Experimental Procedure

Diabetic cardiomyopathy is the condition characterized by congestive cardiac failure or cardiomegaly in diabetic patients in the absence of hypertensive, ischemic or other heart disease. Diabetes mellitus increases the risk of heart failure independent of underlying coronary artery disease and may lead to cardiomyopathy. Several factors contribute to the development of cardiac dysfunction in the absence of coronary artery disease in diabetes mellitus. Thus, cardiovascular complications are the leading cause of diabetes-related morbidity and mortality. Diabetes mellitus is responsible for diverse cardiovascular complications such as increased atherosclerosis in large arteries (carotids, aorta, and femoral arteries) and increased coronary atherosclerosis, which increases the risk for myocardial infarction, stroke and limb loss (Boudina and Abel, 2007).

The overall goal of treatment for cardiomyopathy is to manage the signs and symptoms, prevent the condition from worsening and reduce the risk of complications. Many of the medications that doctors prescribe for cardiomyopathy may have side effects but the herbal medication has less side effect or no side effect. Herbs have been used for medical treatments since the beginning of civilization and some derivatives (eg, aspirin, reserpine, and digitalis) have become mainstays of human pharmacotherapy. For cardiovascular diseases, herbal treatments have been used in patients with congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency, venous insufficiency and arrhythmia. However, many herbal remedies used today have not undergone careful scientific assessment and some have the potential to cause serious toxic effects and major drug-to-drug interactions (Rout et al., 2010). Continuing research is necessary to elucidate the pharmacological activities of many herbal remedies now being used to treat cardiovascular diseases.
Contrary to the synthetic drugs, drugs of plant origin are not associated with side effects and have an enormous therapeutic potential to heal many infectious diseases (Amira and Okubadejo, 2007).

Hence in the present study a survey was carried to find out the prevalence of cardiac disease among diabetic patients in selected hospitals and to study the treatment regimen and complications during medication. The effect of selected medicinal plants on the cardio protective effect was analysed in experimental animals. Phytochemical constituents of the selected samples were also determined. The experimental procedure for the present study entitled “Biochemical studies in diabetic cardiomyopathy patients during drug therapy and the cardio protective effect of selected medicinal plants in Isoproterenol induced swiss albino rats” was conducted in three different phases.

**Phase I**

3.1 Study on the prevalence of cardiomyopathy among diabetic patients in selected hospitals

3.1.1 Survey and screening of the patients

A survey was conducted among diabetic patients to find out the prevalence of cardiac disease. Diabetic patients attending the outpatient ward of two hospitals in Coimbatore were selected for the study. The study was conducted for a period of two years. A survey was taken from the patients using a questionnaire (Appendix I and II). Thousand five hundred diabetic patients were screened for cardiac diseases based on their HbA1C, BP and cholesterol levels. Among them 300 patients were found to be affected with cardiac disorder. The presence of cardiomyopathy in 100 patients were confirmed by angiogram and these were selected for further study. 100 normal persons were taken as control.

3.1.2 Assessment of biochemical parameters in selected patients:

After preliminary screening, the patient’s blood sample was collected and centrifuged to separate the serum. The serum was analysed for Apolipoprotein A and B, High sensitivity C reactive protein, Creatinine kinase, Total Cholesterol,
Triglycerides, HDL-C, LDL-C, VLDL-C and Troponin –T to know the severity of the disease (Table 2).

**Table 2**

Details of the assessment of biochemical parameters in selected diabetic cardiomyopathy patients

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameters</th>
<th>Method of analysis</th>
<th>References</th>
<th>Appendix No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HbA1c</td>
<td>Enzymic Haemoglobin assay</td>
<td>Goldstein et al. (1986)</td>
<td>III</td>
</tr>
<tr>
<td>2</td>
<td>Total Cholesterol</td>
<td>Kit method</td>
<td>Allian et al. (1974)</td>
<td>IV</td>
</tr>
<tr>
<td>3</td>
<td>Triglycerides</td>
<td>Kit method</td>
<td>Jacobs and Van Demark (1960)</td>
<td>V</td>
</tr>
<tr>
<td>4</td>
<td>Low density lipoprotein - cholesterol</td>
<td>Kit method</td>
<td>Rifai and Warnik (1994)</td>
<td>VI</td>
</tr>
<tr>
<td>5</td>
<td>High density lipoprotein - cholesterol</td>
<td>Kit method</td>
<td>Gordon et al. (1977)</td>
<td>VII</td>
</tr>
<tr>
<td>6</td>
<td>Very low density lipoprotein cholesterol</td>
<td>By calculation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Serum glutamic oxaloacetic transaminase</td>
<td>Kit method</td>
<td>Reitman and Frankel, 1957</td>
<td>VIII</td>
</tr>
<tr>
<td>8</td>
<td>Alkaline phosphatase</td>
<td>Kit method</td>
<td>Schlebusch et al. (1974)</td>
<td>IX</td>
</tr>
<tr>
<td>9</td>
<td>Creatine kinase</td>
<td>Kit method</td>
<td>Bonsnes and Taussky (1945)</td>
<td>X</td>
</tr>
<tr>
<td>10</td>
<td>C-reactive protein</td>
<td>Enzyme immune assay</td>
<td>Ridker et al. (2000)</td>
<td>XI</td>
</tr>
<tr>
<td>11</td>
<td>Apolipoprotein – A</td>
<td>Turbidimetry method</td>
<td>Young (1997)</td>
<td>XII</td>
</tr>
<tr>
<td>12</td>
<td>Apolipoprotein – B</td>
<td>Automated turbidimetry immunoassay method</td>
<td>Koga (1990)</td>
<td>XIII</td>
</tr>
</tbody>
</table>

**VLDL Calculation**

The levels of very low density lipoprotein cholesterol (VLDL-C) were calculated using Friedewald equation:

\[
\text{VLDL cholesterol} = \frac{\text{Triglycerides}}{5}
\]
3.1.3 Biochemical and clinical changes during drug therapy in selected patients

The incidence and prevalence of diabetes mellitus are increasing rapidly in our society. The majority of the patients with diabetes often develop atherosclerosis and hypertension both of which are major contributors to the development of heart disease. However, cardiomyopathy can also develop in the absence of established risk factor. Though cardiomyopathy cannot be cured, symptoms can be managed and treated to improve patient’s quality of life. Mild cases may be treated with a change in diet and/or exercise. With more severe cases, a physician may prescribe pharmaceuticals to lower patient’s blood pressure or slow down his/her heart rate. Water pills and blood thinners are also used to remove sodium from cardiomyopathy patients and prevent blood clots. Metacord is also used to reduce the risk of heart complications after a heart attack. Some drugs such as metacord may show side effects such as dizziness, tiredness, diarrhoea, stomach discomfort and difficulty in sleeping (Reiner, 2010).

After the preliminary screening the treatment regimen was studied in 100 selected diabetic cardiomyopathy patients for a period of one year. Levels of HbA1C, total cholesterol, triglycerides, HDL-C, LDL-C, VLDL-C, SGOT, ALP, CK, C-reactive protein, apolipoprotein A and apolipoprotein B were measured initially and after 3, 6 and 12 months period.

Some of the drugs could cause serious complications. When the drugs are given such as diuretics, vasodilators, beta-blockers, these drugs may result in side effects. Alternative treatments are directed towards the control of heart disease. Certain herbs such as foxglove and lily of the valley (Convallaria majalis) contains cardiac glycosides which may be used in herbal therapies (Frishman et al., 2004).

The recent interest on alternative medicine has taken up great dimensions in changing the health care scenario across the globe. Hence the present study was carried out to find out the cardio protective effect of the selected medicinal plants.
3.2 Phase II

Nature has been a source of therapeutic agent for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of these agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care.

Hence the present investigation was undertaken to examine the leaves of *Albizia saman*, which has beneficial effect on glycemia, lipidemia and cardiac dysfunction on streptozotocin (STZ) and Isoproterenol (ISO) induced rats. *Nelumbo nucifera* was also used as a medicinal herb. So in the present study *Albizia saman* leaves and white *Nelumbo nucifera* flowers were selected to evaluate glucose lowering property and cardio protective effect in STZ-ISO induced experimental rats.

3.2.1 Collection of the medicinal plants *Albizia saman* and *Nelumbo nucifera*

The medicinal plant *Albizia saman* was collected from Kumuli at Theni district. The tender leaves were washed in fresh water and allowed to dry in shade. The fresh white lotus flower (*N.nucifera*) was collected from Nagarkoil at Kanyakumari district. Selected samples were authenticated (BSI/SRC/5/23/2012-13/Tech1699, BSI/SRC/5/23/2012-13/Tech 1700) by Botanical Survey of India, Coimbatore.

3.2.2 Extraction, preparation and isolation of the selected medicinal plants

The leaves of *Albizia saman* and flowers of white *Nelumbo nucifera* were dried initially under shade. It was preserved in air tight containers and it was powdered with the use of a mechanical grinder. For the preliminary study, fresh samples of *A.saman* and *N.nucifera* were screened for the presence of phytochemicals. Five grams of *A.saman* leaves and white *N.nucifera* flowers were weighed, mashed and homogenized with 50ml of alcohol, acid (1% HCl) and water separately. These were boiled for one hour, cooled, filtered and used for the analysis of phytochemicals.
3.2.3 Qualitative analysis for the presence of phytochemicals

3.2.3.1 Preliminary phytochemical screening of *Albizia saman* leaves and white *Nelumbo nucifera* flowers

The extract of leaves of *A.saman* and white *N.nucifera* flowers were subjected to preliminary phytochemical screening for the presence of flavonoids, phenols, anthocyanin, tannins, saponins, steroids, alkaloids and terpenoids using standard procedure as per the method given in WHO Guidelines and Ayurvedic Pharmacopoeia (1976) (Appendix XVII).

3.2.3.2 Preparation of the organic extract and phytochemical analysis of selected samples

Dried and powdered leaves and flowers were defatted to remove fatty material. 20 gm of weighed *A.saman* leaves and 20 gm of weighed powdered flowers of *Nelumbo nucifera* were packed in Soxhlet extractor separately and extracted with petroleum ether at 60-80°C for 36 hrs. The completion of extraction was confirmed by pouring a drop of extract from the thimble on a filter paper, which does not show the presence of any oil spot. The column pack of leaves and flowers were removed and dried, subjected separately to continuous hot extraction with absolute methanol in soxhlet apparatus for 24 hrs and completion of extraction was confirmed by pouring a few drop of extract from the thimble which left no residue on evaporation. After complete extraction the solvent was evaporated and concentrated to dry residue for further analysis (Appendix XIV). The qualitative phytochemical tests of various extracts of *A.saman* and *N.nucifera* were carried out using standard procedure. The procedure in detail is given in Appendix XVII.

3.2.3.3 Determination of free radical scavenging activity (DPPH radical scavenging activity) in the selected samples

The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used for the determination of free radical scavenging activity of the extract. Different concentrations (10-100 µg) of various extracts (aqueous, benzene, chloroform, ethanol, ethyl acetate, methanol and petroleum ether) of *A.saman* and *N.nucifera* were added with an equal volume of methanolic DPPH solution (0.5 mM) and
incubated at 37°C for 15 min separately. The absorbance was recorded at 517 nm. The detailed procedure is given in Appendix XVIII. IC₅₀ values denote the concentration of selected plant sample, which is required to scavenge 50 per cent of DPPH free radicals.

3.2.3.4 Evaluation of the Total Antioxidant Activity in the Selected Samples

The phosphomolybdenum method was used to evaluate the total antioxidant activity of the various extracts of A.saman and N.nucifera. Antioxidants can reduce Mo (IV) to Mo (V) and the green phosphate / Mo (V) compounds, which have an absorption peak at 695 nm. The detailed procedure of which is given in appendix XIX.

3.2.4 Characterization of bioactive components present in the plant samples using HPTLC, HPLC, FT-IR and GC-MS analysis

3.2.4.1 Characterization of Phytochemical Constituents by HPTLC Analysis

Thin layer chromatography is a type of liquid chromatography that can separate chemical compounds of differing structure based on the rate at which they move through a support under defined conditions. High performance liquid chromatography is a sophisticated and automated form of TLC. It is an invaluable quality assessment tool for the evaluation of botanical material. With HPTLC the same analysis can be viewed using different wavelength of light thereby providing a more complete profile of the plant sample.

Sample preparation for HPTLC

The 5 ml sample of MeAsL (methanolic extract of A.saman leaves) and MeNnF (methanolic extract of N.nucifera flowers) were taken, centrifuged and collected the supernatant liquid. This portion was used as test solution for HPTLC analysis.

Solvent system

The following mobile phases were used for the analysis of the respective compounds
Table 3

Solvent system for HPTLC analysis

<table>
<thead>
<tr>
<th>Profile</th>
<th>Reference marker</th>
<th>Mobile phase</th>
<th>Spray reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>Quercetin</td>
<td>Ethylacetate-formic acid-Acetic Acid-water (10:1:1:1:2:6).</td>
<td>1% Ethanolic Aluminium chloride reagent dried at 120°C for 10 minutes</td>
</tr>
<tr>
<td>Glycoside</td>
<td>Stevioside</td>
<td>Ethyl acetate-Methanol-Ethanol-Water (8.1:1:1:0.4:0.8)</td>
<td>Phosphoric acid reagent dried at 120°C for 10 minutes</td>
</tr>
<tr>
<td>Phenol</td>
<td>Catechol</td>
<td>Isobutanol-acetic acid-water (14:1:3.5)</td>
<td>5% ferric chloride reagent and dried at 120°C for 10 minutes</td>
</tr>
</tbody>
</table>

2 µl of test solution and 2 µl of standard solution was loaded as 5 mm band length in the 3 x 10 Silica gel 60F<sub>254</sub> TLC plate for flavonoid, glycoside and phenol profile using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm (Table 3).

**Photo-documentation:** The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254nm and UV366nm.

**Derivatization:** The developed plate was sprayed with respective spray reagent and dried at 120 °C in hot air oven for 10 minutes. The plate was photo-documentated in daylight mode using photo-documentation (CAMAG REPROSTAR 3) chamber.
Peak analysis: Finally, the plate was fixed in scanner stage and scanning was done at 366 nm. The peak table, peak display and peak densitogram were noted.

3.2.4.2 Characterization of Phytochemical Constituents by HPLC Analysis

The HPLC analysis of methanolic extracts of *A. saman* leaves and white *N. nucifera* flowers was performed using Shimadzu HPLC system with UV-Vis detector (Model hpv20 A). The separation was performed on a Luna C18 column (2.0 mm i.d. x 150 mm, 3 Qm particle size) with guard column (2.0 mm i.d. x 40 mm, 3 Qm). After the optimization of conditions, mobile phase composed of deionized water containing 0.1% formic acid (mobile phase A) and acetonitrile containing 0.1% formic acid (mobile phase B) was used. Sample injection volume was 20 Ql and UV absorbance was monitored at 340 nm.

3.2.4.3 Characterization of Phytochemical Constituents by FT-IR Analysis

The FT-IR analysis of powder samples was carried out using Shimadzu 8400 model FT-IR and FT Raman Spectrometer using KBr pellet and powder form respectively. The FT-IR was recorded in the range 400-4000 cm\(^{-1}\) for both the samples. The assignments were made assuming Cs point group symmetry. The various modes of vibration were identified and assigned.

3.2.4.4 Characterization of Phytochemical Constituents by GC-MS Analysis

The GC-MS analysis of methanolic extract of *A. saman* and *N. nucifera* was carried out with Thermo Gc-Trace Ultra Ver: 5.0, Thermo MS DSQ II GC-MS instrument (Appendix XX).

Phase III

3.3 Cardioprotective effect of the selected medicinal (*A. saman* and white *N. nucifera*) plants on experimental animals.

The present study was carried out to find out the cardioprotective effect of methanolic extract of *A. saman* leaves and white *N. nucifera* flowers on experimental animals.
Plate 1

(I) Drugs used for the induction of diabetes and cardiomyopathy in experimental animals

a. The drug streptozotocin - sigma product suppliers (Product no S0130-1G)

b. Isoproterenol injection - Samarth Life Sciences PVT.LTD (Unit II), Mumbai

c. The drug metformin - Zanza Healthcare Ltd, UK

d. Metocard XL (50 mg) - Torrent Pharmaceuticals Ltd, Mumbai

(II) Drugs used for the treatment of diabetes and cardiomyopathy in experimental animals
Plate 2

Intraperitoneal injection on experimental animals

Retro-orbital plexus - Blood collection
Plate 3
Dissection of the experimental animals

Dissected heart from experimental animals
Parts of the experimental animal heart

[Images of dissection of experimental animals and heart parts]
3.3.1 Maintenance of the experimental animals

45 male Swiss albino rats between 2 and 3 months of age and weighing between 160–180 g were used for the study. They were bought from a central animal breeding station, Kerala. The rats were maintained under standard laboratory conditions (temperature 25±2°C) with dark/light cycle (14/10h). The animals were kept in neat cages, bottomed with husk and fed standard pellet diet and water ad libitum. The rats were acclimatized to laboratory conditions for 15 days before the commencement of the experiments. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee (Reg no: 623/02/b/CPCSEA).

3.3.2 Grouping of the experimental animals

The animals were randomly divided into nine groups (n = 5, for each) including control group. The groups were explained in protocol (3.3.3).

3.3.2.1 Induction of diabetes and treatment protocol

Diabetes was induced by streptozotocin (STZ) (50 mg/kg intraperitoneally) (group II, III, IV, V, VI, VII) dissolved in normal saline. Forty-eight hours after STZ injection, animals showing glycosuria (>2%) were considered to be diabetic by the analysis of blood glucose in glucometer.

3.3.2.2 Assessment of the body weight and clinical parameters in experimental animals

Food intake, water intake and body weight gain were measured up to eight weeks. At the end of the eight-week treatment period, the animals were kept on an overnight fast, and the blood samples were collected. The blood was allowed to clot for 30 min at room temperature and then centrifuged at 5000 rpm for 20 min. The serum was separated and stored at –20° C until analysis was completed. Serum samples were analyzed spectrometrically (Shimadzu UV-1601, Japan) for glucose, triglyceride, total cholesterol, HDL-C, LDL-C, VLDL-C and CK-MB using their respective kits.
3.3.3 Experimental protocol for the analysis of the cardio protective effect on diabetic induced rats

**Table 4**
Experimental design of the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Non-diabetic control</td>
</tr>
<tr>
<td>Group II</td>
<td>STZ induced diabetic rats were administered with isoproterenol (85 mg/kg body weight for the period of 24 hrs interval for two days).</td>
</tr>
<tr>
<td>Group III</td>
<td>STZ induced diabetic rats were treated with standard drug metacord (200 mg/kg body weight) and Metformin (50 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group IV</td>
<td>STZ induced diabetic rats were treated with MeAsL (100 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group V</td>
<td>STZ induced diabetic rats were treated with MeAsL (150 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group VI</td>
<td>STZ induced diabetic rats were treated with MeNnF (100 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group VII</td>
<td>STZ induced diabetic rats were treated with MeNnF (150 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group VIII</td>
<td>Rats were treated with MeAsL (100 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
<tr>
<td>Group IX</td>
<td>Rats were treated with MeNnF (100 mg/kg body weight) intraperitoneally for a period of 30 days.</td>
</tr>
</tbody>
</table>

STZ - streptozotocin; MeAsL – methanolic extract of *A.saman* leaves; MeNnF - methanolic extract of white *N.nucifera* flowers
Streptozotocin was administrated to induce diabetes in rats from group II to VII over a period of two days. At the end of the experiment isoproterenol was administered in group II to VII (85 mg/kg body weight administered subcutaneously twice at an interval of 24 hrs) on 29th and 30th day. All the rats were anesthetized and then sacrificed by cervical dissection. Blood was collected and serum separated by centrifugation. The heart tissue was excised immediately from the animals, washed off blood with ice chilled physiological saline. A known weight of the heart tissue was homogenized in 5.0 ml of 0.1 M Tris HCl buffer (pH 7.4) solution. The homogenate was centrifuged and the supernatant was used for the estimation of various biochemical parameters.

3.3.4 Biochemical assays

3.3.4.1 Assessment of the selected cardiac markers

The Serum total cholesterol, HDL-C, LDL-C, SGOT, ALP, CK-MB and Troponin T were estimated (Appendix IV, VI, VII, VIII, IX, XIV and XV).

3.3.4.2 Assessment of the lipid peroxidation in heart tissue

Tissue lipid peroxide level was determined as TBARS21. The absorbance was measured photometrically at 532 nm and the concentrations were expressed as nmol malonaldehyde min⁻¹ mg⁻¹ protein (Appendix XXVI).

3.3.4.3 Assessment of the enzymic and non enzymic antioxidant levels

Estimation of catalase

Catalase activity in the heart homogenate was assessed by the method of Luck, (1974) as shown in Appendix XXI.

Estimation of superoxide dismutase (SOD)

The activity of SOD was estimated by the method of Misra and Fridovich, (1972) as shown in Appendix XXII.

Estimation of glutathione Peroxidase (GPx)

The activity of GPx was determined by the method of Rotruck et al., (1973) as shown in Appendix XXIII.
Estimation of glutathione S transferase (GST):

The GST was determined by the method of Habig et al. (1974) as in Appendix XXIV.

Estimation of reduced glutathione (GSH):

The GSH was determined by the method of Moron et al. (1979) as in Appendix XXV.

3.3.5 Biochemical alterations in ISO induced cardiomyopathy rats

Biochemical alteration represents a complex pattern of changes in cardiac marker enzymes, enzymic and non-enzymic antioxidants glycoprotein and decrease in ATP storage in blood vessels as well as in myocardial tissue (Reuter, 1975). Lipolysis is also considered as one of the ISO induced injury (Hu et al., 2006). Degeneration and necrosis of myocardial fibres, accumulation of inflammatory cells and endocardial haemorrhage are visible in histological changes (Lehr, 1972).

3.3.5.1 Assessment of histological status of Heart

The sample of the heart from rat were fixed in 10 percent formalin and then embedded in paraffin. Microtome sections of 6 µm thickness were prepared from each portion of heart and stained with haemotoxylineosin for pathological observation using the method of Culling, (1979) as shown in Appendix XXVII

3.5 Statistical Analysis

Results are expressed as mean ± SD. Between control and untreated patients comparison was made using student “t” test (p<0.05). Statistical significance was evaluated by one way analysis of variance (ANOVA) using SPSS version (17.0) and the individual comparisons were obtained by the Duncan’s multiple range test (DMRT) (Duncan,1957). A value of p<0.05 was considered as significant difference between each groups.