2. MATERIALS AND METHODS

2.1 Preparation of plant extracts

Healthy leaves and bark of *Azadirachta indica* from a full grown tree were collected from the university campus and extracts were prepared by following the method of Bandyopadhyay et al (2000).

2.1.1 *A. indica* leaf extract

One kg of freshly collected, shade dried, powdered leaves of *A. indica* were allowed to soak overnight in 4 liters of distilled water at room temperature. The suspension was then centrifuged at 5000 rpm for 20 min and filtered through Whatman No.1 filter paper. The filtrate was lyophilized to yield 12.9 gm of dry powder and stored at −20°C. A measured amount of the *A. indica* leaf extract (AILE) was dissolved in distilled water at a suitable concentration prior to experiment.

2.1.2 *A. indica* bark extract

Air dried bark from a full grown tree, devoid of external hard wood was cut into small pieces and 100 gm was soaked overnight in one liter distilled water at room temperature with occasional shaking. After filtration, the brown red extract was lyophilized to yield 3.7 gm of dry powder and stored at −20°C. A required concentration of *A. indica* bark extract (AIBE) was prepared in distilled water prior to experiment.

2.1.3 *A. indica* seed oil

*A. indica* seed oil (AISO) extracted by cold pressing was procured from the market and oil of the same company (Vyas Pharmaceuticals, Indore) was used throughout the study.

2.2 Toxicity test with plant extracts

For treatment, effective dosages of the extracts were selected from literature and a sub-acute oral toxicity study was performed to ensure the safety of the extracts for oral consumption. Toxicity study was carried out in Wistar rats (two to three months old) kept at controlled environment and acclimatized to laboratory conditions for one week before study. The study was carried out by following the method of Lorke (1983) with modification. Rats were divided into six groups (n=6, all females). For 14 days oral toxicity study, Group 1 received AILE at a dose of 500mg/Kg body wt, group 2 received AIBE at a
dose of 100mg/Kg body weight and group 3 received AISO at a dose of 5ml/Kg body weight once daily for 14 days. In another set, AILE, AIBE and AISO were given at a single dose of 1000mg/Kg body weight, 200mg/Kg body weight and 10ml/Kg body weight respectively to different groups. The rats were observed for food consumption and behavioral signs such as excitement, nervousness, dullness, ataxia or death, if any. Twenty four hours after last dose, the number of rats survived was recorded. Blood parameters were estimated pre- and post treatment.

2.3 Animals

Healthy female rats of Wistar strain (2-3 months old) weighing between 180-210 g were selected for the study. All animals were kept at 25°C–30°C and 45%-55% relative humidity, acclimatized with standard chow and water ad libitum throughout the study under 12:12 hr light:dark cycle. All animals were carefully monitored and experimental protocols were approved by and in accordance with the recommendations of the Institutional Animal Ethical Committee (IAEC) of Jamia Millia Islamia, New Delhi.

2.4 Induction of Diabetes

Diabetes was induced by following the method of Sochor et al (1985). A group of 70-80 animals were starved for 24 hrs and diabetes was induced by a single subcutaneous injection of alloxan monohydrate, dissolved in a freshly prepared 0.154M sodium acetate buffer (pH 4.5), at a dose of 15 mg /100 g body weight. Each alloxan injected animal was then given 2U of insulin for next 7 days. This procedure decreased the mortality rate of the animals by alloxan. Control animals received only the vehicle. All animals were monitored for plasma glucose level. On seventh day, the urine glucose levels were checked qualitatively by Diastix strips (BAYER, India) for the selection of diabetic rats. Rats with fasting plasma glucose level above 360 mg/dl were selected and randomly divided into following groups of 6 animals each:

- **Group 1**: Normal Control (C) (Healthy rats with normal blood glucose level)
- **Group 2**: Diabetic untreated (D)
- **Group 3**: Insulin treated diabetic group (D+I)
- **Group 4**: *A. indica* leaf extract treated diabetic group (D+AILE)
- **Group 5**: *A. indica* bark extract treated diabetic group (D+AIBE)
- **Group 6**: *A. indica* seed oil treated diabetic group (D+AISO).
2.5 Treatment of diabetic animals

Both control and experimental groups were kept separately in individual cages. All the animals were given normal pellet diet and tap water *ad libitum* until the date of the experiment. The insulin treated diabetic group (D+I) received intraperitoneal (IP) injection of two units of protamine-zinc insulin for 21 days after insulin withdrawal of the diabetic animals.

The *A. indica* leaf extract treated diabetic group (D+AILE) was given orally an aqueous solution of the lyophilized powder of AILE at a dose of 500 mg/Kg body weight, once daily for 21 days. The *A. indica* bark extract treated diabetic group (D+AIIBE) received orally an aqueous solution of the lyophilized powder of AIIBE at a dose of 100 mg/Kg body weight, once daily for 21 days. For each dose, the required amount of the lyophilized powder calculated from the body weight of the animal was weighed, dissolved in distilled water at suitable concentration so that the desired amount for each dose can be administered in 0.5 ml to each animal. The *A. indica* seed oil treated diabetic group (D+AISO) received AISO at a dose of 5 ml/Kg body weight, once daily for 21 days. Effective dosages were selected from the literature. Control group (C) received only vehicle.

2.6 Preparation of Homogenates and separation of subcellular fractions

At the end of the treatment period, rats were sacrificed by cervical dislocation. Tissues were rapidly excised and washed with chilled normal saline. The tissues were then blotted dry and weighed. The 10% (w/v) tissue homogenate was prepared using homogenizing medium containing 0.25 M Sucrose supplemented with 0.12 mM Dithiothreitol (DTT) and buffered with 0.02 M Triethanolamine hydrochloride buffer, pH 7.4. Homogenates were then centrifuged at 1000×g for 10 minutes to remove nuclei and cell debris. The supernatant was further centrifuged at 12,000×g for 40 minutes to obtain cytosolic fraction of the tissue (*Figure 2.1*). All the procedures were carried out at 4°C. The tissue cytosolic fraction was used for determination of enzymes activities.

2.7 Estimation of Enzyme Activities

* Triton treatment to solubilize the enzyme

Extracts from different tissues were treated with Triton X-100 in a final concentration of 1% and kept on ice (4°C) for 30 minutes (Saxena *et al*, 1993), before being used for the assay of enzyme activities.
2.7.1 Superoxide Dismutase

*Superoxide: Superoxide oxidoreductase, EC 1.15.1.1*

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical (O$_2^•$•) to yield hydrogen peroxide and oxygen. The activity of SOD was measured by the method of Marklund and Marklund (1974), with some modification, an assay based on the ability of SOD to inhibit the autoxidation of pyrogallol.

\[
\text{Pyrogallol} + \text{O}_2 \rightarrow \text{O}_2^{••} + \text{product of oxidation}
\]

The assay mixture of 1 ml contained following in final concentration: 50 mM Sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, 0.48 mM Pyrogallol and appropriate amount of tissue extract containing ~7-10 μg of protein.

The reaction was initiated by the addition of extract. The change in absorbance of assay mixture was monitored at 420 nm for 3 minutes at 25°C against blank that contained all the ingredients except the tissue extract.

Enzyme activity is expressed as units/gram tissue and units/mg protein. One unit of enzyme activity is defined as the amount of enzyme that causes 50% maximal inhibition of pyrogallol autoxidation per minute.

\[
\text{Enzyme activity} = \frac{(\text{OD of Blank} - \text{OD of Sample}) \times \text{Total Reaction Volume} \times \text{Dilution factor}}{\text{OD of Blank}/2 \times \text{Sample volume} \times \text{Total time of incubation}} (\text{U/g tissue})
\]

where, OD is the change in optical density in the assay mixture.

2.7.2 Catalase

*Hydrogen peroxide: Hydrogen peroxide oxidoreductase, EC 1.11.1.6*

Catalase (CAT) catalyses the decomposition of hydrogen peroxide to give oxygen and water.

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

**Activation of Catalase**

Samples for Catalase assay were prepared by incubating the supernatant with ethanol (10μ/mdl) for 30 minutes on ice and then Triton X-100 was added to a final concentration of 1%. Ethanol increases the observable CAT level by decomposing complex II, which is an inactive complex of CAT with H$_2$O$_2$. Complex II forms spontaneously in crude tissue homogenates, even when held at 0°C. Ethanol reverses the inactivation that occurs during the time interval from the preparation of tissue homogenates to the subsequent
assay of CAT activity. Triton X-100 also increases observable CAT levels by solubilizing the enzyme.

**Enzyme assay**

The assay of Catalase (CAT) was performed by following the method of Aebi (1974). The assay mixture of 1 ml contained following in final concentration: 50 mM Sodium phosphate buffer (pH 7.0) and 10 mM H2O2. The reaction was started by addition of tissue cytosolic fraction containing ~2-3 μg protein. The decrease in absorbance was monitored at 240 nm for 5 minutes at 25°C against blank containing all ingredients except the tissue sample.

The enzyme activity is expressed as units/gram tissue (units/gram tissue × 10³ for liver and kidney tissues as they have high concentration of CAT and, are the major sites for H2O2 decomposition) and units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to decompose 1μmol of H2O2 per minute.

\[
\text{Enzyme activity} = \frac{\text{OD of Sample} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Sample volume} \times \varepsilon \times \text{Total time of incubation}}
\]

where, OD is the change in optical density per minute, \(\varepsilon\) is 0.04 cm²/μmol (the extinction coefficient of H2O2 at 240 nm).

**2.7.3 Glutathione Peroxidase**

*Glutathione: Hydrogen peroxide oxidoreductase, EC 1.11.1.9*

Glutathione peroxidase catalyses the following reversible reaction:

\[
\begin{align*}
\text{H}_2\text{O}_2 & + 2\text{GSH} \rightleftharpoons 2\text{H}_2\text{O} & + & \text{GSSG} \\
\end{align*}
\]

The activity of Glutathione peroxidase (GPx) was measured using a coupled enzyme assay linked with Glutathione reductase as described by Lawrence and Burk (1976).

\[
\begin{align*}
\text{H}_2\text{O}_2 & + 2\text{GSH} & \xrightarrow{\text{Glutathione peroxidase}} & 2\text{H}_2\text{O} & + & \text{GSSG} \\
\text{GSSG} & + 2\text{NADPH} & \xrightarrow{\text{Glutathione reductase}} & 2\text{GSH} & + & 2\text{NADP}^+ \\
\end{align*}
\]

The assay mixture of 1 ml contained the following in final concentration: 100 mM Potassium phosphate buffer (pH 7.0), 25 mM EDTA, 0.5 mM reduced glutathione, 2 mM Sodium azide, 1.5 U Glutathione reductase, 0.1 mM NADPH and the cytosolic fraction containing ~50 μg of protein. The reaction was started by the addition of t-butyl
hydroperoxide and the decrease in absorbance was monitored for 5 minutes at 25°C following the oxidation of NADPH at 340 nm against blank containing all ingredients except t-butyl hydroperoxide.

The enzyme activity is expressed as units/gram tissue and units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH per minute.

\[
\text{Enzyme activity} = \frac{\text{OD of Sample} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Sample volume} \times 6.22 \times \text{Total time of incubation}} \quad \text{(U/g tissue)}
\]

where, OD is the change in optical density per minute, 6.22 is the extinction coefficient of NADPH at 340 nm.

### 2.7.4 Glutathione Reductase

**NADPH: Oxidized glutathione oxidoreductase, EC 1.6.4.2**

Glutathione reductase catalyses the reduction of oxidized glutathione to reduced glutathione:

\[
\text{GSSG} + 2\text{NADPH} \rightarrow 2\text{GSH} + 2\text{NADP}^+ 
\]

The Glutathione reductase (GR) activity was measured in the Triton X-100 treated tissue extracts by following the method of Erden and Bor (1984). The reaction mixture of 1 ml contained the following in final concentration: 4.1 mM Tris-HCl (pH 7.5), 15 mM MgCl₂, 5.7 mM EDTA, 60 mM KCl, 2.6 mM Glutathione (oxidized) and 0.1 mM NADPH. The reaction was started by the addition of tissue extract containing ~100 μg of protein. The decrease in absorbance was monitored at 25°C at 340 nm.

The enzyme activity is expressed as units/gram tissue and units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of NADPH oxidized per minute.

\[
\text{Enzyme activity} = \frac{\text{OD of Sample} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Sample volume} \times 6.22 \times \text{Total time of incubation}} \quad \text{(U/g tissue)}
\]

where, OD is the change in optical density per minute, 6.22 is the extinction coefficient of NADPH at 340 nm.
2.7.5 Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49

Glucose-6-phosphate dehydrogenase (G-6-PD) converts glucose-6-phosphate into 6-phosphoglucono-δ-lactone and is the rate-limiting enzyme of pentose phosphate pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme Nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn, maintains the supply of reduced glutathione in the cells that is used to scavenge free radicals.

G6PD activity was assayed by following the method described by Cohen and Rosemeyer (1975). The assay mixture of 1ml contained the following in final concentration: 0.05 M trisHCl buffer (pH7.6), 0.8 mM glucose-6-phosphate, 8 mM MgCl2 and 0.1 mM NADP. The reaction was started by adding appropriate amount of tissue extract containing ~100 μg protein. The change in absorbance was monitored at 340 nm for 5 minutes at 25°C against blank that contained all ingredients except the tissue extract.

The enzyme activity is expressed as units/gram tissue and units/mg protein. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1nmol NADP per minute.

\[
\text{Enzyme activity} = \frac{\text{OD of Sample} \times \text{Total reaction volume} \times \text{Dilution factor}}{\text{Sample volume} \times 6.22 \times \text{Total time of incubation}} \quad (U/g \text{ tissue})
\]

where, OD is the change in optical density per minute, 6.22 is the extinction coefficient of NADPH at 340 nm.

2.8 Measurement of lipid peroxidation

The oxidative damage of unsaturated fatty acids can be followed by determining the total consumption of oxygen (Placer et al, 1966). As an index of lipid peroxidation, the method of Armstrong and Al-Awadi (1991) with some modification was employed in the present work using Thiobarbituric acid (TBA) assay for estimating Malondialdehyde (MDA), as described by Koster and Slee (1980). The MDA formation gives a measure of thiobarbituric acid reacting species (TBARS) which is proportional to the lipid peroxidation or formation of lipid peroxides. A chromogen is formed by the reaction of one molecule of MDA with two molecules of TBA (Gutteridge and Halliwell, 1990).

**Preparation of tissue homogenate**

For measurement of lipid peroxidation, 10% tissue homogenate was centrifuged at 1000×g for 10 minutes to obtain the whole homogenate devoid of nuclei and cell debris.
The resulting extract was deproteinized with half volume of 20% Trichloroacetic acid (TCA). Precipitated proteins were removed by centrifugation at 1000×g for 5 minutes and the final supernatant obtained was used for the measurement of lipid peroxidation.

**Reagents**

A stock solution of 0.53% thiobarbituric acid dissolved in glacial acetic acid and adjusted to pH 3.5 was freshly prepared and filtered before use. In addition, a 30% Trichloroacetic acid was prepared for acidification.

**Assay**

The reaction mixture of 3 ml contained following in final concentration: 1.5 ml of 10 mM Potassium phosphate buffer (pH 7.4), 0.5 ml of the homogenate prepared, 0.5 ml of 30% Trichloroacetic acid, 0.5 ml of 0.53% Thiobarbituric acid. The reaction mixture was vortexed and incubated at 80°C in water bath for 15 minutes and cooled. The reaction mixture was then centrifuged at 4000×g for 15 minutes. The supernatant was transferred to another tube and the concentration of MDA-TBA complex was determined spectrophotometrically at 532 nm against blank containing all ingredients except the homogenate prepared. A standard curve (Figure 2.2) was prepared using 1,1,3-tetra ethoxy propane [Malondialdehyde bis (dimethyl acetal)].

Regression equation of standard curve: 

\[ y = 0.016x + 0.003 \]

where, \( y \) = Absorbance; \( x \) = Concentration; 0.016 = Slope of the curve; 0.003 = Intercept on y-axis.

Correlation coefficient (R-squared value) = 0.997

**Calculation**

\[ \text{nmoles of MDA formed/mg protein} = \frac{\text{Concentration} \times \text{Total reaction volume}}{\text{OD of MDA} \times \text{Volume of extract} \times \text{Total protein}} \]

**2.9 Measurement of glycemic index**

**2.9.1 Glucose**

Blood glucose was estimated by using Glucose-kit from Ranbaxy Laboratories Ltd. India, which quantitatively estimates D-glucose, the form that is present in blood plasma, by the Glucose oxidase-peroxidase reaction. Glucose oxidase acts on D-glucose in presence of oxygen to give gluconic acid and hydrogen peroxide into water and oxygen. The oxygen liberated is accepted by the chromogen system to give a red color compound. The red color
so developed is proportional to the glucose concentration and is measured colorimetrically at 505 nm.

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Colored complex} + \text{H}_2\text{O}
\]

\[
\text{Glucose concentration (mg/dl)} = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank}) \times 100}{\text{Absorbance of standard} - \text{Absorbance of blank}}
\]

\[
\text{2.9.2 Glycosylated hemoglobin}
\]

Glycosylated hemoglobin (GHbA1c) was estimated by Ion Exchange Resin method using kit from Asritha Diatech, India. Glycosylated hemoglobin reflects the metabolic control of glucose level over a period of time unaffected by diet, insulin or other drugs and is widely recognized as an important indicator of diabetes mellitus and efficacy of the therapy. The hemolysed preparation of whole blood was mixed with a weakly binding cation exchange resin. The labile fraction and non-glycosylated hemoglobin binds to the resin leaving behind glycosylated hemoglobin in supernatant. Absorbance was taken at 415 nm against blank.

\[
\% \text{GHbA1c} = \frac{\text{Absorbance of Glycosylated hemoglobin} \times \text{4.61 (assay factor)}}{\text{Absorbance of Total hemoglobin}}
\]

\[
\text{2.10 Estimation of blood lipids}
\]

\[
\text{2.10.1 Total Cholesterol and HDL-Cholesterol}
\]

Serum total cholesterol and HDL-cholesterol were estimated by using kit from Span Diagnostics Ltd. India, which quantitatively estimates cholesterol in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction byproducts, H$_2$O$_2$ is measured quantitatively in a peroxidase catalyzed reaction that produces a colored compound. The color intensity is proportional to cholesterol concentration and was measured colorimetrically at 560 nm. The reaction sequence is as follows:

\[
\text{Cholesteryl ester} + \text{H}_2\text{O} \xrightarrow{\text{cholesteryl ester hydrolase}} \text{cholesterol} + \text{fatty acid}
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} 4\text{-}(\text{p-benzoquinone-monoimino)}\text{-phenazone} + 4\text{H}_2\text{O}
\]
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Serum Total Cholesterol (mg/dl) = \frac{\text{Absorbance of sample} \times 200 \text{ (assay factor)}}{\text{Absorbance of standard}}

Serum HDL-Cholesterol (mg/dl) = \frac{\text{Absorbance of sample} \times 50 \text{ (assay factor)}}{\text{Absorbance of standard}}

2.10.2 Triglycerides

Triglycerides were estimated by using kit from Span Diagnostics Ltd. India, which quantitatively estimates triglycerides in serum using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and \( H_2O_2 \), one of the reaction products, is measured quantitatively in a peroxidase catalyzed reaction that produces a colored compound. The color intensity is proportional to triglyceride concentration and was measured colorimetrically at 560 nm. The reaction sequence is as follows:

\[
\text{Triglycerides} + 3H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol-3-phosphate} + \text{ADP}
\]

\[
\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{glycerophosphate oxidase}} \text{dihydroxyacetone phosphate} + H_2O_2
\]

\[
2H_2O_2 + 4\text{-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} 4\text{-}(p\text{-benzoquinone monoimino})\text{-phenazone} + 4H_2O
\]

Serum Triglycerides (mg/dl) = \frac{\text{Absorbance of sample} \times 100 \text{ (assay factor)}}{\text{Absorbance of standard}}

2.11 Protein estimation

Protein estimation was carried out by following the method of Bradford (1976) using BSA as standard. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. BSA concentrations ranging from 2 \( \mu \)g to 15 \( \mu \)g were used to make the protein standards as shown in Figure 2.3.

2.12 SDS-PAGE and Western blotting

SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis) and Western blotting were performed as previously described by Laemmli et al, (1970) and
Towbin et al., (1984). Bio-Rad mini protein gel electrophoresis unit for SDS-PAGE was used for running the gels of the required percentages. Two glass plates were assembled to form a gel mould. Once the separating gel had polymerized, 5% stacking gel was prepared and a comb was fixed to form wells. The gel slab was then suspended into the reservoir tank containing the same reservoir buffer. Protein extract mixed with an equal volume of 2X sample buffer was loaded in each well. Pre-stained markers (19.5 to 118 kD) mix was loaded in the last well. The gel was run at an initial voltage of 50V and once the dye had crossed the stacking layer, the voltage was increased to 100V and allowed to run till the dye reached the bottom.

2.12.1 Recipes for SDS-PAGE gels and buffers

- Tris-HCl 1.5 M, pH 8.8 (required for preparation of separating gel)
- Tris-HCl 0.5 M, pH 6.8 (required for preparation of stacking gel)
- SDS 20% (required for both separating and stacking gels)

**Preparation of 30% Acrylamide**

To 29 g of Acrylamide and 1 g N,N'-methylenebisacrylamide, ~60 ml of distilled water was added and heated to 37°C to dissolve chemicals. Final volume was adjusted to 100 ml with distilled water. Solution was sterilized by filtration through Millipore 0.22 μm vacuum filter. The solution was stored at 4°C.

**10% Ammonium persulfate (APS)**

To 1 g of Ammonium persulfate, distilled water was added to make final volume of 10 ml. The solution was stored at 4°C.

**Resolving gel**

- 30% Acrylamide 16.625 ml
- Tris-HCl (1.5 M, pH 8.8) 15.625 ml
- 10% SDS 0.625 ml
- 10% APS 0.625 ml
- TEMED-100% 0.0625 ml
- Volume made to 50 ml with distilled water.

**Stacking gel**

- 30% Acrylamide 16.625 ml
- Tris-HCl (0.5 M, pH 6.8) 15.625 ml
MATERIALS AND METHODS

- 10% SDS 0.625 ml
- 10% APS 0.625 ml
- TEMED-100% 0.0625 ml
- Volume made to 50 ml with distilled water.

**2X Laemmli sample buffer** (SDS reducing buffer)

- Distilled water 10.4 ml
- 0.5 M Tris pH 6.8 1.2 ml
- Glycerol 1.9 ml
- 20% SDS 1 ml
- β-mercaptoethanol 5 ml
- 1% bromophenol blue 1 ml,

(Total volume 16 ml, solution was stored in aliquots at −20°C)

**5X Reservoir buffer**

To 15 g of Tris base and 72 g of Glycine, distilled was added to make final volume 1 liter.

**2.12.2 Electrophoretic transfer of proteins and Westen blotting** (Towbin, 1984)

**Transfer buffer**

- Tris-HCl 72 g
- Glycine 15 g
- Methanol 200 ml
- Volume made to 1 liter with distilled water.

Once the proteins have been separated, the gel was washed with distilled water and equilibrated with chilled transfer buffer for an hour before it was transferred onto PVDF (polyvinylidene fluoride) membrane (amresco), cut to the size of the gel in a manner so that a sandwich was formed. The gel was in direct contact with the PVDF membrane on one side while on the other side it faced the Whatman sheets no.3. This whole assembly was held in place between two plastic sheets and it was immersed in the transfer buffer. The transfer was carried out at 100V for 2 hours at 4°C.

Once the transfer was complete, the efficiency of transfer was checked by staining with 0.5% ponceau stain for 2-3 minutes followed by de-staining with distilled water. The blots were further processed for Western blot analysis.
Reagents for Western blot analysis

**10X PBS, pH 7.4**

- NaCl 80 g
- KCl 2 g
- Na$_2$HPO$_4$ 14.4 g
- KH$_2$PO$_4$ 2.4 g

(Final volume made to 1 liter with distilled water, pH adjusted to 7.4)

**Blocking reagent**

- Skimmed milk powder 5 g
- Tween 20 50 µl

(Volume made to 10 ml with 1X PBS, pH 7.4)

**Western blot analysis**

Membrane strips with transferred proteins were washed with 1X PBS (pH 7.4) and incubated at 4°C with the blocking reagent for 12-16 hours. This was followed by two rinses of 2 minutes each in 1X PBS. The membranes were then incubated in primary antibody for 4 hours. The membranes were washed twice for 15 minute with 1X PBS and then incubated with secondary antibody for 2 hours. This was followed by two rinses of 10 minute each with 1X PBS. All the procedures were carried out in cold room.

ECL (enhanced chemiluminescence) detection of protein blots were carried out in dark room using luminol and peroxide reagent (Thermo Scientific, USA) as a substrate. The membranes were removed from the substrates and placed in plastic sheet protectors. Each membrane was exposed to Hyperfilm Film (GE Healthcare). The film was kept in developer till the bands developed and scanned. Band densities were estimated using GeneTools analysis software.

**2.13 Subcellular fractionation for measuring GLUT 4 levels**

Membrane fractions from skeletal muscle (from each experimental group) were prepared by following the method of Klip *et al* (2001). 1 g of muscle tissue was minced well and homogenized (1/10, w/v) at 4°C in homogenizing buffer containing 20 mM NaHCO$_3$ (pH 7.0), 0.25 M Sucrose, 5 mM Na$_3$N, 1mM leupeptin, 1 mM aprotinin and 1 mM pepstatin. The homogenate was centrifuged at 1200×g for 10 minutes and the pellet was resuspended in homogenizing buffer, homogenized and recentrifuged to remove debris. The combined supernatants were centrifuged at 9000×g for 10 minutes at 4°C for mitochondria and nuclei
to sediment. The resulting supernatant was then centrifuged at 190,000×g for 60 minutes at 4°C to obtain total membrane fraction. The membrane pellets were resuspended in homogenization buffer and the total membrane protein content were measured by following the method of Bradford (1976) (Figure 2.4).

2.14 GLUT 4 protein analysis

The semi-quantification of GLUT 4 protein in membrane fraction and total homogenate was done by Western blotting as described by Klip et al (1990).

Electrophoresis in 10 % Sodium dodecyl sulphate (SDS)-Polyacrylamide gels of skeletal muscle membrane fractions (10 μg each lane) and whole homogenate (30 μg each lane) was done and resolved proteins were transferred to PVDF membrane. After protein transfer, the membranes were blocked in 5 % fat free milk and incubated with 1:500 dilution of polyclonal anti-GLUT 4 antibody. The antigen antibody complex was detected with horseradish peroxidase conjugated secondary antibody using luminol reagent as substrate. In each case, western blot revealed a single band of 45 kDa, compatible with the molecular weight of the glucose transporter (GLUT 4). Semi-quantification was performed by densitometry after scanning the blots with GeneTools analysis software.

2.15 Immunoblotting for Cu/Zn SOD protein

2.15.1 Extraction

The liver tissues from each experimental group were rapidly isolated and frozen in liquid nitrogen and stored at −80°C. On the day of the experiment, the samples were homogenized in 50 mM Tris (pH 7.5) containing 1mM EDTA and centrifuged at 1000×g for 10 minutes at 4°C. The supernatant was further centrifuged at 10,000×g for 1 hour at 4°C. The resulting supernatant was used as the cytosolic fraction for SOD immunoblotting. Protein concentration was determined by following the method as described by Bradford (1976).

2.15.2 Western blot analysis

Samples of equal protein content (50 μg) were resolved by 12 % SDS-PAGE and electroblotted onto PVDF membrane. After overnight blocking of non-specific binding sites with 5 % BSA, membrane was incubated for 1 hour at 4°C with mouse anti Cu/Zn SOD monoclonal antibody (1:500). Membrane was washed with 1X PBS and then incubated with horseradish peroxidase conjugated secondary antibody (1:5000) for 1 hour at 4°C. The membrane was washed again with 1X PBS. The bound antibody was detected using luminol
reagent as substrate. In each case, western blot revealed a single band of 32 kDa, compatible with the molecular weight of the Cu/Zn SOD protein. Semi-quantification was performed by densitometry after scanning the blots with GeneTools analysis software.

**2.16 Immunoblotting for PKC β2 protein**

**2.16.1 Membrane extraction**

Heart and muscle tissues from control, diabetic and treated rats were frozen in liquid nitrogen, crushed into frozen powder and homogenized in ice cold buffer. The buffer contained the following in final concentration: 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Dithiothreitol (DTT), 0.3 M Sucrose, 25 μg/ml leupeptin. The homogenization was carried out with polytron homogenizer for 20 seconds. Homogenates were centrifuged at 1000×g for 30 minutes at 4°C and the supernatant was ultracentrifuged at 190,000×g for 60 minutes. The pellet obtained was retained as total membrane fraction. The methodology followed is essentially according to the method described by Wakasaki et al (1997).

**2.16.2 Western blotting**

Samples from each group containing approximately 30 μg protein were resolved by 7 % SDS-PAGE and transferred electrically to PVDF membrane. After blocking with 1X PBS containing 0.1 % Tween-20 and 5 % BSA at 4°C overnight, the membrane was incubated with isoform β2 specific anti-PKC antibody (1:500) for 2 hours at 4°C. After washing with 1X PBS, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit IgG antibody. The bound antibody was detected using luminol reagent as substrate. In each case, western blot revealed a single band of 75 kDa, compatible with the molecular weight of the PKC β2 protein. Semi-quantification was performed by densitometry after scanning the blots with GeneTools analysis software.

**2.17 Isolation of genomic DNA**

**2.17.1 Phenol saturation**

Phenol crystals (Qualigens) were melted at 64°C in a water bath. When melted, phenol was taken in dark bottle. To this was added 1.0 M Tris (pH 8.0) and a stirred. After transferring, 0.2 % 8-hydroxyquinoline flakes were added and stirring was performed overnight. The solution was allowed to sit long enough for the phases to separate. The upper phase was removed with a pipette and to this was added equal volume of 0.1 M Tris (pH 8.0) and stirred for 1 hour. After the phase separation, pH was checked. The above
steps were repeated till the pH reached 8.0. Solution was stored in dark bottle in the refrigerator with a layer of buffer on the top. The phenol was drawn off the bottom for use. 8-hydroxyquinoline increases the shelf life of the phenol.

**Digestion buffer**
- 5.0 M NaCl 30 µl
- 2.0 M Tris (pH 7.4) 50 µl
- 10.5 SDS 400 µl
- 0.2 M EDTA 500 µl
- Proteinase K (20 µg/ml) 50 µl
- Distilled water (8.7 ml) to make final volume of 10 ml.

**TE buffer**
10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA in ratio of 10:0.1

2.17.2 DNA isolation
For the isolation of genomic DNA, liver tissues from each experimental group rapidly excised, and washed with chilled normal saline. The tissues were blotted dry and minced well with sterile scissors. Tissues were treated with 5-6 ml of digestion buffer and 40-50 µl of proteinase K essentially according to the method of Kunkel *et al.* (1977).

**Phenol extraction of DNA samples**
The classical phenol method was used to isolate the DNA samples essentially as described by Gupta (1984). An equal volume of Tris EDTA (TE)-saturated phenol was added to an aqueous DNA sample. The mixture was vigorously vortexed, and then centrifuged for phase separation. The upper aqueous layer was carefully removed to a new vial, avoiding the phenol interface and then is subjected to two ether extractions to remove residual phenol.

The above tissue mixture was incubated at 50°C for 10 minutes with gentle shaking. The mixture was then incubated overnight at 37°C in a shaking water bath. An equal volume of TE-saturated phenol was added to the DNA sample and vortexed for 30 seconds. The samples were centrifuged at 5000 rpm for 15 minutes at room temperature to separate the phases. About 90% of the upper phase aqueous layer was transferred to a clean tube, carefully avoiding proteins at the aqueous: phenol interface. At this stage, the aqueous phase was extracted for second time with an equal volume of TE-saturated phenol, isoamyl
alcohol and chloroform (25:24:1). The mixture was kept on rocker for 10 minutes and centrifuged at 5000 rpm for 15 minutes and the supernatant was removed into a clean vial.

**Concentration of DNA by ethanol precipitation**

After phenol extraction, the DNA was concentrated by ethanol precipitation. Most nucleic acid can be precipitated by addition of monovalent cations and two to three volumes of cold 95 % ethanol, followed by incubation at 0 to −70°C. 1/10 volume of 3.0 M sodium acetate, pH 5.2 and two volumes of cold 95 % ethanol was added to the DNA sample to be precipitated. The sample mixture was kept at −20°C for overnight. After incubation, the sample was centrifuged at 5000 rpm for 45 minutes and supernatant was decanted. The DNA sample (pellet) was re-suspended with 85 % ethanol, corresponding to about two volumes of the original sample, incubated at room temperature for 5-10 minutes and centrifuged again at 5000 rpm for 5 minutes. Supernatant was decanted and DNA pellet was dried and dissolved in Tris-EDTA buffer.

The quantity of isolated DNA was checked by adding 5 µl of DNA sample to 1 ml of distilled water and the optical density was read at 260 nm using a UV spectrophotometer against water as blank as described by Ausubel (1995).

2.17.3 Agarose gel electrophoresis

Isolated DNA samples from control and different experimental groups were separated and analyzed by horizontal agarose gel electrophoresis as described by Nagata (2000). A 1.5 % agarose gel was prepared in TE buffer and horizontal gel free from bubbles was cast. The comb was inserted into the gel and the whole assembly was left aside for 30 minutes for the gel to set. The gel was submerged to 2-3 mm depth by pouring TE buffer into the gel tank. Each DNA sample of 1 µl was diluted with 8 µl of DNase free water. 2 µl of gel loading buffer (25 mg bromophenol blue and 4 g sucrose dissolved in 10 ml distilled water) was added to each sample and samples were loaded into the well. The gel was run at 5 V/cm² to separate the DNA fragments until the bromophenol blue tracking dye reached the end of the gel. The gel was stained with 0.5 µg/ml ethidium bromide prepared in TE buffer for 15 minutes. The gel was washed with TE buffer and DNA bands were observed under UV light. A picture of the same was taken using Polaroid camera.

2.18 HPLC analysis

To compare the chemical profile of the *A. indica* extracts, reversed phase HPLC analysis for azadirachtin was done by following the method as described by Epshtein (2004)
and Chimeray et al (2009). An HPLC system (UV-2075 Plus, JASCO, Japan) with two gradient pump systems (PU-2080, JASCO) were used for the analysis. The separation was performed on a C-18 column (Thomascientific, Dim (mm) 250x 4.6, ODS Hypersir). HPLC conditions were as follows: with Solvent A (water) and Solvent B (acetonitrile), gradient elution was used as Solvent A: 0-10 minutes, 30-40%; Solvent B: 10-15 minutes, 40-45%; Solvent A: 20-25 minutes, 50-60%. Flow rate of mobile phase solution was selected at 1.0 ml/min and detection was made at 217nm. 10μl of each sample was injected in to the HPLC machine with low and steady pressure. At the end of the run time (25 minute), the data module plot a real time chromatogram with details of retention time (RT), peak area and relative areas of the peaks (concentration). Azadirachtin in extracts was identified by matching retention time and spectra with that of standard azadirachtin and quantitative data was calculated on the basis of peak area of the compound. To achieve this, a series of standards of known concentration were analyzed and a response factor was calculated on the basis of the relationship between the peak area and the amount analyzed (concentration). From this, quantity of azadiractin was calculated in unknown samples.

2.19 Statistical analysis

All values were calculated as mean ± SEM. The ANOVA test followed by Dunnet Multiple comparison test was employed for statistical comparison between control and various groups. Significance was considered at p<0.05.

2.20 Chemicals

All purified enzymes, substrates, standards and buffers were purchased from Sigma Aldrich, USA. All other chemicals were of analytical grade. Anti Cu/Zn SOD monoclonal antibody was purchased from Calbiochem (Merck). Isoform β2 specific anti-PKC antibody and polyclonal anti-GLUT 4 antibody were purchased from Sigma Aldrich chemicals, USA. Protease inhibitor cocktail was purchased from Calbiochem (Merck).
Figure 2.1: Separation of subcellular fractions by centrifugation

- Tissue homogenate
  - 1000g for 10 minutes
    - Cell debris
    - Supernatent
      - 12,000g for 40 minutes
        - Pellet (Mitochondrial)
        - Supernatent (Cytosolic fraction)
Figure 2.2: Standard curve for estimation of Malondialdehyde (MDA) concentration
**Figure 2.3:** Standard curve for protein estimation

The equation of the line is:

\[ y = 0.026x + 0.008 \]

\[ R^2 = 0.988 \]