Chapter II

Materials and methods

- Chemicals
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- Qualitative analyses of phytochemicals
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Materials and Methods

Chemicals – Alloxan monohydrate, collagenase (Type XI) were procured from Sigma Chemical Co., U.S.A. and all other chemicals were of AR grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Qualigens Fine Chemicals, Mumbai, India, Suvidhanath Laboratories, Vadodara, India and Spectrochem Pvt. Ltd., Mumbai, India. Kits for serum total cholesterol, triglycerides, HDL-cholesterol and GOD-POD kits for blood glucose estimation were purchased from Monozyme India Pvt. Ltd., India and Angstrom Biotech Ltd., India. RIA kits for serum insulin estimation were procured form Board of Radio Isotope and Technology (BRIT), Mumbai, India.

Plant material and extract preparation -

Authentic plant material was procured from local market of Vadodara (Gujarat) and identified at the Botany Department, M. S. University, Baroda. Voucher specimen [Oza 51,51(a)] of *Enicostemma littorale* Blume is present at the Herbarium of Botany Department, M. S. University, Vadodara.

(a) *Enicostemma littorale* aqueous extract: Whole dried plant was used for extract preparation. Plant was cut into small pieces, soaked into water for 2 hours and boiled twice in water for 30 min. Residues were removed by filtration and the combined filtrate was evaporated to obtain a desired concentration (1 g dry plant equivalent extract / ml).

(b) Combined medicinal plants aqueous extract: Whole dried plant of *Enicostemma littorale* Blume, dried rhizome of *Curcuma longa*, dried fruit of *Emblica officinalis* and dried seeds of *Trigonella foenum-graecum* were taken in equal proportions, crushed, soaked and boiled twice for 20 minutes. The filtrates were combined and concentrated to get the concentration as 1g dry plant equivalent extract/ml.

Qualitative analyses for presence of different phytochemicals - Standard qualitative tests were performed to test the presence of various phytochemicals (chemical constituents) like phenolic compounds, tannins, steroids, terpenoids, anthroquinones, flavonoids, alkaloids, glycosides, saponins etc in different extracts and partially purified fractions (Finar, 1989; Ravishankara et al, 2002).

Animals

Diabetic Animals

Male Charles foster rats (body weight 180 – 250g) were used for the study. Alloxan (120 mg/kg body weight, i.p.) was used to induce diabetes. Alloxan monohydrate, a diabetogen, specifically destroys β-cells by generating free radicals and produces diabetes like condition in the rats. After alloxan treatment animals were kept for 15 days to stabilize the diabetic condition and those animals showing blood glucose levels more than 200 mg/dl were considered as diabetic and used for the experiments.

Atherogenic Animals

Male Charles foster rats (body weight 180 – 250g) were used for the study. Animals were made atherogenic by the diet consisting of wheat flour 43.9%, sucrose 20%, casein 20%, groundnut oil 10%, salt mixture 4%, vitamin mixture 1%, cholesterol 1% and cholic acid 0.1%. For control diet cholesterol and cholic acid were replaced by wheat flour (Rathi et.al., 1984).

Diabetic Patients

Information on general clinical history and diet of the patients volunteered were obtained. Diabetics with fasting blood glucose levels between 140 & 230 mg/dl and post prandial blood glucose levels between 230 & 270 mg/dl were selected for treatment with the aqueous extract. Presence of any systemic diseases and body mass index (BMI) less

than 19 were considered as exclusion criteria. Subjects randomized to the treatment groups were to be orally self administered aqueous extract of *E. littorale* in two divided doses, half an hour before meal as 5g of aqueous extract per single dose. The study protocol was approved by the ethics committee of Baroda Medical College, Vadodara, Gujarat, India. All subjects participating in the study gave their written consent.

Assays – During different *in-vivo*, *ex-vivo* and *in-vitro* experiments various glycemic, lipid, antioxidant and toxicity parameters were estimated.

GLYCEMIC PARAMETERS

- **Plasma glucose** (Trinder et.al, 1969)

Glucose is an important source of energy. Glucose concentration fluctuates only in narrow range as insulin and its counter regulatory hormones in the body maintain glucose homeostasis.

Principle: This is an enzymatic method for estimation of serum glucose levels. The aldehyde group of glucose is oxidized by the enzyme glucose oxidase (GOD) in the presence of oxygen (air) to gluconic acid with the liberation of hydrogen peroxide (H_2O_2). Peroxidase splits H_2O_2 into H_2O and active oxygen, which reacts with phenol and a chromogen 4-amino antipyrene to form a pink coloured complex, which can be estimated colorimetrically at 505 nm.

D-Glucose + O_2 + H_2O $\xrightarrow{GOD}$ Gluconic acid + H_2O_2

H_2O_2 + 4 amino antipyrine $\xrightarrow{POD}$ Red coloured quinol + H_2O
+ Phenol

Reagents – commercially available kits contain following reagents

1. Enzymes (glucose oxidase - peroxidase) with chromogen and phenol

2. Glucose standard (100mg/dl) (Range of standard –10 –50 μg)

Procedure

Reagents	Blank	Standard	Test
Enzyme reagent -	1.0 ml	1.0 ml	1.0 ml
Glucose standard -	-	0.01ml	-
Serum -	-	-	0.01 ml

All the tubes were incubated for 15min at RT or for 10min at 37 C and then absorbance was read at 505 nm against blank.

Calculation – Calculation was done according to the slope calculated from the standard graph.

Units – mg/dl

- **Glycosylated haemoglobin** (Parker et.al, 1981)

Protein can universally bind non-enzymatically with glucose or other sugars present in the vicinity. The degree of glycation is directly proportional to the concentration of the sugar present in the surrounding medium. RBC has longer life span (120 days) as compared to other proteins like albumin (4 days). Therefore estimation of Glycosylated Haemoglobin (Gly Hb) gives an accurate reflection of mean plasma glucose concentration over this period.

Principle: This method is specific for ketoamine-linked hexoses which form furfural when heated under strong acidic condition. This furfural reacts with 2-thiobarbituric acid (TBA) and produces a bright yellow coloured compound, which can be estimated colorimetrically at 443 nm.

Reagents

1. 0.5 M Oxalic acid (Stable for 2 weeks at RT)
2. 0.72g % Thiobarbituric Acid (TBA)(pH 6.0, stable for 1 weeks at RT)
3. Saline (0.9g % NaCl)
4. 40 % Trichloroacetic acid (TCA)
5. Drabkin's reagent (pH 9.1) – 0.2g Potassium Ferricyanide [$K_3Fe(CN)_6$], 0.05g Potassium Cyanide (KCN), 0.14g Potassium dihydrogen phosphate (KH_2PO_4) in one litre of distilled water.
6. Hb standard solution (60 mg/dl or 65 mg/dl) was procured from market.
7. Fructose standard 0.2 μM

(Range of the standard – 0.02 - 0.12 μM)

Procedure

Haemolysate preparation –RBC sediments were washed three times with 0.9% saline. Then packed cells were lysed by adding equal amount of distilled water and ¼ part CCl_4. Mixed well and centrifuged at 3000 rpm for 10 min. Haemoglobin (Hb) was estimated with Drabkin's reagent and adjusted to 10g Hb/dl.

Reagents	**Control**	**Test**
haemolysate	-	1ml
D/W	1ml	-
oxalic acid	0.5 ml	0.5ml
	Keep in boiling waterbath for 1 hr	
chilled TCA	1ml	1ml
	Centrifuge the tubes at 2000 rpm for 15min	
Supernatant	1ml	1ml
TBA	0.1ml	0.1ml

Incubated in water bath at 40°C for 30 min and read the absorbance at 443nm against the sample blank.

Calculation – Calculation was done according to the slope calculated from the standard graph.

Units – % of total Hb

- ***Invitro* insulin release from isolated rat pancreatic islets**

Insulin is the major hormone, which regulates glucose metabolism. Glucose is the primary stimulus for the insulin secretion and this process involves various other downstream signaling molecules too. Insulin is secreted from pancreatic β-cells in response to increase in glucose concentration in the surrounding medium. This insulin can be measured by radioimmuno assay (RIA).

Reagents –

1. Hanks Balanced Salt Solution (HBSS) – (0.15 M NaCl, 0.005 M KCl, 0.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 5.5 mM glucose, pH 7.4)
8. Hank's buffer – (HBSS with 0.11 g% $NaHCO_3$ and 0.5 g% BSA, pH 7.4)
9. Krebs ringer buffer (KRB) – (119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2mM KH_2PO_4, 1.2 mM $MgCl_2.7H_2O$, pH 7.4)
10. Washing buffer – (KRB with 0.2 g % $NaHCO_3$, 0.02 g% BSA and 0.27 g% glucose, pH 7.4)
11. Glucose KRB (incubation buffer) – (KRB with 0.2 g% $NaHCO_3$, 0.38 g% HEPES, 0.1 g% BSA with 11.1 mM glucose, pH 7.4)

Sample preparation - **Islet isolation from rat pancreas (Xia et al., 1993)**

Pancreatic islets were isolated from the pancreas of adult rats by collagenase digestion. In brief the pancreas was dissected out, washed in HBSS and cut in small pieces. Before digestion all HBSS was removed and then 5 mg of collagenase (Type XI) was added with 4ml of Hank's buffer. Digestion was carried out at 37°C in shaking waterbath. Digestion was stopped after 10–15 min by adding 30 ml of ice-cold Krebs-Ringer bicarbonate (KRB) washing buffer containing 0.02 % BSA. Tubes were allowed to stand in ice for 15 min then upper layers of solution were discarded leaving 3 – 4ml of solution in the bottom. Remaining solution was washed two times with the same buffer and islets were picked up manually from this solution under stereomicroscope and divided in batches of 10 islets for all the experiments.

Procedure

Glucose-KRB buffer containing either 5.5 mM or 11.1 mM glucose was used as incubation buffer. Islets were picked up in a batch of 10 islets in each tube for all the experiments. Further, in all the *in-vitro* experiments islets were preincubated with 0.5 ml incubation buffer containing 11.1 mM glucose alone or in combination with various concentrations of the plant extract or various fractions at 37°C for 30 min in a shaking waterbath. After the preincubation was over, buffer was removed and all the additions were done in similar fashion and incubated under similar conditions. Aliquots of 50 µl /100 µl were removed form static incubation mixture at 0, 10 and 60 min, and were frozen immediately until insulin assay was performed by RIA.

- **Insulin estimation (RIA method)**

Insulin is a polypeptide hormone, synthesized by pancreatic β-cells. It regulates glucose metabolism and maintain glucose homeostasis in the body.

Principle: Radio immuno assay (RIA) is based upon the competition between unlabelled insulin in the standard or serum sample and radiolabelled ($I-^{125}$) insulin for the limited binding sites on a specific antibody. At the end of the incubation, the antibody bound and free insulin are separated by the second antibody-polyethylene glycol (PEG) aided separation method. Insulin concentration ($I-^{125}$) of the sample is quantitated by measuring the radioactivity (counts/minute, CPM) associated with bound fraction of sample and standards with help of gamma counter.

Reagents

1. Radiolabelled ($I-^{125}$) insulin
2. Insulin free serum
3. Primary antibodies
4. Secondary antibodies
5. Polyethylene glycol (PEG)
6. Insulin standard

(Standard range -12.5 - 200 μU)

All these reagents are available with RIA kit in the form of dry powder. These were reconstituted with double distilled water or assay buffer as described in the kit broacher.

Procedure

Reagents	**Total counts**	**Nonspecific binding**	**Zero binding**	**Standard**	**Test**
Assay buffer	-	0.4 ml	0.3ml	0.2ml	0.3ml
Insulin standard	-	-	-	0.1ml	-
Serum sample	-	-	-	-	0.1ml
Insulin free serum	-	0.1ml	0.1ml	0.1ml	-

Primary antibody	-	-	0.1ml	0.1ml	0.1ml
Mix gently and incubate all the tube at 2-4°C overnight					
(I^{-125}) insulin	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
Mix gently and incubate all the tube at RT for 3hrs.					
Secondary antibody	-	0.1ml	0.1ml	0.1ml	0.1ml
PEG	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Vortex and keep all the tubes at RT for 20 minutes then centrifuge the tubes at 1500g for 20minutes.					

Calculation

T = total counts of 100µl Insulin I^{125} only.

B_0 = CPM of bound with Insulin I^{125} in absence of serum insulin or insulin standard (zero binding)

B = CPM of bound with Insulin I^{125} in presence of serum insulin or insulin standard.

% B/T and %B/ B_0 was calculated for insulin standards and serum samples. A logit-log graph was plotted against %B/ B_0 and concentration of insulin standards. Serum insulin concentration was calculated from this graph.

Unit - µU of insulin/ml of serum or ng insulin/ 10 islets.

LIPID PARAMETERS –

- **Hypercholesterolemic Diet** (Rathi et.al., 1984)

Wheat flour	43.9 %
Sucrose	20.0 %
Casein	20.0 %
Ground nut oil	10.0 %
Salt mixture	4.0 %
Vitamin mixture	1.0 %
Cholesterol	1.0 %

Cholic acid 0.1 %

For control diet cholesterol and cholic acid were replaced by wheat flour.

♦ **Serum Total Cholesterol & HDL cholesterol (Kit Method)** [Allain et.al., 1974]

Serum cholesterol levels is an indicator of liver function, biliary function, intestinal absorption, propensity towards coronary artery disease & adrenal disease. Decreased HDL cholesterol levels associated with increased risk of developing coronary artery disease and other atherosclerotic complications.

Principle

$$\text{Cholesterol ester} \xrightarrow[+\ H_2O]{\text{CHE}} \text{Cholesterol} + \text{Free fatty acids}$$

$$\text{Cholesterol} \xrightarrow[+\ O_2]{\text{CHOD}} \text{Cholest} - 4 \text{ ene } 3 - \text{one} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4- Aminoantipyrine} \xrightarrow{\text{POD}} \text{Red Quinoneimine Complex} + H_2O$$

The coloured complex is read at 505 nm against blank.

HDL Cholesterol: On addition of the precipitating reagent, which contains phosphotungstic acid, to the serum, followed by centrifugation, HDL fraction remains in the supernatant while the other lipoproteins precipitate out.

Reagents

Reagent 1	Enzyme Reagent
Reagent 2	Precipitating Reagent
Reagent 3	Cholesterol standard (200 mg/dl)

Procedure

Pipette into a centrifuge tube:

Serum/Plasma : 0.2 ml

Precipitating reagent : 0.3 ml

Mix and allow to stand for 5 minutes at R.T. Centrifuge at 3000 rpm for 10 minutes to get a clear supernatant.

Pipette into 4 test tubes labeled Blank (B), standard (S), Total Cholesterol (T_C) and HDL cholesterol (T_H) as shown below:

	B	S	T_C	T_H
Enzyme Reagent (1)	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard (3)	-	0.01 ml	-	-
Specimen	-	-	0.01 ml	-
Supernatant (from Step 1)	-	-	-	0.1 ml
D/W	0.1 ml	0.1 ml	0.1 ml	-

Mix well and incubate for 10 minutes at R.T. Read the absorbances of S, T_C, T_H against B at 505 nm.

Calculation

$$\text{Total cholesterol (in mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 200$$

$$\text{HDL Cholesterol (in mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 50$$

♦ **Serum Triglycerides (kit method)** (Fossati and Prencipe, 1982)

Principle:

The TG is determined after enzymatic hydrolysis with lipases. Peroxidase catalyses the conversion of hydrogen peroxide, 4-Aminoantipyrine and ESPAS to a colored quinoneimine complex measurable at 546 nm.

Lipases

TG --------⇒ glycerol + fatty acids

GK

Glycerol + ATP ----------⇒ glycerol 3 phosphate (G 3 P)

GPO

G 3 P + O_2 ----------⇒ H_2O_2 + dihydroxy acetone phosphate

POD

H_2O_2 + 4 amino antipyrine ----------⇒ purple quinoneimine complex + H_2O

+ESPAS (N-ethyl N-sulfoprophyl N-anisidene)

+HCl

Reagents

Reagent 1 Enzyme reagent (Enzyme chromogen)

Reagent 2 Triglycerides standard (200 mg / dl)

Procedure:

Enzyme	Blank	Standard	Test
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Standard	--	0.01 ml	--
Sample	--	--	0.01 ml
Mix well incubate for 10 min. at 37° C for 15 min at R.T.			

Read 546 nm. Final color is brownish purple and stable for 30 min at R.T.

Calculation: Serum triglycerides (mg/dl) = $\frac{\text{Abs. Of Test}}{\text{Abs. Of Std.}} \times \text{Conc. of Std}$

- **LDL cholesterol = (Total Cholesterol – HDL cholesterol – TG/5)**
- **VLDL = TG/5**
- **Tissue Lipid Extraction** (Folch et.al., 1957)

20 % tissue was homogenized in Chloroform : Methanol (2:1) mixture, which was prepared fresh.

- To 1 ml aliquot from above, add 4 ml Chloroform : Methanol (2:1) mixture. Vortex vigorously and centrifuge at 3000 rpm for 10 minutes.
- Remove lower phase by a glass syringe to another tube, marked as A.
- Again add 3 ml Chloroform : Methanol (2:1) mixture, and vortex vigorously and centrifuge at 3000 rpm for 10 minutes.
- Remove lower phase by a glass syringe to the tube marked A.
- Add 0.2 ml magnesium chloride (0.017 %) per ml of extract of tube A (to remove last traces of proteins) and vortex vigorously and centrifuge at 3000 rpm for 10 minutes.
- Take the lower phase with glass syringe, which contains the extracted lipids and keep it for evaporation.
- Dissolve the precipitate in Ethanol and used for further assay.

- **Tissue Cholesterol and Triglycerides**

Lipids were extracted as above and cholesterol and triglycerides were estimated as described in the KIT method.

- **Liver HMG CoA reductase Activity** (Rao and Ramakrishnan, 1975)

HMG CoA reductase is required for the conversion of HMG-CoA to Mevalonate. This is an indirect method to measure HMG CoA reductase activity where HMG-CoA /Mevalonate ratio is taken. This ratio is inversely proportional to the HMG CoA reductase activity.

Principle: HMG CoA was determined by it's reaction with hydroxylamine at alkaline pH and subsequent colorimetric measurement of the resulting hydroxiamic acid by formation of complexes with ferric salts. Because mevalonate interferes in this estimation at acidic

or neutral pH., alkaline hydroxamine was used to estimate specially HMG-CoA only. Read at 540 nm .

Mevalonate was estimated by reaction with the same reagent, but at pH 2.1. At this pH, the lactone form of mavalonate readily reacts with hydroxylamine to form the hydroxamate.

Reagents:

1. Saline arsenate solution (1g/liter) in physiological saline.
2. Dilute perchloric acid (50 ml / liter of D/W).
3. Hydroxylamine hydrochloride reagent (2mol/lit.)

For Mevalonate : Mix equal volumes of hydroxylamine hydrochloride reagent and water freshly before use (pH 2.1)

For HMG-CoA : Mix equal volumes of hydroxylamine hydrochloride reagent and sodium hydroxide solution (4.5 mol/lit) freshly before use.

4. $FeCl_3$ reagent: Dissolve 5.2 gm of trichloroacetic acid (TCA) + 10 gm of $FeCl_3$ in 50 ml of 0.65 mol/liter HCl and dilute to 100 ml with the latter.

Procedure:

10% homogenate (Saline arsenate solution) + equal vol. of perchloric acid diluted (50 ml/liter) and after 5 min, centrifuged for 10 min at 2000 rpm .

	HMG CoA	Mevalonate
Supernatant	1.0 ml	1.0 ml
Hydroxylamine reagent	0.5 ml (alkaline pH)	0.5 ml (acidic pH)

Incubate for 10 min. at RT and read absorbance at 540 nm

Calculation: Ratio of O.D. = $\frac{\text{HMG-CoA}}{\text{Mevalonate}}$

ANTIOXIDANT PARAMETERS

- **DPPH (1,1-Diphenylpicryl-2-hydrazyl) free radical scavenging** (Vani et.al., 1997)

Crude aqueous extract were tested *in-vitro* for DPPH (1,1-Diphenylpicryl-2-hydrazyl) free radical scavenging activity. 3 mM DPPH stock solution was prepared in methanol. All the DPPH (~90%) remains as radical in the solution and gives dark blue-purple colour when dissolved in methanol. In presence of any free radical scavenger, DPPH radical get reduced in to DPPH, which is a colourless compound. So, the DPPH radical scavenging can be measured by reduction in dark blue-purple colour at 516nm. DPPH stock solution (80-90µl) was added to 2.9ml of methanol and initial absorbance was adjusted to 0.9 at 516nm. Various aliquots of aqueous extract were added to this solution and change in the absorbance was recorded after 15 minutes.

- **Superoxide radical scavenging** (McCord and Fridovich, 1969)

Superoxide radical scavenging activity was determined by the nitroblue tetrazolium reduction method. Various concentrations of the extracts were added into the reaction mixture containing 0.1 M EDTA, 0.0015% sodium cyanide (200 µl), 1.5 mM NBT (100 µl), 0.12 mM riboflavin (50 µl) and 0.2 M phosphate buffer (pH 7.8) in a final volume of 3 ml. The tubes were then illuminated uniformly under an incandescent lamp for 15 min and the OD at 530 nm was taken. The percentage inhibition was calculated using the following formula:

$$\frac{\text{OD of control tube} - \text{OD of treated tube}}{\text{OD of control tube}} \times 100$$

- **Hydroxyl radical scavenging** (Elizabeth and Rao, 1990)

Hydroxyl radical generated from a Fe^{2+}/ascorbate/H_2O_2 system degrades deoxyribose producing TBARS. The efficacy of the extracts to inhibit TBARS was

evaluated. Various concentrations of the extracts were added into the reaction mixture containing 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, 20 mM KH_2PO_4 – KOH buffer (pH 7.4) in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37 °C. The TBARS formed was measured by the method Beuge and Aust (LPO) as mentioned below and the percentage inhibition was calculated using the following formula:

$$\frac{\text{OD of control tube} - \text{OD of treated tube}}{\text{OD of control tube}} \times 100$$

- **Nitric oxide radical scavenging** (Sreejayan and Rao, 1997)

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide with interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions.

Various concentrations of the extracts were added into the reaction mixture containing 10 mM sodium nitroprusside in PBS and incubated at R.T. for 150 minutes. After incubation period, 0.5 ml of Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm the percentage inhibition was calculated using the following formula:

$$\frac{\text{OD of control tube} - \text{OD of treated tube}}{\text{OD of control tube}} \times 100$$

- ***Ex-vivo* antioxidant experiments in isolated rat pancreatic islets**

Islets were isolated as mentioned earlier and divided into the following groups, each having 50 islets /0.5 ml 2.8 mM glucose KRB in eppendorfs. Incubation with

aqueous extracts of *E. littorale* (EL) and herbal combination (ALL) at concentrations of 5 and 20 µg was carried out in a shaking water bath at 37°C and the alloxan (A) concentration used was 0.5 mM.

1) Control	8) A + EL-20
2) Alloxan	9) ALL-5
3) EL-5	10) ALL-20
4) EL-20	11) ALL-5 + A
5) EL-5 + A	12) ALL-20 + A
6) EL-20 + A	13) A + ALL-5
7) A + EL-5	14) A + ALL-20

Alloxan was incubated for 10 minutes duration (pre- and post-incubation) and the extracts were for 30 minutes (pre- and post-incubation). After the incubation, the tubes were kept on ice.

For the GSH and LPO, the islets were sonicated whereas NO was estimated in the supernatant by the methods described in this chapter.

Sonication : 20 blasts for 20 duty cycles for 2 minutes.

- **Reduced glutathione (GSH)** (Beutler et.al., 1963)

Glutathione (g-glutamylcysteinylglycine, GSH) is highly concentrated intracellular antioxidants, accounts for 90% intracellular non-protein thiol content. Highest concentration of GSH is present in liver. Glutathione exists in two forms: reduced glutathione (GSH) and the oxidized form glutathione disulfide (GSSG). The GSSG/GSH ratio may be a sensitive indicator of oxidative stress (Parris MK 1997). Glutathione status is homeostatically controlled both inside the cell and outside, being continually self-adjusting with respect to the balance between GSH synthesis (by GSH

synthetase enzymes), its recycling from GSSG (by GSH reductase), and its utilization (by peroxidases, transferases, transhydrogenases, and transpeptidases).

Principle: Red cell contains GSH as a non – protein sulfhydryl compounds. 5-5' Dithiobis (2–nitrobenzoic) acid (DTNB) is a disulfide compound, which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which can be read at 412 nm.

Reagents

1. Precipitating (Ppting) reagent: glacial metaphosphoric acid (1.67 g), EDTA (0.20 g), NaCl (30 g) and total volume was made up to 100 ml with distilled water (D/W)
2. Na_2HPO_4. (0.3M)
3. DTNB: 40 mg DTNB dissolved in 100 ml 1% sodium citrate.
4. PBS (0.1M, pH 7.4).
5. Standard GSH solution (2 mM GSH).

(Standard range 10-100 μg)

Sample preparation – Anticoagulated whole blood or 10% tissue homogenate in Phosphate buffered saline (PBS) (0.1M, pH 7.4).

Procedure:

Reagents	**blank**	**Blood**	**Tissue**
Sample	-	0.1ml	1.0ml
D/W	1.0ml	0.9ml	-
Ppting reagent	1.5ml	1.5ml	1.5ml
Keep the tubes for five minutes then centrifuge at 3000 rpm for 15 min			
Supernatant	0.5ml	0.5ml	0.5ml
Na_2HPO_4 solution	2.0ml	2.0ml	2.0ml
DTNB	0.25ml	0.25ml	0.25ml

Read absorbance at 412 nm within a minute after adding DTNB

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit –GSH mg % (blood) and mg/g of tissue

- **Protection of GSH Auto-oxidation**

Liver homogenate (10%) was prepared in PBS and incubated with various concentrations of extract. 250 µl of incubation mixture at different time intervals, were transferred to a tube containing 0.5 ml of precipitating reagent. The sample was centrifuged at 3000 rpm for 10 minutes. Supernatant was taken and mixed with 2.5 ml of Na_2HPO_4 solution and colour was developed by adding 100 µl DTNB (0.01 %). GSH levels were determined as described above (Beutler et.al., 1963)

- **Lipid peroxidation levels (LPO)** (Beuge and Aust, 1978)

Polyunsaturated fatty acids (PUFA) are vulnerable to oxidative damage. ROS generated during various biochemical reactions initiates a chain reaction by abstracting H atom from PUFA and forms primary stable peroxyl radical and lipid hydroperoxide. Lipid peroxides generate secondary stable products lipid aldehydes, malondialdehydes, 4-OH alkenals, alkanals, 2-alkanls and 2-4 alkanals etc. Compared to free radicals these aldehydes are stable and can diffuse within or even escape from the cell and attack targets far from the site of their generation. LPO is a good indicator of oxidative damage to the tissues, especially the membrane lipids.

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives

thiobarbituric reactive substance (TBARS). TBARS gives a characteristic pink color that can be measured colorimetrically at 532 nm.

Reagent

1. TBA reagent: TBA (100mg), EDTA (46mg), 20%TCA (10ml), 2.5 N HCl (5 ml) total volume was made up to 20ml with D/W.
2. 0.1M Phosphate buffered saline (PBS) - pH 7.4:
3. Drabkin's reagent: described earlier
4. 10mM Tetra methoxy propen (TMP) for standard solution.

(Standard range 1-10 nmoles)

Sample Preparation

Blood – RBC sediments were prepared and washed thrice with phosphate buffered saline (PBS, 0.1M). Haemolysate of Hb concentration 2.65g/dl (PCV 7.5) was prepared

Tissue - 10% tissue homogenate was prepared in PBS.

Procedure:

Reagents	Blank	Blood	Tissue
Sample	-	1.0ml	1.0ml
D/w	1.0ml	-	-
TBA reagent	1.0ml	1.0ml	1.0ml

Keep in boiling waterbath for 20min, cool the tubes, centrifuge at 3000 rpm for 15min and read the absorbance at 532nm.

Calculation – Calculation was done according to the slope calculated from the standard graph.

Units – nmoles of MDA formed/ g Hb or g tissue

- **Inhibition of lipid peroxide formation in liver homogenate**

Liver homogenate (10%) was prepared in PBS and incubated with various concentrations of extract. The incubation mixture contained liver homogenate (0.5 ml), ferric ion (0.1 mM), ADP (1.7 mM), ascorbic acid (0.5 mM) and the final volume was

made upto 1.5 ml with KCl (0.15 M). Mixture was incubated for 20 minutes at 37 °C in presence and absence of extracts. After incubation TBARS was determined as mentioned above (Beuge and Aust, 1978)

- **Superoxide dismutase (SOD)** (Kakkar et.al, 1984)

SOD is present in all the aerobic organisms. It provides an essential defense against the potential toxicity of molecular oxygen. SOD helps to prevent tissue damage by superoxide radicals ($O_2^{\bullet -}$). It is a metalloenzyme, which catalyzes dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen (O_2).

$$2\,O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Two isoenzymes i.e. Cu-Zn SOD (cytosol) and Mn-SOD (mitochondria) are present in eukaryotic animals, which are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments, but how it is communicated at molecular level is unknown.

Reagents

1. 0.89%KCl
2. PBS (0.1M, pH 7.4)
3. Sodium pyrophosphate (pH 8.3) - 0.052 mM
4. PMS - 186 µM
5. NBT - 300 µM
6. NADH - 780 µM
7. D/W

Sample Preparation

Erythrocyte - Erythrocyte sediment were washed thrice with PBS. Haemolysate was prepared with Hb concentration 1-2g Hb/dl.

Tissue - 4% tissue homogenate in 0.89% KCl was prepared and centrifuged at 3000 rpm for 15 min. Supernatant was used for SOD estimation.

Principle: Mixtures of NADH and phenazine methosulfate (PMS) generate to superoxide under non – acidic conditions via the univalent oxidation of reduced PMS. NBT serve as a detector molecule for superoxide through reduction in to a stable, blue coloured formazone product, which can be measured at 560nm.

Procedure

Reagents	Test	Control
Sodium pyrophosphate buffer	- 1.2 ml	1.2 ml
PMS	- 0.1 ml	0.1 ml
NBT	- 0.3 ml	0.3 ml
Diluted enzyme	- 0.01ml /0.02ml	---
D / W	- 1.2 ml / 1.18 ml	1.2 ml
NADH	- 0.2ml	0.2 ml

All tubes were incubated for 90 seconds at 37°C then reaction was terminated by adding 1.0 ml glacial acetic acid and shaken vigorously. Reduced NBT was extracted in 4 ml of n-Butanol. Tubes were centrifuged and absorbance was read at 560 nm against butanol blank.

Calculation –

$$\text{SOD (U/g Hb)} = \frac{OD^{control} - OD^{test}}{OD^{control}} \times \frac{100}{0.01} \times \frac{60}{90} \times \frac{1}{\text{Hb conc}^{n}\text{ (g/dl) or tissue wt(g/dl)}}$$

Unit – One unit of SOD is defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

- **Catalase (CAT)** (Hugo, 1987)

Catalase (CAT) is a heme protein contains four ferriprotoporphyrin groups per molecules. This enzyme is also found in all aerobic organisms and is important in removal of H_2O_2 generated in peroxisomes (microbodies). Highest CAT activity is found in liver and kidney and lowest in connective tissue. In tissue it is mainly present bound to the membranes of mitochondria and peroxisomes, whereas it exist in soluble state in erythrocyte (Hugo, 1987).

Principle: Catalase is a heme-containing enzyme, which catalyzes dismutation of hydrogen peroxide in to water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

Reagents

1. Phosphate Buffer: (50 mM, pH 7.0)
   a) 1.70 g KH_2PO_4 in 200 ml D/W.
   b) 2.13 g Na_2HPO_4 in 300 ml D/W.

Mix both and adjust pH to 7.0

2. Hydrogen peroxide (30 mM)
3. Absolute alcohol (ethanol)
4. Triton X-100 (10 %)
5. PBS (0.1M, pH 7.4)

Sample Preparation

Blood is centrifuged at 3000 rpm for 10 minutes and plasma separated out. Erythrocyte sediment was washed thrice with PBS. Haemolysate was prepared and Hb

concentration adjusted to 5g/dl.. Just before assay, 0.02 ml haemolysate was diluted up to 10 ml with phosphate buffer. This diluted solution was used for enzyme analysis.

Tissue sample was prepared as 10 % tissue homogenate in PBS, centrifuged at 1000 rpm to remove cell debris. Supernatant was used for enzyme analysis. 10 µl ethanol was added to 1.0 ml supernatant and these tubes incubated in ice water bath for 30 minutes. Just before the assay 10 µl of Triton X-100 and 9 ml of phosphate buffer were added.

Procedure:

Reagents	**Blank for blood**	**Blood**	**Blank for tissue**	**Tissue**
Sample	2.ml	2 ml	0.2ml	0.2ml
Phosphate buffer	1.0ml	-	2.8ml	1.8ml
H_2O_2	-	1.0ml	-	1.0ml

Immediately after adding H_2O_2 decrease in the absorbance was recorded at every 5 seconds interval for 15 seconds at 240nm.

Calculation –

$$\text{CAT activity} = \frac{2.303}{\Delta t} \times \log \frac{E1}{E2} \times \text{dilution factor}$$

Unit - mmoles of H_2O_2 decomposed /sec/g Hb or g tissue

- **Glutathione Peroxidase (GPx)** (Paglia and Valentine, 1976)

Glutathione peroxidase catalyzes the reduction of various organic hydroperoxides, as well as hydrogen peroxide, with glutathione as hydrogen donor.

Reagents

1. Precipitating Reagent, DTNB – Prepared similarly as that of GSH assay.
2. GSH (2mM)- Dissolve 3.07mg GSH in 5 ml D/W.
3. Phosphate buffer 0.4 M (pH 7.0) –

a) Dissolve 5.44g KH_2PO_4 in 100 ml D/W.

b) Dissolve 6.96g K_2HPO_4 in 100 ml D/W. Mix the two together.

4. Sodium azide (10 mM) for tissue / Sodium cyanide (15 mM) for haemolysate
5. H_2O_2 (10 mM) – Dissolve 10 µl 30% H_2O_2 in 8.8 ml phosphate buffer (3).
6. Na_2HPO_4 (0.4 M)

Procedure

Prepare 10% homogenate in PBS (pH 7.4) OR haemolysate as mentioned in earlier methods. Centrifuge, use supernatant (tissue) as Enzyme source. Proceed as follows:

0.4 M phosphate buffer	-	0.10 ml
2 mM GSH	-	0.10 ml
10 mM NaN_3/KCN	-	0.10 ml
Enzyme	-	0.01 ml
D/W	-	0.09 ml
Incubate at 37°C for 10 minutes. ***Now add***, 10 mM H_2O_2	-	0.10 ml
Incubate at 37°C for 3 minutes. ***Add***, Precipitating Reagent	-	0.40 ml

Wait for 5 minutes and centrifuge. ***Use supernatent*** and proceed as follows:

Supernatent	-	0.6 ml
0.4 M Na_2HPO_4	-	0.6 ml
DTNB	-	0.03 ml

Run control tube without Enzyme.

Run blank tube without GSH.

Take reading at **412** nm.

Units – µmoles of GSH utilized / gHb/min OR mmoles of GHS utilized/mg protein/min

TOXICITY PARAMETERS

- **Glutamate pyruvate trans-aminase (GPT)** (Reitman and Frankel, 1957)

Liver tissue is rich in GPT. Normally serum GPT levels are low but in case of extensive liver damage this enzyme is liberated into the serum and hence a specific indicator of liver damage.

Principle – GPT transfers amino group from alanine to α-keto gluterate (α-KG) and convert it in to pyruvate. This pyruvate gives dark brown colour by reacting with 2,4-dinitro phenyl hydrazine (DNPH), which can be measured at 546 nm.

$$\alpha\text{-KG} + \text{Alanine} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{Glutamate}$$

$$\text{Pyruvate} + \text{DNPH} \xrightarrow{\text{Hydrazine}} \text{dark brown colour in alkaline medium}$$

Reagents

1. Buffered substrate (pH 7.4) - 15g K_2HPO_4, 2.0g KH_2PO_4, 300mg α-Keto Gluterate, 17.8 g Alanine. pH was adjusted with NaOH and volume was made up to 1 liter. This is stable at 2-8°C for one month.
2. 2,4-Dinitro-phenyl hydrazine (DNPH) - 20mg% in 1 N HCl.
3. 0.4M NaOH
4. Sodium pyruvate standard - 44mg%
5. Sample – serum/plasma or 10% tissue homogenate (in PBS) after cell debris removal.

(Standard range-22-110 µg)

Procedure

For sample -

Reagents	Control	Test
Serum/ tissue homogenate	-	0.05

Buffered substrate	0.25	0.25
	Incubate for 30 min at 37C	
DNPH	0.25	0.25
	Keep at RT for 20 min	
NaOH	2.5	2.5
	Keep at RT for 5 min	
Serum	0.05	-

Read absorbance at 540nm

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit - µmole of pyruvate formed/ min/L of serum or g of tissue

- **Alkaline phosphatase (ALP)** (Bowers and McComb, 1975)

ALP is present in high concentrations in liver, bone (osteoblast), placenta and intestinal epithelium. Raised ALP levels are frequently encountered as a clinical evidence of bone or liver damage.

Principle – ALP reacts with p-nirtophenyl phosphate (PNPP) and converts it in to p-nitrophenol (PNP), which is yellow in colour. PNP in alkaline medium gives bright yellow coloured phenolic ions, which can be measured at 405 nm.

Reagents –

1. PNPP - 0.4g% (freshly prepared)
2. 0.05N NaOH
3. Glycine buffer – 7.5g Glycine, 0.095g $MgCl_2$, 85ml 1N NaOH and total volume was made up to 1 liter
4. Working buffered substrate – equal volume of glycine buffer and stock PNPP mixed and pH was adjusted to 10.4.
5. Standard PNP solution – Stock 1mM in 0.05N NaOH (keep in dark).
   Working standard 0.04mM made 0.05n NaOH

(Standard range 0.04-0.16 μ moles)

Sample preparation – serum / plasma or 10% tissue homogenate (in PBS) after cell debris removal.

Procedure -

Reagents	**Control**	**Test**
Working buffered substrate	0.4 ml	0.4ml
	Placed in water bath at 37C for 5 min	
Serum/tissue homogenate	-	0.02ml
	Incubated at 37C for 30 min	
0.05M NaOH	4.0 ml	4.0 ml
Serum/ tissue homogenate	0.02ml	-
	Absorbance was recorded at 405nm	

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit - μ moles PNP formed/ min/L of serum or g of tissue

Precautions – always use serum or heparinised blood. Avoid EDTA coated bulbs.

- **Creatinine** (Bonsnes and Taussky, 1945)

Creatinine is the end product of creatine metabolism. It is largely formed in the muscle by irreversible and non-enzymatic removal of water from creatine phosphate. It is a waste product and excreted out from the kidney. An increased serum creatinine level is virtually a diagnostic of renal disease.

Principle – Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex. Intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 540 nm.

Reagents –

1. Saturated Picric acid

2. NaOH (0.75M)
3. Creatinine standard solution - Stock – 100mg/dl

Working – 10mg/dl

(Standard range 10-50 μg)

Sample - 1.5 ml picric acid was added to 0.5ml serum/plasma, and tubes were centrifuged at 2000 rpm for 10min. Supernatant was taken out for creatinine estimation.

Procedure –

Reagents	**Control (ml)**	**Test (ml)**
Supernatant	-	1.0
D/W	2.0	1.0
Picric acid	1.0	1.0
0.75M NaOH	1.0	1.0

Tubes kept for 20 min at RT and absorbance was recorded at 540nm

Calculations – $\frac{\text{O.D. of test}}{\text{OD of std}}$ X conc^{n} of std

Unit – mg/dl

- **Statistical analysis** – Results were subjected to student's-test, one-way analysis of variance (ANOVA), ANOVA followed by Shiffe'e multiple comparison test using prism 3.03 software to test the difference among different treated and untreated groups.