Methodology
Chemicals

Sigma Chemical Co, St. Louis, MO, USA

Acetyl-CoA, Adenosine 5-monophosphate from yeast, Adenosine-5'-triphosphate dipotassium salt \textit{bacterial source}, D-Fructose-1, 6-diphosphate tetra sodium salt, DL-Glyceraldehydes, Malonyl-CoA, P-Nitro phenyl butyrate, Phosphoglucoisomerase Type \textit{XI} from rabbit muscle, 304 Units/mg, Streptozotocin, Thiobarbituric acid (TBA).

SISCO Research Laboratories Private Ltd., Mumbai, Maharashtra, India

Adenosine-5'-monophosphate sodium salt, Adenosine-5'-diphosphate disodium salt, Adenosine-5'-triphosphate disodium salt (ATPNa$_2$), Albumin, Bovine serum fraction V powder, 1-Amino-2-naphtho-4-sulphonic acid, 1-Chloro-2, 4-dinitro benzene (CDNB), Cysteine, 2,4-Dinitro phenyl hydrazine (DNPH), DL-Aspartic acid, D(-) Fructose, D-Glucose-1-phosphate dipotassium salt, D-Glucose-6-phosphate disodium salt extra pure, Glucose-6-phosphate dehydrogenase from \textit{Leuconostoc mesenteries} lyophilized powder 410 $U/mg$ soli, Glutathione reduced (GSH), Glutathione oxidized (GSSG), Glycogen, $\alpha$-Ketoglutaric acid, Lactate dehydrogenase from rabbit muscle lyophilized powder 400-600 $U/mg$ protein, Maleic acid, Maltose, $\alpha$-Mercaptoethanol, Nicotinamide adenine dinucleotide phosphate disodium salt (NADPNa$_2$), Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPHNa$_4$), Nicotinamide adenine dinucleotide reduced disodium salt (NADHNa$_2$), Phosphoenol pyruvate, Potassium sodium tartrate, Pyruvate kinase from rabbit muscle lyophilised powder 168 $U/mg$ solid, Pyrogallol, Sodium pyruvate, Sodium sulphate, Triethanolamine hydrochloride, Triton X-100.

Qualigen chemicals, Mumbai, Maharashtra, India

Ammonium molybdate, Citric acid, Disodium hydrogen orthophosphate, Dipotassium hydrogen orthophosphate, Ether, 3-Ethylene diamine tetra acetic acid disodium salt (EDTANa$_2$), Ferric chloride, Folin \& Ciocalteu reagent(2N), Hydrogen peroxide, Magnesium sulphate, Potassium chloride, Potassium dihydrogen ortho phosphate, Potassium hydroxide, Potassium iodide, Sodium carbonate, Sodium chloride, Sodium dihydrogen orthophosphate, Sodium fluoride, Sodium hydroxide, Sucrose, Trichloro acetic acid, Trisodium citrate.

Span Diagnostics

Hematoxyline Stain (ready to use)
All other chemicals used were of analytical grade from Merck (India) and SISCO Research Laboratories (India).

**Plant material:** Aqueous extract of *Moringa oleifera* leaf (AEMO) was purchased from Chemiloids (manufacturers and exporters of herbal extracts, Vijayawada, Andhra Pradesh, India). Herb-to-product ratio was 10:1. The extract was dissolved in distilled water prior to use.

**Information about the plant Extract as given by Chemiloids**

- **Name of the product:** *Moringa oleifera* Extract Dry Powder
- **Product Code:** P/DSM/MOOL- 01
- **Batch Number:** P8060947
- **Mfg Date:** June, 2008
- **Botanical Name:** *Moringa oleifera*
- **Part of the Plant:** Leaf
- **Extraction Medium:** Water
- **Loss on Drying:** 6.59%
- **Water Soluble Extracts:** 93.26%

**FLOW CHART:**

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RAW MATERIAL ↓ extraction with water
EXTRACT ↓ concentration under vacuum
RESIDUE ↓ drying under vacuum
DRY FLAKES ↓
PULVERISATION
MULTIMILL/ MICRO PULVERISATION ↓
SIEVING ON SHIFTER AND PACKING
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Qualitative screening for phytochemicals

The extract was qualitatively tested for the presence of various phytochemical constituents (Brain and Tunfer, 1975; Sofowora, 1982; Trease and Evans, 1983; Harborne, 1991).

Alkaloids

The aqueous extract was tested for the presence of alkaloids using the procedures of Smolenski et al. (1972).

A portion of the aqueous extract was made alkaline with 10% ammonium hydroxide and treated with ether. The ether extract was extracted with 10% hydrochloric acid and the acidic aqueous solution was collected and tested for alkaloids. The resulting acidic solution was divided into three portions. Of these, two portions were tested for alkaloids by adding Mayer’s reagent (1.35 g HgCl₂ and 5 g KI /100 ml distilled water), and Wagner’s reagent (2 % KI in distilled water) respectively, while the third was used as blank. The formation of a faint turbidity or precipitation after the addition of the above reagents indicates the presence of alkaloids.

Anthracene glycosides

Presence of anthracene glycosides was tested by Borntrager’s test (Peyer, 1931). To 1.0 ml of the aqueous extract, 1.0 ml of chloroform was added and the separated chloroform layer was collected. To this, an equal volume of 2.5 % ammonium hydroxide solution was added. Appearance of red color indicates the presence of anthracene glycosides.

Flavonoids

To 5.0 ml of the aqueous extract, 1.0 ml of alcohol was added and subjected to the following tests:

1. Ferric chloride test: To 1.0 ml of the above solution, 2-3 drops of neutral ferric chloride solution was added. Appearance of blackish red color indicates the presence of flavonoids.

2. Lead acetate test: To 1.0 ml of the above alcoholic solution, a few drops of aqueous basic lead acetate solution was added. Formation of reddish brown bulky precipitate indicates the presence of flavonoids.

Gallic-tannins and Catecholic compounds

To 1.0 ml of the aqueous extract, 2.0 ml of ethanol and 2-3 drops of dilute ferric chloride solution were added. Formation of bluish black color indicates the presence of
Gallic-tannins, while a greenish black colour indicates the presence of catecholic compounds.

**Phenols**

One ml of the aqueous extract was treated with a few ml of neutral ferric chloride solution. Change in colour indicates the presence of phenols.

**Saponins**

The aqueous extract was tested for the presence of saponins (Cambie et al., 1961). Five ml of the aqueous extract was mixed with 10 ml of distilled water and agitated in a graduated cylinder for 15 min. Formation of persistent foam indicates the presence of saponins.

**Steroids and Triterpenoids**

The aqueous extract was tested for the presence of steroids and triterpenoids by Libermann-Burchard test (Harborne, 1976). The aqueous extract was dissolved in 5.0 ml of chloroform and subjected to the following tests:

**Salwoski Test:** To 1.0 ml of the above solution, 1.0 ml of concentrated sulphuric acid was added, mixed and allowed to stand for 5 min. Appearance of golden yellow colour in lower layer indicates the presence of steroids.

**Libermann-Burchard Test:** One ml of the above solution was treated with a few ml of acetic anhydride and 1.0 ml of concentrated sulphuric acid from the sides of the test tube and allowed to stand for 5 min. Formation of brown ring at the junction of the two layers and appearance of green color in the upper layer indicates the presence of steroids.

**Trace elements**

Trace elements in AEMO were estimated by particle induced X-ray emission (PIXE) technique. Aqueous extract of *M. oliefera* was taken, and thin pellets were made using a 10-ton motorized pellet press machine. The maximum thickness of the pellet was about 2 mm. Care was taken to avoid any kind of contaminations. The PIXE experiment was carried out using a 2 MeV proton beam from a 1.7-MV Tandetron accelerator available at Indira Gandhi Center for Atomic Research (IGCAR), Kalpakkam, India. NIST reference material, (Apple leaves NIST SRM 1515), was used for standardization and calibration. The beam current was kept low to avoid charge buildup, and the spectrum was acquired for a fixed charge of 10 μC, twice for each sample. The X-ray spectra were collected using a Canberra Si (Li) detector, which had a nominal resolution of 180 eV at 5.9 keV. The data were
analysed using GUPIXWIN® (V2.1, 2008). The 2-MeV proton beam was used to identify and characterize major and minor elements namely Cl, K, Ca, Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Br, and Sr.

Animal ethical clearance

Local Institutional Animal Ethical Committee of our university, obtained ethical clearance for conducting experiments on animals from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Regd.no.470/01/a/CPCSEA, dt.24th Aug 2001). The present work was carried out with prior permission from Local Institutional Animal Ethical Committee.

Procurement of animals and maintenance

Male albino Wistar rats of age 4-5 weeks with a body weight of 150-160 g were procured from Sri Venkateswara Enterprises, Bangalore. Animals were maintained as per the guidelines of NIN Animal User’s Manual (Raghuramulu et al., 2003). Animals were acclimatized for 7 days to animal house maintained at a temperature of 22 ± 2° C. The animal room was regulated by a 12 h light: 12 h dark schedule. Three to four animals were housed per cage (sized 41 cm length, 28 cm width and height of 14 cm). Paddy husk was used for bedding, which was changed every day and washed thoroughly with water along with Domex, a disinfectant and detergent. All rats were fed on a standard pellet diet before dietary manipulation, unless otherwise stated.

Experimental animal models

Insulin resistance animal model

Insulin resistance was induced by a fructose-enriched diet (Reaven and Banting, 1988). This diet contained 66% fructose, 18% protein, 8% fat, 4% cellulose, 3% mineral and 1% vitamin mix, which was procured from National Centre for Animal Science, National Institute of Nutrition (Hyderabad, India).

STZ induced diabetic animal model

Diabetes was induced in rats by a single intraperitoneal injection of freshly prepared STZ with a dosage of 55 mg/ kg body weight, in 0.05 M citrate buffer pH 4.5 in a volume of 0.1 ml. STZ was first weighed individually in Eppendorf tubes for each animal according to the bodyweight and then solubilized in buffer just prior to injection. Seventy-two hours after STZ administration, plasma glucose level of each rat was determined for confirmation of diabetes. They were allowed for a window period of 5 days before commencement of
treatment. Rats with fasting plasma glucose greater than 300 mg/dl were considered diabetic and included in the present study.

**Experimental design**

In the present study fifty four male albino Wistar rats aged about 4-5 weeks with average body weight of 150-160 g were acclimatized to our animal house before induction of IR/ type-1 diabetes. Insulin resistance was induced in 16 rats by feeding fructose-enriched diet throughout the experimental period. About twenty two rats were made diabetic by STZ injection and maintained on standard pellet diet and used for further studies in the present investigation. The remaining 16 rats served as controls and were maintained on standard pellet diet. Each set of animals (Control, IR and type-1 DM,) was further subdivided into two groups thus comprising a total of six groups: control group (C-group), control rats administered with AEMO (C+MO-group), fructose fed rats (F-group), fructose fed rats administered with AEMO (F+MO-group), STZ diabetic group (D-group) and STZ diabetic rats administered with AEMO (D+ MO -group). Rats in the Groups-C+MO, F+MO and D+MO were administered with the aqueous extract of *M. oleifera* leaf at a dose of 200 mg/kg body weight through gastric intubation for a period of 60 days.

Body weight, plasma glucose, plasma insulin, lipid profile were measured at 15- day interval during experimental period. At the end of the experimental period (60 days), an oral GTT was performed in all groups of rats. Plasma urea, creatinine, transaminases (GPT & GOT), alkaline phosphatase, total protein, albumin, calcium and magnesium were assayed at the beginning and end of the experimental period.

**Sample collection and preparation for biochemical estimations and assays**

**Blood collection and plasma separation**

Blood was collected in heparinised Eppendorf tubes from 12 h fasted rats by means of a heparinised capillary tube through retro-orbital plexus. Plasma was separated immediately by centrifugation at 4°C using REMI- 24 model centrifuge. Plasma was used for biochemical parameters and insulin assay.

**Sacrification of rats and organ collection**

After the experimental period of 60 days, rats from all six groups were sacrificed by cervical dislocation following 12 h fasting. Liver, pancreas, kidneys, adipose tissue, testes, heart and thigh muscle were dissected immediately and suspended in ice cold 0.15 M KCl
in properly labelled polypropylene containers, sealed with parafilm, and frozen at -80° C until assays were carried out. Small intestines of rats from all groups (nearly 20-25 cm length) were cut and immediately suspended in ice-cold 0.9% saline and stored at -80°C till further assay.

Parts of liver and pancreas, whole kidney and testes and a portion of adipose tissue of three rats from each group were suspended in properly labelled polypropylene containers with 10% formalin immediately after dissection.

Plasma glucose, urea, creatinine, transaminases (GPT and GOT), alkaline phosphatase, total proteins, albumin, total cholesterol, triacylglycerols, HDL-C, LDL-C, calcium and magnesium were assayed on fully automated chemistry Analyser AU 400.

OLYMPUS AU 400 is a fully automated, random access chemistry analyser from the manufacturers:-OLYMPUS CORPORATION, JAPAN. This system allows us to use the reagents of our choice, either OLYMPUS SYSTEM PACKS from the machine manufacturers or reagents from any other competitor. The reagent carousel contains 80 slots for reagents, which can be loaded in the system simultaneously. The test procedures are programmed in the system, which are specific for the test and the reagent used. The minimum quantity of sample required for an assay is 3 μl and the minimum quantity of reagent, 200 μl. Before performing the tests, the reagents are calibrated with a calibrator (standard) of known concentration. Further to assess the quality of the reagents and the accuracy of the machine’s performance, BIORAD controls are assayed simultaneously along with the test samples. The samples/calibrators/controls are loaded in specific racks. The reaction carousel of AU 400 contains 80 small quartz cuvettes into which the sample and the reagent are added and mixed. The reaction mixture is maintained at a constant temperature. The absorbency of the reaction mixture is measured at two different wavelengths (bichromatic measurement) which facilitates a far more better accuracy when compared to the monochromatic measurement. Both kinetic and end point reaction tests can be performed in this system. For end point chemistries, the results are calculated from the calibration curve. In case of the kinetic /rate reactions, the Δabs/min of the reaction is multiplied by the factor which is calculated from the calibration. The final results are displayed on the monitor screen.

**Glucose**

Plasma glucose was determined by glucose oxidase/ peroxidase method using IDENTI kit. Glucose is oxidized in presence of glucose oxidase to produce gluconate and
hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with 4- amino antipyrine and catalysed by phenol peroxidase to form a coloured complex quinoneimine, which is bichromatically measured at 520/800 nm. The intensity of the color is directly proportional to the concentration of glucose (mg/dL) in the sample (Trinder, 1969)

Reagents: phosphate buffer 100 mmol/l, pH 7.5, phenol 5 mmol /L: glucose oxidase \( \geq 10 \text{ U/ml} \), peroxidase \( \geq 1 \text{ U/ml} \), 4- amino antipyrine 0.4 mmol/L.

**Urea**

Plasma urea was determined by urease-GLDH enzymatic UV test described by Talke and Schubert, 1965) using DIASYS UREA- FS kit. Urea in the sample is hydrolysed by urease to produce ammonia and carbon dioxide. The ammonia formed, reacts with 2-oxoglutarate to form L- glutamate while NADH is simultaneously oxidized to NAD\(^+\). The resulting decrease in absorbance due to oxidation of NADH was measured bichromatically at 340/380 nm and the rate of decrease in absorbance is proportional to the concentration of urea (mg/dL) in the sample.

Reagents: Tris buffer pH 7.8 150 mmol/ L, adenosine-5' diphosphate 0.75 m mol/L, 2-oxoglutarate 9 mmol/L, NADH 1.3 mmol/L, urease \( \geq 7 \text{ kU/L} \), glutamate dehydrogenase, \( \geq 1 \text{ kU/L} \).

**Creatinine**

Plasma creatinine was measured by alkaline picrate method. (Bartels and Bohmer, 1971) using IDENTI kit. Creatinine reacts with picric acid in alkaline conditions to form a yellow complex. The rate of color formation was measured bichromatically at 520/800 which is proportional to the creatinine concentration (mg/dL) in the sample.

Reagents: Sodium hydroxide 0.4 mmol/L, picric acid 25 mmol/L.

**TRANSAMINASES**

**Glutamate pyruvate transaminase/Alanine aminotransferase** (GPT/ALT).

Plasma GPT activity was measured by IFCC (International Federation for Clinical Chemistry) recommended method (Bergmayer, 1986) using the OLYMPUS system pack. It transfers the amino group from alanine to 2-oxoglutarate to form pyruvate and glutamate. The pyridoxal phosphate in the reaction mixture ensures maximum catalytic activity of
GPT. The pyruvate formed is coupled to lactate dehydrogenase catalysed reaction with NADH to produce lactate and NAD⁺. The decrease in absorbance due to oxidation of NADH was measured bichromatically at 340/660 nm and is proportional to the GPT activity (U/L) in the sample.

\[
\text{GPT/ALT} \quad \begin{align*}
\text{Alanine} + 2\text{-ketoglutarate} & \rightarrow \text{Pyruvate} + \text{glutamate} \\
\text{LDH} & \\
\text{Glutamate} + \text{NADH} + H^+ & \rightarrow \text{Lactate} + \text{NAD}^+
\end{align*}
\]

Reagents: Tris buffer 100 mmol/L pH 7.15 (37°C), L-alanine 500 mmol/L, 2-oxoglutarate 12 mmol/L, LDH > 1.8 kU/L, NADH 0.20 mmol/L, pyridoxal phosphate 0.1 mmol.

**Glutamate oxaloacetate transaminase/Aspartate aminotransferase (GOT/AST)**

Plasma GOT activity was measured by IFCC (International Federation for Clinical Chemistry) recommended method (Bergmayer, 1985) using the OLYMPUS system pack. It catalyses the transamination of aspartate and 2-oxoglutarate forming L-glutamate and oxalacetate. The pyridoxal phosphate in the reaction mixture ensures maximum catalytic activity of GOT. The oxalacetate is reduced to L-malate by malate dehydrogenase (MDH), while NADH is simultaneously oxidized to NAD⁺. The decrease in absorbance due to the consumption of NADH was measured bichromatically at 340/660 nm which is proportional to the GOT activity (U/L) in the sample.

\[
\text{GOT/AST} \quad \begin{align*}
\text{Aspartate} + 2\text{-ketoglutarate} & \rightarrow \text{L- Glutamate} + \text{Oxaloacetate} \\
\text{MDH} & \\
\text{Oxaloacetate} + \text{NADH} + H^+ & \rightarrow \text{L-Malate} + \text{NAD}^+
\end{align*}
\]

Reagents: Tris buffer 80 mmol/L pH 7.65 (37°C), L-aspartate 240 mmol/L, 2-oxoglutarate 12 mmol/L, LDH > 0.9 kU/L, MDH > 0.6 kU/L, NADH 0.20 mmol/L, pyridoxal phosphate 0.1 mmol.
Hepatic and Renal tissue transaminases

Preparation of tissue homogenate for Transaminases.

The frozen tissues (liver and kidney) were thawed, and a 10% tissue homogenate was prepared in ice cold 0.1 M Tris-HCl buffer, pH 7.4 and centrifuged at 12,000 rpm for 45 min at 4°C. Transaminases of cytosolic fraction were assayed by the method described by Reitman and Frankel (1957).

GPT

\[ \alpha\text{-keto glutarate} + L\text{-alanine} \leftrightarrow L\text{-glutamate} + pyruvate \]

GOT

\[ \alpha\text{-keto glutarate} + L\text{-aspartate} \leftrightarrow L\text{-glutamate} + oxaloacetate \rightarrow \text{Pyruvate} \]

The Pyruvate formed gives a brown coloured compound with 2, 4-dinitrophenyl hydrazine (DNPH) which was measured colorimetrically at 520 nm (Reitman and Frankel, 1957).

To 1.0 ml of GOT buffered substrate (0.19 M of DL-aspartic acid, 0.02 M of \(\alpha\)-ketoglutarate in 0.1M disodium and monopotassium phosphate buffer, pH 7.4) or GPT buffered substrate (0.202 M of L-alanine and 0.02 M of \(\alpha\)-ketoglutarate in 0.1M disodium and monopotassium phosphate buffer, pH 7.4), 0.2 ml of enzyme source were added and incubated at 37°C for 60 min. The reaction was arrested by the addition of 1.0 ml of 1 mM 2,4-DNPH in 1.1 N HCl. After 20 min, 10 ml of 0.4 N NaOH was added and left at room temperature for another 10 min. A series of pyruvate standards (10-50 \(\mu\)g) were also treated in a similar manner. The reddish brown colour developed was read at 520 nm against the reagent blank. The enzyme activities were expressed as \(\mu\)g of pyruvate liberated/min/mg protein.

Alkaline phosphatase (ALP)

The activity of ALP in plasma was determined by measuring the rate of conversion of p-nitro-phenyl phosphate (pNPP) to p-nitrophenol (pNP) in the presence of magnesium and zinc ions and 2-amino-2-methyl-1-propanol (AMP) as phosphate acceptor at pH 10.4 (Tietz et al., 1983) using the OLYMPUS system pack. The rate of change in absorbance due to the formation of pNP was measured bichromatically at 410/480 nm and is directly proportional to the ALP activity (U/L) in the sample.
Methodology-50

Reagents: 2-Amino-2-Methyl-1-Propanol (AMP) 0.35 mol/L pH 10.4, p-nitrophenyl phosphate 16 mmol/L, HEDTA (N-(2 hydroxy ethyl) ethylenediamine triacetic acid) 2 mmol/L, zinc sulphate 1 m mol/L, magnesium acetate 2 mmol/L.

**Plasma total protein**

Plasma total protein was estimated by biuret method using the OLYMPUS system pack. Cupric ions in an alkaline solution react with proteins and polypeptides containing at least two peptide bonds to produce a violet colour complex (Weichselbaum, 1946). The intensity of the colour of the complex was measured bichromatically at 540/660 nm which is directly proportional to the concentration of protein (gm/dL) in the sample.

Reagents: sodium hydroxide 200 mmol/L, potassium sodium tartrate 32 mmol/L, copper sulphate 18.8mmol/L, potassium iodide 30 mmol/L.

**Albumin**

Plasma albumin was measured by BCG (bromocresol green) method using the OLYMPUS system pack. A coloured complex is formed when bromocresol green reacts with albumin. The absorbance of the albumin–BCG complex was measured bichromatically at 600/800 nm which is directly proportional to the albumin concentration (gm/dL) in the sample (Doumas et al., 1971).

Reagents: succinate buffer (pH 4.2) 100 mmol/L, bromocresol green 0.2 mmol/L.

**Total Cholesterol (TC)**

Plasma TC was measured by cholesterol oxidase/ peroxidase method using the OLYMPUS system pack. The cholesterol esters in a sample are hydrolysed by cholesterol esterase (CHE). The free cholesterol produced is oxidised by cholesterol oxidase (CHO) to cholestene- 3-one with the simultaneous production of hydrogen peroxide which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidise (POD) to form a chromophore (Allain,1974). The red quinoneimine dye formed was measured bichromatically at 540/600 nm and the intensity of the colour is directly proportional to the concentration (mg/dL) of cholesterol in the sample.

Reagents: phosphate buffer (pH 6.5) 103 mmol/L, 4-aminoantipyrine 0.31 mmol/L, phenol 5.2 mmol/L, cholesterol esterase $ \geq 0.2$ kU/L, cholesterol oxidase $ \geq 0.2$ kU/L, peroxidase $ \geq 10.0$ kU/L.
Triacylglycerols (TAG)

Plasma TAG were measured by glycerol phosphate oxidase (GPO)/ peroxidase method using the OLYMPUS system pack. The procedure is based on a series of coupled enzymatic reactions. The triacylglycerols in the sample are hydrolysed by a combination of microbial lipases to give glycerol and fatty acids. The glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to produce glycerol--3-phosphate. The glycerol--3-phosphate is oxidised by molecular oxygen in the presence of GPO to produce hydrogen peroxide and dihydroxyacetone phosphate. The formed hydrogen peroxide reacts with 4-aminophenazone and N, N-bis(4-sulfobutyl)-3, 5-dimethylaniline, disodium salt in the presence of peroxidase (POD) to produce a chromophore, (Jacobs,1960; Koditschek, 1969; Trinder,1969) which was read bichromatically at 660/800 nm. The resulting increase in absorbance is proportional to the triacylglycerol concentration (mg/dl) of the sample.

Reagents: PIPES buffer (pH 7.5) 50 mmol /L; N, N-bis(4-sulfobutyl)-3, 5-dimethylaniline, disodium salt (MADB) 0.25 mmol/L; 4- aminoantipyrine 0.5mmol/L; ATP 0.5mmol/L; lipases 1.5 kU/L; glycerol kinase 0.5 kU/L; peroxidase 0.98 kU/L; glycerol-3-phosphate oxidase 1.48kU/L.

High density lipoprotein cholesterol (HDL-C)

Plasma HDL-C was estimated by Third generation Direct Homogeneous assay, which does not need any off-line pretreatment or centrifugation steps, using ACCUREX direct HDL-C kit. The HDL-C in the sample is solubilized by a detergent, which is specific for HDL-C. Cholesterol esterase and chromogenic coupler react with this solubilized HDL-C to develop colour. The intensity of the colour was measured bichromatically at 600/700nm which is directly proportional to the concentration of HDL-C (mg/dL) in the sample (Warnick; 2001).

Low density lipoprotein cholesterol (LDL-C)

Plasma LDL-C was estimated by Third generation Direct Homogeneous assay, which does not need any off-line pretreatment or centrifugation steps, using ACCUREX direct LDL-C kit. The reagent consists of a detergent capable of solubilizing LDL specifically. Cholesterol esterase and chromogenic coupler react with this solubilized
LDL-C to develop colour. The intensity of the colour was measured bichromatically at 540/660 nm which is directly proportional to the concentration of LDL-C (mg/dL) (Gotto, 1988).

**Very low density lipoprotein cholesterol (VLDL-C)**

VLDL-C was calculated using the Friedewald (1972) formula which is as follows:

\[ \text{VLDL-C} = \frac{\text{TG}}{5} \]

The values are expressed as mg /dL.

**Antiatherogenic Index (AAI)**

The antiatherogenic index was calculated according to the method of Guido and Joseph, 1992, from total cholesterol and HDL-C as follows:

\[ \text{AAI} = \frac{\text{HDL-C} \times 100}{\text{TC} - \text{HDL-C}} \]

The values are expressed as percentage.

**Calcium**

Plasma calcium was measured by Arsenazo III method with OLYMPUS reagent. The procedure is based on calcium ions (Ca^{2+}) reacting with Arsenazo III (2, 2'-[1, 8-Dihydroxy-3, 6-disulphonaphthylene-2, 7- bisazo]-bisbenzenearsonic acid) to form an intense purple coloured complex which was measured bichromatically at 660/700 nm (Bauer, 1981; Michalylova, 1971). The resulting increase in the absorbance of reaction mixture is directly proportional to calcium concentration (mg/dL) in the sample. Reagents: imidazole (pH 6.9), arsenazo III 0.1-0.2 %, triton X-100.

**Magnesium**

Plasma magnesium was measured with OLYMPUS magnesium reagent which utilises a direct method in which magnesium ions form a coloured complex with xylidyl blue in a strongly basic solution. The colour produced was measured bichromatically at 520/800 nm and is proportional to the magnesium concentration (mg/dL) in the sample. Calcium interference is eliminated by glycoletherdiamine- N, N, N', N'-tetraacetic acid (GEDTA)(Mann, 1957). Reagents: E-Amino-n caproic acid 450 mmol/L, Tris buffer 100mmol/L, glycoletherdiamine-N, N' N', N'tetraacetic acid 0.12mmol/L, xylidyl blue 0.18 mmol/L.
Determination of plasma Insulin

Insulin was assayed by means of insulin RIA kit obtained from BARC, Mumbai, India. The RIA method is based on the competition of unlabelled insulin, in the standard or samples, and radioiodinated insulin (125I insulin), for the limited binding site on insulin specific antibody. At the end of incubation, the antibody bound and free insulin were separated by precipitation with second antibody and free polyethylene glycol (PEG). Radioactivity associated with the bound fraction of sample and standards was measured. Insulin concentration of sample was quantified by measuring the radioactivity associated with the bound fraction of sample and standards (Berson and Yalow, 1968).

Reagents: 1. EDTA-phosphate (0.01M) buffer containing 0.1% bovine albumin pH 7.4; human insulin standard (200 µU/ml); 125I radio iodinated insulin containing 1.5 ng/ml with specific radio activity 100 µCi/µg 125I-insulin; insulin free serum; anti-insulin serum; second antibody (anti rabbit gamma globulin), 25 % (w/w) polyethylene glycol solution.

The flow chart given in table-1 shows the procedure of the assay. At the end of incubation, the bound and free insulin were separated by precipitation with second antibody and PEG. The radioactivity of the bound fraction was counted in a gamma counter.

Calculation: percent of B/Bo was calculated as follows:

\[
\text{Corrected average counts of standard or sample} \times 100
\]

\[
\text{Corrected average counts of zero standard} \times 100
\]

where B is sample or standard binding, Bo is zero standard binding.

A standard curve was plotted on a logit log graph sheet with % of B/Bo on the logit scale and standard insulin concentration µU/ml on the logarithmic scale (Fig-6). The B/Bo % values for the samples were extrapolated from the standard curve as µU insulin /ml. The lowest detection limit of the assay was 2 µU/ml. Inter assay coefficients of variation was 5.6 -7%. Intra assay coefficients of variation were 7-8 %. Control sera showed 92.0% to 108.0% recovery.
After centrifugation decant and count Radioactivity in the precipitate. Calculate and plot the standard curve. Determine patients sample value.

### Insulin assay flow chart

<table>
<thead>
<tr>
<th>Tube No</th>
<th>Assay Buffer (ml)</th>
<th>Insulin Standard (ml)</th>
<th>Serum Sample (ml)</th>
<th>Insulin Free Serum (ml)</th>
<th>Insulin Anti Serum (ml)</th>
<th>I-125 Insulin (ml)</th>
<th>Second antibody (ml)</th>
<th>PEG (ml)</th>
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</tr>
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<td>0.1F</td>
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Vortex and keep all the tubes at room temp. for 20 min. Centrifuge the tubes at 1500g for 20 min.

After centrifugation decant and count Radioactivity in the precipitate. Calculate and plot the standard curve. Determine patients sample value.
Fig. 6. Insulin standard curve

Insulin concentration μIU/ml
**HOMA**

Homeostasis Model Assessment (HOMA) used as an index to measure the degree of insulin resistance (IR), was calculated by the formula:

\[
\text{Fasting plasma insulin (μU/ml) × Fasting glucose (mmol/L) / 22.5}
\]

(Pickavance *et al.*, 1999).

**Oral Glucose Tolerance Test (OGTT)**

At the end of the experimental period an oral GTT was performed on overnight fasted animals of C, C+MO, F and F+MO groups. The fasted animals were challenged with a glucose solution at a dose of 2 g/kg body weight by oral intubation. Blood samples were collected from retro orbital plexus at 0 (before glucose administration), 30, 60 and 120 min after glucose administration.

**Measurement of glucose-insulin index**

The action of insulin on glucose disposal rate was measured using the glucose-insulin index, which is the product of the areas under the curve (AUC) of glucose and insulin during the glucose tolerance test.

**Isolation and purification of tissue total lipid:**

Tissue total lipids were extracted in purified form according to the method of Folch *et al.* (1957) which involved the following two successive operations.

1. Extraction of Lipids: The tissue (250 mg) was homogenized with 2:1 chloroform methanol mixture (v/v) to a final volume of 5 ml. After temperature equilibration and final volume adjustment, the homogenate was filtered through a fat-free filter paper into a glass-stoppered vessel.

2. Washing of Crude Extract: The crude extract was mixed thoroughly with 1 ml of water and the mixture was allowed to separate into two phases, without any interfacial fluff, by centrifugation. The upper phase was removed as completely as possible. The inside wall of the tube was rinsed with about 1.5 ml of 3:48:47 proportions of chloroform, methanol, water mixture, which was allowed to flow gently from a pipette so that the washing fluid
Methodology collects on top of the lower phase without any mixing of the two phases. The tube was rotated gently to ensure mixing of the rinsing fluid with the remaining upper phase and the mixture was removed. This rinsing of the tube wall and the interphase were repeated twice. Finally, the lower phase was diluted to a final volume of 10 ml by the addition of 2:1 chloroform-methanol mixture. This water washing entailed the loss of about 1 % of the tissue lipids and completely removed non-lipid substances. Total cholesterol, triacylglycerols, free fatty acids and phospholipids in this extract were estimated.

**Total cholesterol**

Total cholesterol in the extract was measured by cholesterol oxidase/peroxidise method using IDENTI kit. The cholesterol esters in a sample are hydrolysed by cholesterol esterase (CHE). The free cholesterol produced is oxidised by cholesterol oxidase (CHO) to cholestene-3-one with the simultaneous production of hydrogen peroxide which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidise (POD) to form a chromophore. The red quinoneimine dye formed was measured at 505 nm and the intensity of the colour is directly proportional to the concentration of cholesterol in the sample (Allain et al., 1974).

Reagents: PIPES buffer (pH 7.0) 35 mmol/L; sodium cholate 0.5 mmol/L; Phenol 28 mmol/L; cholesterol esterase >0.2 U/ml; cholesterol oxidase >0.1 U/ml; peroxidase >0.8U/ml; 4-aminoantipyrene 0.5 mmol/L.

Sixty µl of lipid extract was taken in an eppendorf tube and was allowed to evaporate in an incubator. To this 1.0 ml of the cholesterol reagent was added, mixed and incubated at 37° C for 10 min. Cholesterol standard (200 mg %) and water blank were also treated in a similar manner. After incubation, absorbance was read at 505 nm and values are expressed as mg/g tissue.

**Triacylglycerols**

Triacylglycerols in the extract were measured by glycerol phosphate oxidase (GPO)/peroxidase method using IDENTI kit. The procedure is based on a series of coupled enzymatic reactions. The triacylglycerols in the sample are hydrolysed by a combination of microbial lipases to give glycerol and fatty acids. The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK) to produce glycerol.
3-phosphate. The glycerol – 3-phosphate is oxidised by molecular oxygen in the presence of GPO to produce hydrogen peroxide and dihydroxyacetone phosphate. The formed hydrogen peroxide reacts with 4-aminophenazone and 3,5-dichloro-2-hydroxy benzene sulfonate in the presence of peroxidise (POD) to produce a chromophore, which is read at 505 nm. The intensity of colour is proportional to the triglyceride content of the sample. (Trinder, 1969).

**Reagents:** ATP 0.7 mmol/L; magnesium aspartate 4.0 mmol/L; 4-aminoantipyrene 0.4 mmol/L; 3,5-dichloro-2-hydroxybenzene sulfonate(DHBS) 0.8mmol/L; lipases; glycerol kinase 1000U/L; glycerol phosphate oxidase 3500U/L; peroxidase 10,000 U/L.

Sixty µl of the lipid extract was taken in an eppendorf tube and was allowed to evaporate in an incubator. To this 1.0 ml of the triacylglycerol reagent was added, mixed and incubated at 37° C for 10 min. Triglyceride standard (200 mg %) and water blank were also treated in a similar manner. After incubation, absorbance was read at 505 nm and values are expressed as mg/g tissue.

**Free fatty acids**

The free fatty acids form a complex with cupric ions when mixed with copper reagent which is soluble in chloroform. Diethyl dithiocarbamate is used as a colour developer (Duncombe, 1963).

One ml of the lipid extract was placed in centrifuge tubes, then 4 ml of chloroform solution, 2.5 ml of copper reagent (this consists of 9 volumes of aqueous 1 M triethanolamine, 1 volume of 1 N acetic acid and 10 volumes of 6.45 % Cu(NO₃)₂·3HzO) were added. The tubes were stoppered and shaken vigorously for 1 min and centrifuged for a few minutes. The supernatant aqueous phase was removed by suction with a fine hypodermic needle. The surface of the chloroform phase can easily be left clean with only traces of aqueous phase adhering to the wall of the tube. 2.5 ml of chloroform layer was taken into a clean dry tube. Care was taken such that the pipette did not touch the inner wall of the tube, as traces of copper containing aqueous phase might be transferred. Then 0.5 ml of diethyl dithiocarbamate reagent (0.1% (w/v) of sodium diethyl dithiocarbamate in redistilled secondary butanol, stored in refrigerator) was added to the chloroform solution and mixed. The extinction was read at 440 nm against blank in a spectrophotometer. A standard curve was prepared by taking myristic acid as standard fatty acid in the range of 10-100 µM. The values of tissue free fatty acids are expressed as mg/g tissue.
Phospholipids

The total phospholipids were estimated by the method of Connerty et al. (1961). Phospholipids were digested with hydrogen peroxide and sulphuric acid and the liberated inorganic phosphate was estimated by the method of Fiske and Subbarow (1925).

To 0.2 ml of lipid extract 1.0 ml of 10N sulphuric acid was added and heated in an incubator at 150-160°C for 3 h. Then 20 µl of 30% H₂O₂ was added and the solution was returned to the incubator for at least 1 h more to complete combustion and to decompose all the peroxide. The tubes were cooled and the phosphorus was estimated by Fiske Subbarow method. To the above digest 1.0 ml molybdate II and 0.4 ml ANSA reagents were added and the volume was made up to 10 ml with distilled water mixed thoroughly and incubated for 15 min. The blue colour formed was read at 660 nm against blank. The phospholipids content was calculated by multiplication of phosphate value by 25. The results are expressed as mg/gm tissue.

Estimation of inorganic phosphate

Inorganic phosphate (Pi) was estimated by the method of Fiske and Subbarow (1925). Molybdate reagent reacts with Pi to form phosphomolybdic acid. The hexavalent molybdenum of phosphomolybdic acid is reduced by means of 1, 2, 4-aminonaphthosulphonic acid (ANSA) to give a blue color complex having maximum absorbance at 660 nm.

To 0.8 ml of 10% TCA, 0.2 ml of the test sample was added and centrifuged at 5,000 rpm for 15 min. To 0.5 ml of the supernatant, 1.0 ml of molybdate II reagent (2.5% ammonium molybdate in 3N H₂SO₄) was added and mixed. Then 0.4 ml of ANSA (0.25 % ANSA, 15 % NaHSO₃ in 195 ml and 5 ml 20 % Na₂SO₃) was added and made up to 10 ml with distilled water and mixed well. After standing for 5 min, extinction was read at 660 nm against blank. The standard phosphate solution (potassium dihydrogen phosphate standard) in suitable range was also run simultaneously. The results are expressed as µmoles Pi/mg protein.

ASSAY OF KEY ENZYMES IN LIPID METABOLISM

Sample preparation

The frozen tissues were slowly thawed at 4°C. Ten percent tissue homogenate was prepared in ice cold 0.1 M potassium phosphate buffer, pH 8.0 and centrifuged at
12,000 rpm for 45 min at 4°C. The clear supernatant was carefully collected and used for enzyme assays.

**Fatty acid synthase (E.C 2.3.1.85)**

The FAS activity was estimated by measuring the malonyl CoA-dependent oxidation of NADPH at 37°C (Gibson and Hubbard, 1960).

One ml of assay mixture contained 100 μmol of potassium phosphate buffer (pH6.5), 1 μmol of EDTA, 10 μmol of cysteine, 0.3 mg of bovine serum albumin, 0.04 μmol of acetyl-CoA, 0.075 μmol of NADPH and 0.01 ml enzyme source. The reaction was initiated by the addition of 10 μl of 0.075 μM malonyl-CoA and change in the absorbance was measured at 340 nm. A control was run to measure background activity. Activity is expressed as μmol of NADPH utilized/min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹.

**Malic enzyme (E.C 1.1.1.40)**

The malic enzyme activity was estimated by measuring the reduction of NADP (Geer et al., 1980).

The assay system contains 2.0 ml of 100mM triethanoamine HCl buffer (pH7.4), 0.01 ml of 100 mM L-malic acid solution, 0.05 ml of 20 mM NADP, 0.75 ml 20 mM MnCl₂. The reaction was initiated by adding 0.10 ml enzyme source. The increase in absorbance was monitored at 1 min interval for 5 min at 340 nm. The activity is expressed as μmol of NADP reduced /min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹.

**Lipoprotein lipase (E.C 3.1.1.34)**

Lipoprotein lipase activity was estimated by measuring the formation of p-nitrophenol from p-nitrophenyl butyrate (Quinn et al., 1982).

The assay system contained 0.9 ml buffer (100 mM sodium phosphate buffer with 150 mM sodium chloride and 0.5 % triton X-100 pH7.2) and 0.1 ml enzyme source. The reaction was initiated by addition of 0.01 ml of 50 mM p-nitrophenyl butyrate in acetonitrile. The increase in absorbance was monitored at 1 min interval for 5 min at
400nm. The activity was expressed as μmol of p-nitophenol formed /min/mg protein using extinction co-efficient as 1.48 cm⁻¹ mmol⁻¹.

carbohydrate metabolic studies

Preparation of tissue extracts for glycogen estimation

The excised liver and muscle tissues were blotted with Whatman filter paper and weighed. Ten per cent tissue homogenate was made in 5% TCA and centrifuged at 6,000 ×g for 15 min and the supernatant was used for glycogen estimation.

Glycogen

Glycogen released from protein free supernatant of TCA homogenized tissues was precipitated with alcohol. The precipitated glycogen was then hydrolyzed under acidic condition and the liberated glucose was estimated by anthrone method as adapted by Carrol et al., (1956).

To 1.0 ml of protein free supernatant, 5.0 ml of 95 % ethanol was added and allowed to stand over night at 4° C. After precipitation was completed, the sample was centrifuged at 6000 ×g for 15 min. Supernatant was decanted and the residue was dissolved in 1.0 ml of distilled water. Five ml of anthrone reagent (0.4 g of anthrone in 200 ml of H₂SO₄) was added by constant mixing and incubated in a boiling water bath for 15 min with a marble on top to prevent loss of water by evaporation, cooled and the blue-green colour developed was read at 620 nm against reagent blank. A system devoid of glycogen was chosen as blank and a series of standard glucose (20-100 μg) were treated in a similar manner. Values are expressed as mg glucose/g tissue weight.

Protein

Tyrosine and tryptophan present in the proteins reacts with Folin-Ciocalteau reagent in the presence of alkaline copper to give coloured complex with a maximum absorbance at 750 nm (Lowry, 1951).

An aliquot of the test sample was made up to 1.0 ml with distilled water and 5.0 ml of alkaline copper sulphate reagent (50 ml of 2 % Na₂CO₃ in 0.1 M NaOH mixed with 1.0 ml of 0.5% CuSO₄ in 1% sodium potassium tartrate) was added, mixed thoroughly and allowed to stand at room temperature for 10 min. 0.5 ml of 1.0 N Folin-Ciocalteau reagent was added rapidly with immediate mixing and left at room temperature for 30 min. The
intensity of the colour developed was read at 750 nm. The concentration of protein was calculated from the BSA standard curve. A series of standards (40-200 µg) were also treated in a similar manner along with a blank.

**Sample preparation for carbohydrate key metabolic enzymes assay**

The frozen liver, kidney and muscle tissues were slowly thawed at 4°C. Muscle tissue was powdered using liquid nitrogen. Ten per cent tissue homogenate was prepared in ice cold 0.1 M Tris-HCl buffer (pH 7.4) and was centrifuged at 12,000 rpm at 4°C for 45 min. The clear supernatant containing the cytosolic fraction was used for assay of key enzymes of carbohydrate metabolism.

**Glycogen Phosphorlyase (E.C 2.4.1.1)**

Phosphorylase activity was assayed by measuring the rate of liberation of inorganic phosphate from glucose-1-phosphate in the presence of glycogen (Sutherland, 1955).

The assay mixture containing 0.1 ml of 250 µM NaF, 0.05 ml of 20 µM AMP, 0.15 ml of enzyme source and 0.2 ml of water was incubated at 37°C for 10 min. The reaction was started by adding 1.0 ml of solution (pH 6.1) containing 50 µM of glucose-1-phosphate dipotassium salt, 50 µM of NaF and 5.7 mg glycogen. At zero and 10 min of incubation at 37°C, 0.5 ml of reaction mixture withdrawn and added to centrifuge tubes containing 0.5 ml of 6 % TCA, 1.0 ml of water and centrifuged at 4000 rpm for 10 min. Aliquots of the supernatant were taken and the liberated inorganic phosphate was estimated by the method of Fiske and Subbarow, (1925).

**Hexokinase (E.C 2.7.1.1)**

The glucose-6-phosphate produced in the hexokinase reaction is coupled with glucose-6-phosphate dehydrogenase. The reaction is followed by measuring the increase in absorbance at 340 nm due to the NADPH formation. A sufficient excess of glucose-6-phosphate dehydrogenase is provided to minimize the transit time and to avoid inhibition of hexokinase by glucose-6-phosphate (Easterby and Qadri, 1973).

To 1.4 ml of assay buffer (30 mM Tris-HCl buffer, pH 7.5 containing 50 mM glucose and 30 mM MgCl₂), 0.04 ml of enzyme source, 0.2 ml each of 1mM NADP, glucose-6-phosphate dehydrogenase (2 U/ml in 30 mM Tris-HCl pH 7.5) were added and
incubated at 30° C for 5 min. The reaction was initiated by the addition of 0.2 ml of 100mM ATP pH 7.5 and increase in absorbance at 340 nm was measured at 1 min intervals for 5 min. The activity is expressed as μmol of glucose-6-phosphate formed /min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹.

**Phosphofructokinase (E.C 2.7.1.11)**

Fructose 1, 6-bisphosphate formation is coupled with the oxidation of NADH using pyruvate kinase and lactate dehydrogenase and decrease in absorbance at 340 nm was monitored spectrophotometrically (Sadava et al., 1997).

To 1.1 ml of 0.1M Tris-HCl buffer (pH 8.5), 0.1 ml each of 28 mM MgSO₄, 90mM KCl, 14 mM phosphoenol pyruvate, 20 mM ATP- potassium salt, 8 mM NADH, 18U LDH and enzyme source were added, and reaction was initiated by the addition of 0.1 ml of 36 mM fructose 6-phosphate. The decrease in optical density of the system was measured at 1 min interval for 5 min at 340 nm. The activity is expressed as μmol of fructose 1, 6-bisphosphate formed/min/mg protein using extinction co-efficient of NADH as 6.22 cm⁻¹ mmol⁻¹

**Pyruvate kinase (E.C 2.7.1.40)**

Pyruvate kinase activity is measured by coupling with LDH, which transforms pyruvate into lactate and oxidizes NADH into NAD⁺ Oxidation of NADH is followed at 340 nm (Sadava et al., 1997)

To 0.8 ml of 0.1 M Tris-HCl buffer, pH 7.6, 0.2 ml each of 0.1M KCl, 45 mMADP-Na₂, 25 mM MgSO₄, 2.0 mM NADH and 0.1 ml of 10 U LDH and 0.1 ml of enzyme source were added, and the reaction was initiated by adding 0.2 ml of 5 mM PEP. The decrease in optical density of the system was measured at 1min interval for 5min at 340 nm. The activity is expressed as μmol of NADH oxidized /min/mg protein using extinction coefficient of NADH as 6.22 cm⁻¹ mmol⁻¹.

**Fructokinase (E.C 2.7.1.3)**

Fructokinase activity was measured by coupling adenosine diphosphate formation with phosphoenol pyruvate, pyruvate kinase and lactate dehydrogenase, according to the method of Adelman et al. (1966).
A typical reaction mixture of 3 ml contained 2 ml of 150 μM triethanolamine buffer (pH 7.0), 0.1 ml of 3mM KCl, a suitably diluted enzyme preparation, 0.1 ml of 90μM fructose, 10 units of lactate dehydrogenase (in 0.1 ml), 10 units of pyruvate kinase (in 0.1 ml), 0.1ml of 1.5 mM NaF (to inhibit ATPase), 0.5 mg of NADH, 0.1 ml of 60 μM phosphoenol pyruvate, 0.1 ml of 360 μM ATP, and 0.1 ml of 360 μM MgCl₂. The reaction was started by the addition of ATP-Mg, and absorbance change due to oxidation of NADH was measured at 340 nm. An invariably small blank absorbance was found in control cuvettes without fructose; this was subtracted.

Glucose-6-phosphate dehydrogenase (E.C 1.1.1.44)

Glucose-6-phosphate dehydrogenase activity was measured by following the rate of reduction of NADP in the presence of glucose-6-phosphate (Beutler, 1975)

The assay system containing 0.2 ml each of 1.0 M Tris-HCl buffer, pH 8.0 containing 5 mM EDTA, 0.1 M MgCl₂, 2 mM NADP, 0.04 ml of enzyme source (100μg protein) and 1.16 ml of water, was incubated at 25° C for 10 min. After incubation, 0.2 ml of 6 mM glucose-6-phosphate was added and the increase in optical density was measured at 1 min interval for 5 min at 340 nm. The activity is expressed as △mol of NADP reduced /min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹

Glucose-6-phosphatase (E.C 3.1.3.9)

Glucose-6-phosphatase was assayed by incubating the whole homogenate with glucose-6-phosphate and estimating the liberated Pi (King, 1965).

To 0.5 ml of 0.01 M glucose-6-phosphate,0.4 ml of 0.1M citrate buffer, pH 6.5 and 0.1 ml of enzyme source were added and incubated at 37° C for 60 min. The reaction was arrested by the addition of 1 ml of 10 % TCA. For controls, enzyme source was added after arresting the reaction. Aliquots of supernatant obtained after centrifugation at 4000 rpm for 15 min were used for the estimation of liberated inorganic phosphate by the method of Fiske and Subbarow (1925). The activity is expressed as nmol of Pi formed/min/mg protein.

Fructose-1, 6-bisphosphatase (E.C 3.1.3.11)

Fructose-1, 6-bisphosphatase catalyzes the hydrolysis of fructose-1, 6-bisphosphate to fructose-6-phosphate and Pi. Enzyme activity was determined spectrophotometrically by
following the rate of formation of NADPH at 340 nm in the presence of excess phosphoglucone isomerase and glucose-6-phosphate dehydrogenase (Sadava et al., 1997).

To 1.0 ml of 0.05M Tris-HCl buffer (pH 7.5), 0.1 ml each of 10 mM EDTA, 40 mM (NH₄)₂SO₄, 100 mM MgSO₄, 200 mM β-mercaptoethanol, 8 mM NADP, 6 U phosphoglucone isomerase, 2 U glucose-6-phosphate dehydrogenase were added and the reaction was initiated by the addition of 0.2 ml of 1mM fructose-1,6-biphosphate. The increase in absorbance at 340 nm was measured at 1 min intervals for 5 min. The activity was expressed as μ mol of F6P formed/min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹.

INTESTINAL DISACCHARIDASES

Collection of Intestinal mucosa and preparation of enzyme source for disaccharidases

Intestinal mucosa was collected by following the procedure outlined by Ravinder et al. (1989). A long segment 20-25 cm of small intestine between jejunum and caecum leaving about 5 cm on either side was excised. The intestine was flushed with ice-cold saline and cut open longitudinally. The mucosa scraped with a microscopic slide was homogenized in ice cold saline, filtered and used as enzyme source for maltase, sucrase and lactase assays.

Maltase, Sucrase and Lactase

The intestinal disaccharidases were assayed by following the method outlined by Dahlqvist (1968) with slight modification.

After incubation of the enzyme with the appropriate substrate, the reaction was arrested by the addition of Tris buffer and the liberated glucose was assayed by GOD/POD enzymatic method using Identi kit (Trinder, 1969). The reaction was started by mixing 0.1 ml of enzyme source with 0.1 ml of appropriate substrate (0.056 M maltose/sucrose/lactose in 0.1M sodium maleate buffer, pH 6.0). After incubation for 60 min at 37°C, 2.0 ml of 0.5M Tris buffer, pH 7.0 was added to arrest the reaction. To all the tubes 1.0 ml of glucose oxidase reagent was added, mixed well and incubated at 37°C for 10 min and the color intensity was read at 505 nm against reagent blank. A series of standard glucose (20-100μg)
were treated in a similar manner. The activity is expressed in terms of nmol of disaccharide hydrolyzed/min/mg protein.

OXIDATIVE STRESS and ANTIOXIDANT STUDIES

Preparation of tissue extracts for Glutathione and Lipid peroxidation

Immediately after separation of liver, pancreas, heart and testes, a 10% tissue homogenate was prepared in 0.15 M KCl using pestle and mortar at 4°C. The whole homogenate was used for estimation of glutathione and lipid peroxidation.

Lipid Peroxidation

The extent of lipid peroxidation was determined by assaying malondialdehyde (MDA) formation according to method of Utley et al. (1967).

To 1 ml of the liver/pancreas/heart/testes homogenate, 2 ml of 10% TCA and 4 ml of 0.67% TBA were added and heated in a water bath for 30 min. After cooling and centrifuging, the absorbance of the supernatant was read at 535 nm. A reagent blank was prepared using water instead of tissue homogenate. The extent of lipid peroxidation is expressed as nmole MDA formed/mg protein, using a molar extinction co-efficient of MDA as 1.56 x 10^5 M^-1 cm^-1

Reduced Glutathione

Total reduced glutathione content was measured following the method of Ellman's, (1959). This method is based on the development of a yellow colour, when 5,5.-dithio-2-nitro benzoic acid (DTNB) reacts with the compounds containing sulphydryl groups with a maximum absorbance at 412 nm.

Tissue homogenate 0.5 ml was deproteinized with 3.5 ml of 5% TCA and centrifuged. To 0.5 ml of the supernatant, 3.0 ml of 0.2 M phosphate buffer, pH 8.0 and 0.5 ml of freshly prepared Ellman's reagent (19.8 mg DTNB in 100 ml of 0.1% sodium citrate) were added and the yellow colour developed was read at 412 nm. A series of standards (4 - 20 µg) were treated in a similar manner along with a blank. Values are expressed as µg GSH/mg protein.
Sample preparation for antioxidant enzyme assays

Ten per cent tissues homogenate in 0.15 M KCl was prepared using pestle and mortar at 4°C and centrifuged in cold (4°C) at 12,000 rpm for 45 min. The clear supernatant was used for antioxidant enzymes assay.

Glutathione peroxidase (E.C 1.11.1.9)

A known amount of the enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period according to the method of Rotsruck (1973) and the remaining GSH was measured by following the method of Ellman (1959).

To 0.5 ml 0.4M phosphate buffer (pH 7.0) containing 0.4 mM EDTA, 0.2 ml enzyme source, 0.2 ml of 2 mM GSH, 0.1 ml of 0.2 mM H₂O₂ were added and incubated at room temperature for 10 min along with a control tube containing all reagents except the enzyme source. The reaction was arrested by adding 0.5 ml of 10 % TCA, centrifuged at 4000 rpm for 5 min and GSH content in 0.5 ml of supernatant was estimated. The activity is expressed as μg of GSH consumed/min/mg protein.

Glutathione reductase (E.C 1.6.4.2)

Glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) by NADPH to GSH. The activity of the enzyme was measured following the oxidation of NADPH spectrophoto metrically at 340 nm according to the method of Pinto and Bartley (1969).

The assay system contained 0.5 ml of 0.25M potassium phosphate buffer of pH7.4, 0.1 ml of 25 mM EDTA, 0.1 ml of 1mM NADPH, 0.96 ml of distilled water and 0.1ml of enzyme source (150 μg proteins). The reaction was initiated by addition of 0.24 ml of 50 mM GSSG. The decrease in absorbance was recorded at 1 min intervals at 340 nm for 5 min. The activity is expressed as μmol of NADPH oxidized/min/mg protein using extinction co-efficient of NADPH as 6.22 cm⁻¹ mmol⁻¹.

Glutathione-S-transferase (E.C 2.5.1.18)

Glutathione-S-transferase activity was measured by monitoring the increase in the absorbance at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al. (1974).
The assay system contained 1.7 ml of 0.14M sodium phosphate buffer pH 6.5, 0.2 ml of 30 mM GSH and 0.04 ml enzyme source (40 μg protein). The reaction was initiated by adding 0.06 ml of 0.01M CDNB dissolved in 50 % ethanol. The change in absorbance was monitored at 1 min intervals at 340 nm for 5 min and the activity is expressed as mmoles of CDNB-GSH conjugate formed /min/mg protein using extinction co-efficient of CDNB-GSH conjugate as 9.6 cm⁻¹ mmol⁻¹.

**Catalase (E.C 1.11.1.6)**

Catalase catalyses the break down of H₂O₂ to H₂O and O₂ and the rate of decomposition of H₂O₂ was measured spectrophotometrically at 240 nm following the method of Beers and Sizer (1952).

The assay system contained 1.9 ml of 0.05 M sodium phosphate buffer, pH7.0 and 1.0 ml of 0.059 M H₂O₂ in buffer. The reaction was initiated by addition of 0.1 ml enzyme source (45 μg protein). The decrease in absorbance was monitored at 1 min interval for 5 min at 240 nm and activity was expressed as mM of H₂O₂ decomposed/min/mg protein using a molar extinction co-efficient of H₂O₂ as 43.6 cm⁻¹ mol⁻¹.

**Superoxide dismutase (E.C 1.15.1.1)**

Superoxide dismutase activity measurement is based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. A modified procedure described by Marklund and Marklund (1974) and followed by Soon and Tan (2002) was adopted.

The assay system contained 2.1 ml of 50 mM phosphate buffer, pH 7.8 containing 1mM EDTA buffer, 0.02 ml of enzyme source (35 μg protein) and 0.86 ml of distilled water. The reaction was initiated by the addition of 0.02 ml of 10 mM pyrogallol in 0.01N HCl and change in absorbance was monitored at 420 nm for 5 min. The per cent inhibition was calculated on the basis of comparison with a blank assay system. One unit of SOD was defined as that amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50% in standard assay system of 3 ml. The specific activity is expressed as units/min/mg protein.

**Sorbitol dehydrogenase (E.C 1.1.1.14)**

Sorbitol dehydrogenase activity was measured by the method of Asada and Galambos (1963). Sorbitol dehydrogenase catalyses the reduction of fructose to sorbitol in
Methodology

presence of NADH as reducing agent. The activity was measured by monitoring the decrease in the absorbance at 340 nm using fructose as substrate.

The assay system contained 1.6 ml of 107 mM triethanolamine buffer (pH 7.4), 0.1 ml of 0.4 mM NADH. 0.1 ml of enzyme source and incubated at 25° C for 30 min. The reaction was initiated by adding 0.3 ml of 4 M fructose. The decrease in absorbance per min at 340 nm was monitored for 5 min. The activity is calculated using extinction co-efficient of NADH as 6.22 cm⁻¹ mmol⁻¹.

**Calculation of per cent recovery**

Per cent recovery from fructose diet and STZ induced alterations in F+MO was calculated by the following formulae:

\[
\frac{F - F+MO}{F - CON} \times 100
\]

Similarly for D+MO,

\[
\frac{D - D+MO}{D - CON} \times 100
\]

**Histological Examinations**

Immediately after scarification of the rats, the organs/ piece of organs were fixed in 10% formalin. Embedding was done in paraffin. Embedding is the orientation of the tissue in melted paraffin which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut. Melted, filtered paraffin was poured in properly labelled metal blocks of size 1-2 square inches and the piece of tissue was placed in position in to the paraffin. When the tissues were oriented and in place, the paraffin was hardened by cooling. The hardened paraffin blocks with tissues (tissue blocks) were separated from the metal blocks. Sections of 3 microns thickness were cut from the tissue blocks by microtome. Using a heated tissue separator (water bath), the sections were uniformly separated from each other and were carefully placed on a clean glass slide. Sections were properly drained at approximately 60°C for 30 minutes. A small drop of Mayer’s egg albumin was used to attach sections to the glass slides.

Harris’ Hematoxylin and Eosin stain was used for staining the tissue sections following the procedure from the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (Third Edition), American Registry of Pathology
(Luna and Lee) Progressive Stain. Harris' Hematoxylin stain contains hematoxylin crystals in 100% alcohol, ammonium alum and mercuric oxide (as oxidizing agent to hasten the oxidation of hematoxylin to hematein). The Staining was done as follows:

1. Harris' hematoxylin stain was added to sections on the slides and left for 15 minutes.
2. Rinsed in tap water.
3. Differentiated in acid alcohol with 3-10 quick dips.
4. Washed in tap water.
5. Dipped in ammonia water until sections were bright blue.
6. Washed in running tap water.
7. Stained with eosin for 15 seconds.
8. Dehydrated in 95% and absolute alcohol until excess eosin was removed.
9. Dipped in absolute alcohol for 3 min, changed, again dipped in absolute alcohol for another 3 min.
10. Dipped in xylene for 2 min.
11. Mounted in paramount.

Under microscope the sections showed distinct nuclei with light background.

Statistical Analysis

The changes in the studied biochemical parameters of D and F groups were calculated using the corresponding values of C, F+MO and D+MO groups and these values were subsequently used for calculation of per cent recovery. The results are expressed as means ±S.E.M. Data were analyzed for significant differences using Duncan's Multiple Range (DMR) test (Duncan, 1955). (P < 0.05).