg/dl (diabetic control) to 0.34±0.12 mg/dl (Glibenclamide treated groups), 0.48±0.62 in methanolic extract of *A. monilifer* extract. The methanolic extract was found to be more effective in reducing the Biliruin level, but less effective when compared to Glibenclamide.

The effect of *A. monilifer* on pancreatic function in STZ-induced diabetic rats. Serum Amylase was found to be decreased from 112.52±19.56 (diabetic control) to 55.80±11.22 (Glibenclamide treated groups), 60.51±10.01 in methanolic of *A. monilifer* extract. The methanolic *A. monilifer* extract was found to be more effective in reducing the Serum Amylase level, but less effective when compared to Glibenclamide.

**Estimation of plasma urea, uric acid and creatinine**

The levels of urea, uric acid and creatinine in the plasma are elevated remarkably in the plasma of diabetic rats as compared with normal control rats. Diabetic rats treated with methanolic group of *A. monilifer* showed the reversal of these parameters to near normal levels.

**SGOT and SGPT**

Across all the extract treatment groups, STZ + pet. ether group showed highest value, whereas STZ + methanolic group showed lowest value. Although, dose dependent values were observed with all the extract treatment groups, ethanolic extract of high
dose of both the plants showed near baseline values. When compared with STZ induced group, methanolic extract treated Group showed significant values.

**Estimation of TBARS and conjugated dienes**

The changes in the levels of TBARS and CD in diabetic rats had elevated levels of TBARS and CD. Diabetic rats treated with methanolic extract of *A. monilifer* and glibenclamide brought back TBARS and CD.

**Effect of superoxide dismutase (SOD) and catalase (CAT) in liver and kidney of control and experimental groups rats**

The activities of SOD and CAT in the tissues of control and diabetic rats. Diabetic rats had decreased activities of SOD and CAT in the tissues as compared with normal control rats. Diabetic rats treated with methanolic extract of *A. monilifer* and glibenclamide showed reversal of these parameters to near normalcy. The activities of superoxide dismutase (SOD) and catalase (CAT) in the liver and kidney of normal control and experimental groups of rats. The activity of SOD and CAT in liver were significantly lower in diabetic control rats compared to normal control group of rats. After administration of methanolic extract of *A. monilifer* as well as glibenclamide in diabetic rats were significant increase the antioxidant enzymes like SOD&CAT.

**Effect of glutathione peroxidase (GPX) and glutathione reductase (GSH-Rx) in liver and kidney of control and experimental groups rats**

The activities of glutathione peroxidase (GPx) and glutathione reductase (GSH Rx) and in the liver, kidney and pancreas of normal control and experimental groups of
rats. The activity of GST and GPx in liver, kidney and pancreas were significantly lower in diabetic control rats compared to diabetic induced rats. The GPx & GSH Rx levels in liver, kidney and pancreas were significantly enhanced in methanolic extract of *A. monilifer* treated rats as well as glibenclamide.

**Effect of glutathione-S-transferase (GST) in liver and kidney of control and experimental groups rats**

The activities of glutathione-s-transferase(GST) in the liver, kidney and pancreas of normal control and experimental groups of rats. The activity of GST in liver, kidney and pancreas were significantly lower in diabetic control rats compared to diabetic induced rats. The GST levels in the liver, kidney and pancreas were significantly enhanced in methanolic extract of *A. monilifer* treated rats as well as glibenclamide.

**Effect of various extracts of *A. monilifer* on vitamin C in the plasma and tissues of control and diabetic rats.**

The levels of vitamin C and E and GSH in the plasma and tissues of diabetic rats showed high level of vitamin E and low level of vitamin C and GSH in the plasma and tissues when compared with normal control rats. Treatment with methanolic extract of *A. monilifer* and glibenclamide brought back these parameters to near normalcy.
Effect of various extracts of *A. monilifer* on (Na\(^+\))-ATPase, (Ca\(^{2+}\))-ATPase, (Mg\(^{2+}\))-ATPase in the erythrocytes and tissues of control and diabetic rats

The activities of total ATPase and (Na\(^+\))-ATPase in the erythrocytes and tissues (liver, kidney and pancreas) of diabetic rats had decreased activity of total ATPase and (Na\(^+\))-ATPase in the erythrocytes and tissues as compared with control rats. Treatment with methanolic extract of *A. monilifer* and glibenclamide restored the activities of total ATPase and (Na\(^+\))-ATPase to near normalcy.

The activity of Ca\(^{2+}\)-ATPase and Mg\(^{2+}\)-ATPase in the erythrocytes and tissues (liver, kidney and pancreas) of control and diabetic rats. The activities of Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase decreased in the erythrocytes and tissues (liver, kidney and pancreas) of diabetic rats when compared with control rats, treatment with methanolic extract of *A. monilifer* and glibenclamide brought back Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase to near normalcy.

Effect of various extracts of *A. monilifer* on histopathological examination

In our study, histopathological examination of diabetic pancreas showed shrinkage of islet cells and growth of adipose tissue in the pancreas. Treatment with *A. monilifer* and glibenclamide reduced the changes in the pancreas, which supports the biochemical analysis.

Histopathological examination of diabetic liver showed fatty changes and inflammatory cell infiltration around the portal triad in the liver. Treatment with *A. monilifer* and glibenclamide showed marked reduction in fatty changes and inflammatory cell infiltration around the portal triad.
Histopathology of diabetic kidney showed large area of hemorrhage, lymphocyte infiltration and fatty infiltration, which upon treatment with *A. monilifer* and glibenclamide showed marked reduction of hemorrhage and fatty infiltration.

**Isolation of compounds by column chromatographic separation**

The methanolic extract of *A. monilifer* was subjected to column chromatographic separation using normal phase silica gel column. The dark brown solid (20 g methanolic extract of *A. monilifer*) was adsorbed on silica gel (20 g) and transferred to a column of silica gel (200g equilibrated with benzene). Elution was performed with hexane (100%), hexane: chloroform (90:10), hexane: chloroform (80:20), hexane: chloroform (50:50), hexane: chloroform (30:70), chloroform (100%), chloroform: ethyl acetate (70:30), chloroform: ethyl acetate (50:50), chloroform: ethyl acetate (30:70), ethyl acetate (100%), ethyl acetate: methanol (80:20), ethyl acetate: methanol (70:30) ethyl acetate: methanol (50:50), ethyl acetate: methanol (30:70) and methanol (100%). Fractions of 100ml were collected every time, distilled off the solvent and the homogeneity of the resulting residues were examined on TLC by using different solvent systems and similar fractions, identified by their TLC behaviour were mixed together.

Fractions 11-12 (eluted with hexane: chloroform 80:20) gave a solid designated as compound 1 (135 mg), fractions 65-68 (eluted with ethyl acetate: methanol 70:30) gave a semi solid designated as compound 2 (195mg).

**Structure and identification of isolated compounds**

The structure and identification of isolated compounds were analyzed by FT-IR, $^1$H NMR, $^{13}$C NMR and mass spectrophotometry. As per the spectral analysis the structure of compound 1 was proposed to acetate of $3\beta$-Hydroxy-5α-Cholanic acid and its molecular
formula is deduced as $\text{C}_{26}\text{H}_{38}\text{O}_3$. Compound 2 was proposed to 1- (2- hydroxy-5-methylphenyl) ethanone and its molecular formula deduced as $\text{C}_9\text{H}_{10}\text{O}_2$.

On the basis of the finding in present study clearly showed the significant anti diabetic and antioxidant activities were found in methanolic extract of $A.monilifer$ However, these actions are may be due to the presence of Acetate of 3β-Hydroxy-5α-Cholanic acid and 1- (2- hydroxy-5-methylphenyl) ethanone from methanolic extract of $A.monilifer$. The above evidences provide some biochemical basis for the use of methanolic extract of $A.monilifer$ as a potent anti diabetic agent having preventive diabetic diseases.
SCOPE FOR FUTURE STUDY

The present study scientifically established the antidiabetic activity of *A. monilifer* can be further investigated on

- To assess the dose dependent and time dependent (Longer duration of experiment) activity of acetate of 3β-Hydroxy-5α-Cholanic acid and 1- (2-hydroxy-5-methylphenyl) ethanone isolated from *A. monilifer*.
- A detailed pharmocodynamic and pharmacokinetic studies of the compounds.
- Feasibility aspects of formulation of the crude drug and also the active constituents.
- Furthermore, the *in-vivo* antioxidant activities to be carried out for acetate of 3β-Hydroxy-5α-Cholanic acid and 1- (2- hydroxy-5-methylphenyl) ethanone isolated from the *A. monilifer*.
- Taking the present trends of herbal technology, the process can be developed to make herbal products of *A. monilifer* may be carried out for the management of diabetes diseases.