Chapter -II

MATERIALS AND METHODS

Experimental Animals

Male Albino Wistar rats, aged 4 months (~180-200g) were used for the present study. The rats were maintained on standard pellet diet and provided access to water *ad libitum*. They were housed in clean, dry polypropylene cages and maintained in a well-ventilated animal house with 12 h light- 12 h dark cycle, as per the guidelines of Institute Animal Ethics committee.

Chemicals

Streptozotocin was purchased from Sigma Aldrich, St. Louis, USA. Thiobarbituric acid, Adenosine Triphosphate, Diphenyl amine, Drabkin's solution, Nicotinamide Adenine Dinucleotide Phosphate oxidised ($NADP^+$), reduced (NADPH), Ferric chloride, 2,6-dichlorophenol indophenol, Phenazine methosulphate, Glucose-6-Phosphate, Fructose-1, 6-bisphosphate, Aminonaphthol sulphonic acid, Trichloroacetic acid, orthophosphoric acid, Alumina (neutral), Potassium hydroxide, Ammonium acetate, Sodium Fluoride, Phosphotungstic acid, Magnesium Chloride, Sodium Hydroxide, Acetyl acetone, N-acetyl neuraminic acid (Sialic acid), Periodic acid, Sodium meta arsenate, Cysteine hydrochloride, Fucose, Galactose, Mannose, p-dimethyl amino benzaldehyde (Ehrlisch's reagent), Galactosamine hydrochloride, Cholesterol, Triolein, Sodium meta periodate, Triethanol amine, Diphenyl Carbazide, Copper reagent, Palmitic acid, Hydroxylamine Hydrochloride, 1,1',3,3'-tetra methoxy propane, Butylated hydroxyl Toluene, Xylenol orange, 2, 4-Dinitro phenyl hydrazine, Ascorbic acid, α- Tocopherol, 2,2'-Dipyridyl-5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), Reduced Glutathione, Hydrogen peroxide, 1-chloro-2,4-dinitro benzene, Ethylene diamine tetra acetic acid (EDTA), α -keto glutarate, DL-alanine, sodium pyruvate, DL- aspartic acid, para-nitrophenyl phosphate etc, chemicals of Analytical Grade were purchased from SRL, Merck, Sd. Fine and Hi Media. Organic solvents of highest purity grade were obtained from the above mentioned Scientific companies. Glibenclamide (Daonil ®), an oral hypoglycemic agent, was obtained from pharmacy. I^{125}- Insulin and Insulin RIA kit were purchased from BARC, Mumbai, India.

Collection of Plant Material

A. echioides was collected from Tirumala hills. The plant material was taxonomically identified and authenticated by the taxonomist, Dr. Madhava chetty, Department of Botany, S.V.University, Tirupati. The voucher specimen (Herbarium accession number: SVUBH/589) was deposited in the herbarium, Department of Botany, S.V.University, Tirupati for further references. The leaves of *A. echioides* were a shade dried, powdered which was used further for the preparation of the extracts for screening of antidiabetic activity.

Preparation of Crude aqueous suspension of *A. echioides* leaves (CASAE)

The leaf powder (250 g) of *A. echioides* was soaked in distilled water (3 volumes) in a glass jar for 48 hrs at room temperature and the solvent was filtered. This was repeated until the filtrate gave no coloration. These filtrates were pooled up and concentrated under reduced pressure by using Buchi rotavapour R-200 and finally freeze dried. The yield was found to be 8% (w/w).

Preparation of AETOF fraction of *A. echioides*

The AETOF fraction was prepared by using the procedure described by Kilani *et al.*, (2008) and Sunil *et al.*, (2011). The leaves of *A. echioides* were shade a dried and made into coarse powder. The leaf powder (250 g) was macerated in water and acetone (1:2 ratio). The solvent mixture was incubated in dark for 6 hrs with continuous stirring and the extract was filtered. In order to obtain aqueous phase, acetone was evaporated under reduced pressure by using Buchi rotavapour R-200. In aqueous phase tannins were separated by precipitation with excess of NaCl for 24 h at 5^0 C and then supernatant was recovered. The supernatant was extracted with ethyl acetate, concentrated and precipitated with excess amount of chloroform. The precipitate was separated and lyophilized to yield the AETOF fraction. The yield of the fraction was 0.52% (w/w). AETOF fraction was dissolved in water for assessment of its antihyperglycemic activity.

Estimation of Total Flavonoid contents in AETOF fraction

The total flavonoid content of AETOF fraction was determined by standard methods as described by Delcour and Varebeke, (1985). The total flavonoid content was expressed in μg quercetin equivalents (QE) /mg of AETOF fraction.

LC-ESI-MS/MS analysis of AETOF fraction

LC-ESI-MS/MS analysis of AETOF fraction was carried out by the method of (Hemanth kumar *et al.*, 2013) with slight modifications. LC-ESI-MS/MS analysis of AETOF fraction was performed on model 6520 Precise Q-TOF mass spectrometer coupled to HPLC equipped with UV-Vis detector (Agilent Santa Clara, CA). Specifications of the column: Zorbax SB C18 Rapid resolution, 4.6 mm × 150 mm, 3.5 µ particle sizes and the conditions were : Binary solvent system was used , solvent A contains 0.1 % v/v formic acid + 10mM ammonium phosphate and solvent B contains acetonitrile + 0.1 % formic acid gradient: the total run time was 40 min (i) 35 % from 0 to 0.5 min (ii) 55 % from 10 min (iii) 95 % from 25 to 33 min (iv) 35 % at 35-40 min. The flow rate was 0.2 ml/min. 3 µl of Sample was injected. Electro spray ionization (ESI) parameters: both positive and negative ion modes; mass range 100-m/z value; spray voltage 4 KV; gas temperature 325^0 C ; gas flow rate 10 L/min; 40 psi nebulizer and mass was analyzed by using Agilent technologies Mass-hunter software.

Induction of diabetes

Diabetes was induced in overnight fasted rats by a single intraperitoneal administration of Streptozotocin (50 mg/kg bw) dissolved in freshly prepared 0.01 M ice cold citrate buffer. After the 6^{th} hr of Streptozotocin administration the animals were given 15% glucose solution to prevent drug induced hypoglycemic mortality. After 72 h rats with marked hyperglycemia ≥250 mg/dl were considered diabetic and used for the study. The whole experimental protocol was approved by the Institutional Animal Ethics Committee vide resolution no: 35/2012-2013/(i)/a/CPCSEA/ IAEC/ SVU/CAR-MVJK/dt. 01-07-2012.

Evaluation of antihyperglycemic effect of Crude aqueous suspension of *A. echioides* leaves powder (CASAE) in normal and STZ-induced diabetic rats

The animals were divided in to three groups and each group consisted of six rats

Group 1: Normal untreated rats.

Group 2 : Diabetic untreated rats.

Group 3: Diabetic rats treated with 250 mg CASAE/kg bw.

After an overnight fast, all the respective groups' blood samples were collected from the tail vein and the FBG levels were measured. Group 3 received 250 mg of CASAE/Kg bw orally by gastric intubation using force feeding needle. While, group 1 and group 2 have received water only. Blood samples were drawn from tail vein for the measurement of blood glucose at 1, 2,3,4,5 and 6 h after the oral administration of CASAE/water by using Accu-Check Active Glucometer (Kesari *et al.*, 2006).

Evaluation of antihyperglycemic effect of different doses of an AETOF fraction in normal and STZ-induced diabetic rats.

The rats were divided into nine groups, each group consisted of six animals

Group 1: Normal untreated rats.

Group 2: Diabetic untreated rats.

Group 3: Normal rats treated with 30 mg AETOF/kg bw.

Group 4: Normal rats treated with 50 mg AETOF/kg bw.

Group 5: Normal rats treated with 70 mg AETOF/kg bw.

Group 6: Diabetic rats treated with 30 mg AETOF/kg bw.

Group 7: Diabetic rats treated with 50 mg AETOF/kg bw.

Group 8: Diabetic rats treated with 70 mg AETOF/kg bw.

Group 9: Diabetic rats treated with 0.02 g glibenclamide/kg bw.

After an overnight fast the normal treated and diabetic treated rat groups were administered with different doses of an AETOF fraction by gastric intubation using a force feeding needle. Normal untreated and diabetic untreated rats were fed distilled water alone. Blood samples were collected for the measurement of blood glucose from the tail vein at 0, 1, 2, 3, 4, 5, & 6 hr after the administration of AETOF fraction and the fasting blood glucose levels were determined by using glucose oxidase-peroxidase reactive strips and a Glucometer (Accu-Chek, Roche Diagnostics, USA).

Effect of AETOF fraction on oral glucose tolerance (OGT)

The oral glucose tolerance test was performed in five groups of normal and diabetic rats, each group consisted of six animals.

Group 1: Normal untreated rats.

Group 2: Normal rats treated with 50mg ATEOF/kg bw.

Group3: Diabetic untreated rats.

Group4: Diabetic rats treated with 50mg ATEOF/kg bw.

Group5: Diabetic rats treated with 0.02 g glibenclamide/kg bw.

After an overnight fast the FBG levels of all groups of rats were measured and then glucose at a dosage of 2 g/ Kg b.w was administered orally to all groups of rats using a force feeding needle. The group 2, group 4 rats were administered orally AETOF fraction while the group 5 rats were administered glibenclamide. Group 1 and group 3 were fed with water alone. The blood samples were collected from the tail veins of all animals from 0 min (before glucose administration) to 180 min after glucose administration for estimation of blood glucose using glucose oxidase peroxidase reactive strips and a Glucometer (Accu-Chek, Roche Diagnostics, USA).

Plasma Insulin Assay

The insulin was assayed by using the modified method of Herbert *et al* (1965) using Insulin RIA (radio immuno assay) kit obtained from BARC, Mumbai, India.

Blood samples were collected in EDTA coated vials and plasma was separated immediately and kept frozen at -20^0C till the assay. The flow chart given in the Fig. 12 shows the procedure of the assay. The RIA method is based on the competition of unlabelled insulin in the standard or samples and labeled insulin (I^{125}-Insulin) for the limited binding sites on insulin specific antibody. At the end of incubation, secondary antibody and polyethylene glycol were used to precipitate antibody bound and free insulin. Radioactivity of bound fractions of samples and standards were measured and the insulin levels of samples were obtained by interpolating from the graph plotted using the known concentrations of standard insulin.

Reagents

1. EDTA – phosphate buffer (0.01 M) containing 0.1% bovine albumin (pH 7.4).
2. Human Insulin standard 200 μU/ml
3. ^{125}I– Insulin containing 1.5 ng/ml with specific radioactivity of 100 μ Ci/μg ^{125}I – insulin
4. Insulin free serum
5. Anti-insulin serum
6. Secondary antibody (anti-rabbit gamma globulin)
7. 25% (w/w) PEG solution.

Calculations

Percentage of B/Bo was calculated as follows where B is sample or standard binding, Bo is zero standard binding.

$$\% = \frac{Corrected \text{ average counts of standard or sample}}{Corrected \text{ average counts of zero standard}} x100$$

A standard curve was plotted on a log it log graph sheet with % of B/Bo on the log it scale and standard insulin concentration (μU/ml) on the logarithmic scale. The IRI (immuno reactive insulin) values for the samples were interpolated from the standard curve as micro units of insulin/ml. The lowest detection limit of the assay was 2 μU/ml. Inter assay coefficients of variations was 7-8% control sera showing 92% - 108% recovery.

Fig.12: Insulin Assay Flow Chart

Tube no.	Assay buffer (ml)	Insulin standard (ml)	Serum sample (ml)	Insulin free serum (ml)	Insulin anti serum (ml)		I-125 insulin (ml)		Second antibo dy (ml)	PEG	
1,2	----	---	---	---	---	Mix gently, Incubate all tubes at +4°C overnight	0.1	Mix gently, Incubate all tubes for 3hrs at room temp	---	---	Vortex & keep all tubes at room temp for 20 min. Centrifuge the tubes at 1500g for 20 min
3,4	0.4	---	---	0.1	---		0.1		0.1	0.1	
5,6	0.3	---	---	0.1	0.1		0.1		0.1	0.1	
7,8	0.2	0.1F	---	0.1	0.1		0.1		0.1	0.1	
9,10	0.2	0.1E	---	0.1	0.1		0.1		0.1	0.1	
11,12	0.2	0.1D	---	0.1	0.1		0.1		0.1	0.1	
13,14	0.2	0.1C	---	0.1	0.1		0.1		0.1	0.1	
15,16	0.2	0.1B	---	0.1	0.1		0.1		0.1	0.1	
17,18	0.2	0.1A	---	0.1	0.1		0.1		0.1	0.1	
19,20	0.3	---	0.1	---	0.1		0.1		0.1	0.1	
21,22	0.3	---	0.1	---	0.1		0.1		0.1	0.1	

After centrifugation decanted and counted radioactivity in the precipitate

Estimation of Blood Glucose

Blood glucose estimation was done by the Glucose Oxidase–Peroxidise method using the Accu chek active of Roche Diagnostics, Germany.

Estimation of Haemoglobin

Haemoglobin content of blood was estimated by the cyanomethaemoglobin method of Drabkin and Austin, (1932). When blood is diluted with an alkaline solution of potassium cyanide and potassium ferricyanide, haemoglobin is oxidised to methaemoglobin and combines with cyanide to form cyanomethaemoglobin, which is measured calorimetrically at 540 nm.

Reagents

1. Drabkin's reagent: This reagent contains 0.05 g of potassium cyanide, 0.20 g of potassium ferricyanide and 1 g of sodium bicarbonate in 1 liter of distilled water (pH 9.6).
2. Cyanomethaemoglobin standrard: The solution was obtained commercially and had a concentration of 15 g/dl.

Procedure

To 5 ml of Drabkin's solution (potassium cyanide and ferricyanide solution), 20µl of blood was added, mixed and the contents were allowed to stand at room temperature for 5 minutes. The cyanomethoglobin formed in the reaction was measured and compared with the cyanomethaemoglobin standard at 540 nm against a blank containing only reagent.

Estimation of Glycosylated Haemoglobin

Glycosylated haemoglobin was estimated by the method of Eross *et al.*, (1984).

Reagents

1. Hydroxymethyl furfural (Standard)
2. Haemoglobin reagent
3. Trichloroacetic acid: (40%)
4. Orthophosphoric acid: 2.5M
5. Thiobarbituric acid: 0.05 M

Procedure

0.05 ml of the blood was taken in screw capped Kimax tubes (13 x 108 mm) and 2.0 ml of distilled water was added. Mixed and allowed to stand for 5 minutes for complete lysis of RBC. To the solution 0.2 ml of 40% TCA was added, mixed and centrifuged at 3000 x g for 20 minutes. The supernatant was discarded and to the pellet 2 ml of 2.5 M orthophosphoric acid was added. The tubes were tightly capped with Teflon coated caps and placed in a boiling water bath at 100°C. The tubes were

taken out after 1 hour and cooled in ice-cold water. Again the protein was precipitated by adding 1 ml of 40% TCA and the tubes were centrifuged at 3000 x g for 20 minutes. The supernatant was separated and to this added 1 ml of 0.05M TBA, mixed well and incubated at 40^0C for 30 minutes. The tubes were cooled to room temperature and the absorbance was read at 443 nm in a spectrophotometer. Reagent blank was run simultaneously by adding 1 ml of TBA to 1 ml of TCA and 2 ml of orthophosphoric acid. The values were expressed as percentage of haemoglobin glycosylated.

Estimation of Glycogen

The estimation of glycogen in tissues was carried out by the method of Kemp and Van Hejnigen, (1954).

Reagents

1. Glucose standard (0.1mg/ml)
2. Concentrated sulphuric acid
3. Trichloroacetic acid 15%
4. Methanol 90%

Procedure

5 ml of 10% tissue homogenate was prepared in 90% methanol and the contents were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and to the pellet added 5 ml of deproteinising solution i.e., TCA and the level was marked on the tubes. The tubes were capped and kept in boiling water bath for 15 min. After boiling the tubes were cooled and the contents were made up with TCA upto the mark and centrifuged at 3000 rpm for 15 min. 1ml of the supernatant was taken and to this 3 ml of H_2SO_4 was added. The tubes were shaken well and kept in boiling water bath for 6.5 min. Then the tubes were cooled and the colour developed was read at 520 nm. A blank and a standard were run similarly taking 1 ml of distilled water and 1 ml of glucose standard (respectively).

Glycogen content was calculated from the amount of glucose present in the sample and was expressed as mg/g tissue.

Estimation of Protein

The estimation of protein was carried out by the method of Lowry *et al.*, (1951).

Reagents

1. Bovine serum albumin (BSA) was used as a standard (0.1mg/ml)
2. Sodium Carbonate (Na_2CO_3), 4% in 0.1 N Sodium hydroxide (NaOH)
3. Sodium potassium tartarate: 2%
4. Copper sulphate: 1%
5. Lowry alkaline Copper solution: Reagents 2, 3 and 4 are mixed in the proportions of 100:1:1 just before use.
6. Folin's Phenol reagent: Commercially available Folin ciocalteau's reagent was diluted with distilled water in 1:1 proportion.

Procedure

25 µl of the tissue homogenate was made up to 1 ml with distilled water and to this added 5 ml of alkaline copper reagent and the contents were kept at room temperature for 20 min. After the incubation, to the test tubes added 0.5 ml of Folin ciocalteu's reagent and the tubes were kept at room temperature for 10 min. The colour developed was read at 650 nm. Blank and standards were also run simultaneously.

Estimation of Cholesterol

Estimation of cholesterol was carried out by the method of Zlatkis *et al.*, (1953).

Reagents

1. Standard Cholesterol in aldehyde free acetic acid (2mg/ml)
2. Concentrated Sulphuric acid
3. Ferric chloride and acetic acid reagent: 0.05% in aldehyde free acetic acid.

Procedure

9.9 ml of ferric chloride-acetic acid reagent was added to 0.1 ml of serum for deproteinization. The contents were centrifuged at 3000 rpm for 15 min. 5 ml of the supernatant was taken and to this added 3 ml of conc. sulphuric acid and kept for 20

min at room temperature. The pink colour formed was read at 540 nm against a blank containing 5 ml of ferric chloride-acetic acid reagent. A set of standards were also performed in the similar manner.

Estimation of Triglycerides

Serum triglycerides were measured by the method of Foster and Dunn, (1973).

Reagents

1. Standard Triolein in isopropanol was used as standard (0.1 mg/ml).
2. Activated Alumina (neutral)
3. Isopropanol
4. Alcoholic KOH : 50 g of KOH in 1 litre of mixture of Iso-propanol and water (2:3)
5. Acetyl Acetone reagent: To 200 ml of Isopropanol added 7.5 ml of acetyl acetone and the contents were made up to 1 litre with distilled water.
6. Sodium meta periodate: 60 ml of acetic acid and 77g of anhydrous ammonium acetate were added to 700 ml of water. 650 mg of sodium meta periodate was dissolved in this solution and the final volume was made up to 1 litre with distilled water.

Procedure

0.1 ml of serum was taken in a glass stoppered centrifuge tube and to this added 4 ml of isopropanol and 400 mg of alumina. The tubes were tightly capped and shaken vigorously for 10 min. Then the tubes were centrifuged at 3000 rpm for 15 min and 2 ml of the supernatant was pipetted into clean, dry test tubes. To these added 0.6 ml of alcoholic KOH and kept at 70^0C for 15 min. The tubes were cooled to room temperature. To this added 0.5 ml of acetyl acetone reagent, 1.0 ml of meta periodate reagent and incubated at 50^0C for 30 min. Standard was also run in the same fashion with triolein instead of serum. The colour developed was read at 405 nm against the reagent blank.

Estimation of HDL-Cholesterol (HDL-C)

Determination of serum HDL-cholesterol was carried out by the method of Burstein *et al.*, (1970).

Reagents

1. Phosphotungstic acid reagent: To 200 ml of distilled water added 22.5 g of phosphotungstic acid and 80 ml of 1M sodium hydroxide and the volume was made up to 500ml with distilled water.
2. Magnesium chloride solution: 101.7g of $MgCl_2$ was dissolved in 250 ml of distilled water.

Procedure

0.5 ml of serum was taken in a centrifuge tube and to this added 0.25 ml of phosphotungstic acid reagent and 0.25 ml of $MgCl_2$ and was centrifuged at 1500 x g for 30 min in a refrigerated centrifuge and the amount of cholesterol was determined in the supernatant by the method of Zlatkis *et al.*, (1953).

Estimation of VLDL and LDL Cholesterol (VLDL-C and LDL-C)

By using Freidwald formula the concentration of VLDL and LDL cholesterol in serum were calculated (Friedwald *et al.*, (1972).

$$VLDL-C = \frac{Triglycerides}{5}$$

LDL-C= (Total Cholesterol – VLDL-C) - (HDL-C)

Enzymes of Carbohydrate Metabolism

Tissues were rinsed with ice cold saline, and blotted with tissue paper and weighed. For the enzyme assays tissues were homogenized (10% homogenate) in Tris HCl buffer (0.1 M, pH 7.0) at 0^0C (10% homogenate). The homogenates were used for the enzyme assays. The enzyme assays were carried out within 48 hrs and till the assay the homogenates were kept at 4^0C.

Assay of Hexokinase

The assay of Hexokinase was carried by the method of Branstrup *et al.*, (1957).

Reagents

1. Tris-HCl buffer: 0.10 M, pH.8
2. Glucose: 0.005 M
3. ATP: 0.72 M
4. Mg Cl_2: 0.05 M
5. KCl: 0.1 M
6. Sodium Fluoride: 0.15 M
7. KH_2PO_4: 0.0125 M
8. o-Toluidine reagents for glucose estimation

Procedure

The incubation mixture of test (5 ml) contains 2.5 ml of buffer, 1 ml of substrate, 0.5 ml of ATP, 0.1 ml of $MgCl_2$, 0.1 ml of NaF, 0.4 ml of KH_2PO_4 and 0.4 ml of KCl. The mixture was pre-incubated at 37^0C for 5 min. The reaction was initiated by the addition of 0.2 ml of tissue homogenate. The mixture was incubated for 30 minutes at 37^0C and the reaction was arrested by adding 1 ml of 10% TCA. Along with the test, a control was also run in the same manner but to the control the TCA was added at zero time i.e. before the incubation with enzyme. The reaction mixture was centrifuged at 3000 rpm for 15 min and 0.5 ml of the supernatant was used for the estimation of residual glucose by o-toluidine method. The activity of enzyme was expressed as micromoles of glucose phosphorylated/ min/mg protein.

Assay of Glucose-6-phosphatase

The activity of glucose-6-phosphatase was assayed according to the method of King, (1965).

Reagents

1. Citrate buffer: 0.1 M, pH 6.5
2. Glucose-6-phosphate: 0.01 M
3. TCA: 10%
4. Fiske Subbarow reagents for inorganic phosphate estimation.

Procedure

The incubation mixture of test, in a total volume of 1 ml contained 0.5 ml of substrate, 0.4 ml of buffer and 0.1 ml of homogenate. The reaction mixture was incubated at 37^{0}C for 60 min. The enzyme action was terminated by the addition of 1 ml of 10% TCA. Along with the test a control was also run in the same manner but to control the TCA was added at 0 time i.e. before the addition of enzyme. The supernatant obtained after centrifugation was used for the estimation of inorganic phosphate by the method of Fiske and Subbarow, (1925). The enzyme activity was expressed as the micromoles of inorganic phosphate liberated per min/ mg protein.

Assay of Fructose-1, 6-bisphosphatase

The activity of Fructose-1, 6-bisphosphatase was assayed by the method of Gancedo and Gancedo, (1971).

Reagents

1. Tris-HCL buffer: 0.1 M, pH.7.
2. Fructose-1, 6-bisphosphate: 0.05 M
3. Magnesium Chloride: 0.1 M
4. Potassium Chloride: 0.1 M
5. Ethylenediamine tetra acetic acid: 0.001 M
6. Trichloro acetic acid: 10%

Procedure

The assay mixture of test (2.5 ml) contained 1.7 ml of Tris-HCl buffer, 0.1 ml of KCl, 0.1 ml of fructose-1, 6-bisphosphate, 0.25 ml of EDTA, 0.25 ml of $MgCl_2$ and 0.1 ml of tissue homogenate. The reaction mixture was incubated at 37^{0}C for 15

minutes and the enzyme reaction was terminated by the addition of 1 ml of 10% TCA. Along with the test a control was also run in the same manner but to the control TCA was added at 0 time i.e. before the incubation with enzyme. The contents were centrifuged and the supernatant was used for the estimation of inorganic phosphate by Fiske Subbarow method. The activity was expressed in micromoles of inorganic phosphate liberated/min/mg protein.

Assay of Glucose-6-phosphate Dehydrogenase

The assay of Glucose-6-phosphate dehydrogenase was carried out by the method of Langdon, (1966).

Reagents

1. Tris-HCl buffer: 1M, pH 7.5.
2. Glucose-6-phosphate: 2.5 x 10^{-2}M
3. $NADP^+$: 2 x 10^{-3}M
4. Magnesium chloride: 0.2 M

Procedure

The assay mixture consisted of 2.5 ml of buffer, 0.1 ml of glucose-6-phosphate, 0.2 ml of $NADP^+$, 0.1 ml of $MgCl_2$ and 0.1 ml of homogenate. Immediately after the addition of the homogenate the rate of change in the absorbance was measured at 340 nm for 2 minutes with the time interval of 30 seconds, in a spectrophotometer against a blank without the enzyme. One unit of enzyme activity is defined as that quantity which catalyzes the reduction of 1 micromole of $NADP^+$ per minute under the above assay conditions.

Extraction of Lipids from Tissues

Lipids were extracted from tissues by the method of Folch *et al.*, (1957) using chloroform – methanol mixture (2:1 v/v).

A known weight of tissue was homogenised in 7.0 ml of chloroform-methanol using potter Elvehjam homogeniser. The contents were filtered into a previously weighed side arm flask, residue on the filter paper was scrapped off and homogenised with 14.0 ml of chloroform-methanol mixture. This was again filtered into the side

arm flask and the residue was successively homogenised in chloroform – methanol (2:1 v/v) and each time this extract was filtered. The pooled filtrates in the flask was adjusted to a final volume ratio using chloroform-methanol (2:1 v/v) and evaporated to dryness to a constant weight.

The dried residue of lipid was dissolved in 5.0 ml of chloroform-methanol mixture (2:1 v/v) and transferred into a centrifuge tube; 2.0 ml of 0.1 M potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides was discarded. The chloroform layer was mixed with 1.0 ml of chloroform, methanol, potassium chloride mixture (1:10:10 v/v) and then centrifuged. This washing was repeated thrice and each time, the upper layer was discarded. The lower layer was made up to 5.0 ml and used for the analysis of total cholesterol, triglycerides and α-tocopherol.

Estimation of Total Tissue Lipids

Tissue cholesterol in the lipid extract was estimated by the method of Zlatkis *et al.*, (1953) as mentioned earlier for the plasma cholesterol estimation. Tissue triglycerides in the lipid extract were estimated by the method of Foster and Dunn as mentioned earlier for the plasma cholesterol estimation.

Indirect Assessment of β-hydroxy-β-methylglutaryl-Coenzyme A (HMG – CoA) reductase activity

The ratio between HMG-CoA and mevalonate in tissues was taken as an index of the activity of HMG-CoA reductase as described by Philip and Shapiro, (1979).

Reagents

1. Saline-arsenate: 1 g of sodium arsenate per litre of physiological saline.
2. Diluted perchloric acid: 50ml/L
3. Hydroxylamine hydrochloride reagent for Mevalonate: Equal volumes of hydroxylamine hydrochloride (2M) and water were mixed freshly before use.
4. Hydroxylamine Hydrochloride reagent for HMG-CoA: Equal volumes of hydroxylamine hydrochloride (2M) and sodium hydroxide (4.5M) solution were mixed freshly before use.

5. Ferric chloride reagent: 5.2 g of trichloroacetic acid and 10 g of ferric chloride were dissolved in 50.0 ml of 0.65 N HCl and diluted to 100ml with water.

Procedure

Equal volumes of fresh 10% tissue homogenate and diluted perchloric acid were mixed. Kept for 5 min and centrifuged at 2000 rpm for 10 min. To 1.0 ml filtrate, 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG-CoA) was added, mixed and after 5 min, 1.5 ml of ferric chloride was added and shaken well and similarly hydroxylamine hydrochloride reagent for estimated mevalonate was added to 1.0 ml of filtrate and processed as for HMG-CoA. Readings were taken after 10 min at 540 nm against a similarly treated saline-arsenate blank. The ratio of HMG-CoA to Mevalonate was calculated. Lower ratio indicated higher enzyme activity and vice-versa.

Estimation of Lipid Peroxidation

Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

The levels of TBARS in tissues and plasma were estimated by the method of Fraga *et al.*, (1988).

Reagents

1. Tris-HCl buffer: 0.025 M, pH 7.5
2. Tricholro acetic acid (TCA): 15%
3. Hydrochloric acid (HCl): 0.25 N
4. Thiobarbituric acid (TBA): 0.38%
5. TBA – TCA-HCl reagent: (1:1:1 v/v)
6. Standard (1,1',3,3' tetramethoxy propane): 0.16 ml of 3 M solution of standard tetramethoxy propane was made upto 100 ml with double distilled water. 1.0 ml of this was taken and made upto 100 ml with double distilled water, which served as working standard.

Procedure

10% tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1.0 ml of the tissue homogenate/0.5 ml of plasma was treated with 2.0 ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. A series of standard solution in the range 2-10 nmole concentrations were treated in the similar manner. The absorbance of chromophore was read at 535 nm against the reagent blank.

Estimation of Non-enzymatic Antioxidants

Estimation of ascorbic acid

Ascorbic acid was estimated by the method of Omaye *et al.*, (1979).

Reagents

1. 2, 4 DNPH reagent: 2.0g of DNPH was dissolved in 100 ml of 9N sulphuric acid. To this 4.0 g thiourea was added and mixed.
2. Trichloroacetic acid: 6%
3. Sulphuric acid: 85%
4. Standard ascorbic acid: 10mg of L-ascorbic acid was dissolved in 100ml of 4% TCA. This was diluted to prepare a working standard of concentration 100 µg/ml.
5. Activated charcoal.

Procedure

0.5 ml of plasma/0.5 ml of tissue homogenate was mixed thoroughly with 1.5 ml of 6% TCA and centrifuged for 20 min at 3500 g. To 0.5 ml of the supernatant, 0.5 ml of DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3 hrs. Removed, placed in ice-cold water and added 2.5 ml of 85% sulphuric acid and allowed to stand for 30 min. A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 ml of 4% TCA. The colour developed was read at 530 nm.

Estimation of α-Tocopherol

α-tocopherol was estimated by the method of Baker *et al.*, (1951).

Reagents

1. Petroleum ether 60-80^{0}C
2. Double distilled ethanol
3. 0.2% of 2,2' dipyridyl in ethanol
4. 0.5% ferric chloride in ethanol
5. Stock standard: 100mg of α-tocopherol in 100 ml of distilled ethanol.
6. Working standard: stock solution was diluted to a concentration of 10 μg/ml.

Procedure

To 0.1 ml of plasma / 0.1 ml of lipid extract, 1.5 ml of ethanol and 2.0 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80^{0}C. To this was added 0.2 ml of 2, 2'dipyridyl solution and 0.2 ml of ferric chloride solution. Mixed well and kept in dark for 5 min and added 2.0 ml of butanol. The intense red colour developed was read at 520 nm. Standard tocopherol in the range of 10-100 μg were taken and treated similarly along with blank containing only the reagent.

Estimation of Reduced Glutathione

The estimation of total glutathione (reduced) was carried by the method of Ellman, (1959).

Reagents

1. Standard glutathione (10mg /ml)
2. 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB): 19.8 mg of DTNB in 100ml of 1% sodium citrate solution.
3. Trichloroacetic acid 5% TCA.
4. Phosphate buffer – 0.2 M, pH 8.0.

Procedure

To 0.5 ml of tissue homogenate added 2 ml of 5% TCA and the protein were precipitated. The contents were centrifuged at 3000 rpm for 15 min and the precipitate was discarded. To 1 ml of the supernatant 0.5 ml of DTNB reagent & 3 ml of phosphate buffer were added. The yellow colour developed was read at 412 nm against a blank containing TCA instead of sample. A series of standards were treated in a similar manner.

Assay of Enzymatic Antioxidants in tissues

Assay of Superoxide Dismutase

Superoxide dismutase (SOD) activity was determined by the method of Kakkar *et al.*, (1984).

Reagents

1. Sodium pyrophosphate buffer: 0.025 M, pH 8.3
2. Phenazine methosulphate: 186μM
3. Nitroblue tetrazolium: 300μM
4. NADH: 780μM
5. Glacial acetic acid
6. n-butanol
7. Chloroform
8. Absolute Ethanol

Procedure

0.5 ml of tissue homogenate was diluted to 1.0 ml with water. Then 2.5ml of ethanol and 1.5 ml of chloroform (all reagents chilled) were added. This mixture was shaken for 1 min at 4^0C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025M, pH 8.3), 0.1 ml of 186 μM phenazine methosulphate, 0.3 ml of 300 μM nitroblue tetrazolium, 0.2 ml of 780 μM NADH, appropriately diluted enzyme preparation and

water in a total volume of 3.0 ml. Reaction was started by the addition of NADH. After incubation at 30^0C for 90 seconds, the reaction was stopped by the addition of 1.0 ml of glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560 nm against butanol blank. A system devoid of enzyme served as control.

One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one min under the assay conditions and expressed as specific activity in units/mg protein.

Assay of Catalase

The activity of catalase was determined by the method of Sinha, (1972).

Reagents

1. Phosphate buffer: 0.01 M, pH 7.0
2. Hydrogen peroxide: 0.2 M
3. Potassium dichromate: 5%
4. Dichromate-acetic acid: 1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1.0 ml was diluted again with 4.0 ml acetic acid.
5. Standard H_2O_2: 0.1 ml of 0.2 M H_2O_2 was diluted to 100 ml using distilled water.

Procedure

To 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of hydrogen peroxide were added. After 60 seconds, 2.0 ml of dichromate-acetic acid mixture was added. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620 nm. Standards in the range of 2-10 µmol were taken and preceded as test with blank containing reagent alone. The activities were expressed as µ moles of H_2O_2 consumed /min/mg protein.

Assay of Glutathione Peroxidase

Glutathione peroxidase was estimated by the method of Rotruck *et al.*, (1973).

Reagents

1. Tris buffer: 0.4 M, pH 7.0.
2. Sodium azide: 10 mM
3. Trichloroacetic acid : 10%
4. Ethylene diamine tetra acetic acid: 0.4 mM
5. Hydrogen peroxide solution: 20 mM
6. Glutathione solution: 2m/M.
7. Ellman's reagent: 19.8 mg of 5.5'-dithiobis (2-nitrobenzoic acid) in 100 ml of 1% sodium citrate solution.

Procedure

To 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate were added. To the mixture, 0.2 ml of glutathione followed by 0.1 ml of hydrogen peroxide was added. The contents were mixed well and incubated at 37^0C for 10 min along with a tube containing all the reagents except sample. After 10 min. the reaction was arrested by the addition of 0.5ml of 10% TCA, centrifuged and the supernatant was assayed for glutathione by the method of Ellman, (1959). The activities were expressed as µg of GSH consumed/min/mg protein.

Assay of Glutathione-S-Transferase

Activity of Glutathione-S-transferase was measured in tissue homogenate by following the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitrobenzene as substrate by the method of Habig, (1974).

Reagents

1. Phosphate buffer: 0.3 M, pH 6.5
2. Reduced glutathione: 30 mM
3. 1-chloro-2, 4-dinitrobenzene (CDNB): 30 mM was prepared in 95% ethanol.

Procedure

The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of CDNB, 0.1 ml of tissue homogenate and 0.7 ml of distilled water. The reaction mixture was

incubated at 37^{0}C for 5 min and then the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance change was read at 340 nm for 5 min. Reaction mixture without the enzyme was used as the blank.

Calculations

$$Activity = \frac{OD}{9} x \frac{3}{5} x \frac{100}{mg \text{ protein}} units/mg \text{ protein}$$

9.61 was the difference in the micromolar extinction co-efficient between CDNB-GSH conjugate.

Assay of Serum Glutamic Pyruvate Transaminase (SGPT) or Alanine Amino Transferase (ALT).

SGPT activity was determined by the method of Reitman & Frankel (1957).

Transamination is the process in which an amino group is transferred from amino acid to a α-keto acid. The enzymes responsible for transamination are called transaminases. The substrates in the reaction are α Ketoglutaric acid plus L-alanine for ALT. The products formed by enzyme action are glutamate and pyruvate. Addition of 2, 4, dinitrophenyl hydrazine results in the formation of hydrazone complex with the ketoacids. A red colour produced on the addition of sodium hydroxide is read at 510 nm. The intensity of colour is related to enzymic activity. One unit/L of AST or ALT is defined as the liberation of 1 mmol of pyruvate formed per minute at 37^{0}C incubation per litre of serum.

Reagents

1. Phosphate buffer, pH 7.4: Dissolved 14.9 g of disodium hydrogen phosphate dihydrate ($Na_2HPO4\ 2H_20$) and 2.2g of anhydrous potassium dihydrogen phosphate (KH_2PO_4) in distilled water and made up to one litre.
2. ALT Substrate: Dissolved 1.78 g of DL-alanine and 30 mg of α -keto glutarate in 20 ml of phosphate buffer containing 1.25 ml of 0 .4 M NAOH. Made up to 100 ml with buffer and adjusted to pH 7.4 if necessary.
3. Pyruvate standard 2 m mol/ml: Dissolved 220 mg of sodium pyruvate in phosphate buffer and made up to 100 ml. Diluted 10 ml of this solution to 100

ml with phosphate buffer to obtain the working standard containing 2 m mol pyruvate per ml.

4. Colour reagent: Dissolved 200 mg of 2, 4 -dinitro-phenylhydrazine (2,4 DNPH) in hot 1M HCl and made up to 1 litre with 1M HCl.
5. 0.4 M Sodium hydroxide: Dissolved 16 g of sodium hydroxide in about 800 ml of distilled water and made up to 1 litre with distilled water.

Procedure

Two test tubes were appropriately labelled as Control and Test respectively. 0.5 ml of substrate was added to both the test tubes. 0.1 ml of serum was added only to the 'Test' test tube. The sample was mixed and incubated in a water bath at 37° C for 1 hour. The tubes were removed and 0.5 ml of 2,4 DNPH was added to each of the test tubes. 0.1 ml of serum was now added to the 'control' test tube. Mixed well and left the tubes at room temperature for 20 minutes. Then 5.0 ml of 0.4 M NaOH was added to each of the tubes. Mixed well and left the tubes at room temperature for 5 minutes. The spectrophotometer/filter photometer was adjusted to zero using distilled water at 510 nm/yellow green filter and measured the absorbance of Control and Test in the order.

Construction of calibration curve

In the measurement of both serum AST & ALT, only pyruvate is used as the standard. Theoretically speaking, oxaloacetate should be used as the standard for AST assay and pyruvate as the standard for ALT assay. Oxaloacetate formed in the AST assay is unstable and immediately gets converted into pyruvate; hence the use of pyruvate standard for AST assay. One unit/L of AST or ALT is defined as the liberation of 1m mol of pyruvate per minute at 37^0C incubation per litre of serum.

Pippetted different volumes of pyruvate (0.1, 0.2, 0.3, 0.4 ml) in appropriately labeled test tubes. The final volume in each respective tube was adjusted to 1.0 ml by addition of corresponding volume of ALT/AST substrate. Then 0.2 ml of water was added to each test tube and was followed by 1.0 ml of 2,4 DNPH. The tubes were left for 20 minutes at room temperature and 10.0 ml of 0.4M NaOH was added to each test tube.

Constructed a calibration curve by plotting the corresponding absorbance of standards with concentrations.

Assay of Serum Glutamic Oxaloacetate (SGOT) or Aspartate Amino Transferase (AST).

SGPT activity was determined by the method of Reitman & Frankel, (1957).

Reagents

1. Phosphate Buffer, pH 7.4: Dissolved 14.9 g of disodium hydrogen phosphate dihydrate ($Na_2HPO4\ 2H_20$) and 2.2g of anhydrous potassium dihydrogen phosphate (KH_2PO_4) in distilled water and made up to one litre.
2. AST Substrate: Dissolved 2.66 g of DL-aspartic acid and 30 mg of α-keto glutarate in 20.5 ml of 1 M NAOH. Adjusted the pH to 7.4 by adding 1 M NAOH drop wise while stirring. Made up to 100 ml with phosphate buffer.
3. Pyruvate standard 2 mmol/ml. Dissolved 220 mg of sodium pyruvate in phosphate buffer and made up to 100 ml. Diluted 10 ml of this solution to 100 ml with phosphate buffer to obtain the working standard containing 2 m mol pyruvate per ml. The working standard should be stored in small aliquots of 2 ml in the freezer. One aliquot of working standard should be used for preparing a calibration graph.
4. Colour reagent: Dissolved 200 mg of 2, 4-dinitro-phenylhydrazine (2,4 DNPH) in hot 1M HCl and made up to 1 litre with 1M HCl.
5. 0.4 M Sodium hydroxide: Dissolved 16 g of sodium hydroxide in about 800 ml of distilled water and made up to 1 litre with distilled water.

Procedure

Two test tubes were appropriately labelled as Control and Test respectively. 0.5 ml of substrate was added to both the test tubes. 0.1 ml of serum was added only to the 'Test' testtube. The sample was mixed and incubated in a water bath at 37° C for 1 hour. The tubes were removed and 0.5 ml of 2,4 DNPH was added to each of the test tubes. 0.1 ml of serum was now added to the 'control' test-tube. Mixed well and left the tubes at room temperature for 20 minutes. Then 5.0 ml of 0.4 M NaOH was

added to each of the tubes. Mixed well and left the tubes at room temperature for 5 minutes. The spectrophotometer/filter photometer was adjusted to zero using distilled water at 510 nm/yellow green filter and measured the absorbance of Control and Test in the order.

Construction of calibration curve

Pippetted different volumes of pyruvate (0.1, 0.2, 0.3, 0.4 ml) in appropriately labelled test tubes. The final volume in each respective tube was adjusted to 1.0 ml by addition of corresponding volume of ALT/AST substrate. Then 0.2 ml of water was added to each test tube and was followed by 1.0 ml of 2,4 DNPH. The tubes were left for 20 minutes at room temperature and 10.0 ml of 0.4M NaOH was added to each test tube.

Constructed a calibration curve by plotting the corresponding absorbance of standards with concentrations.

Assay of Serum Alkaline Phosphatase (ALP)

Activity of serum alkaline phosphatase was determined by p-nitro phenyl phosphate method (Bessey, 1946).

Para-nitrophenyl phosphate, which is colorless, is hydrolysed by alkaline phosphatase at pH 10.5 & 37^0 C to form free para-nitrophenol, which is coloured yellow. The addition of NaOH stops the enzyme activity and the final colour shows maximum absorbance at 410 nm.

Reagents

1. 2-amino 2- methyl 1-propanol (AMP) buffer pH 10.5: Added 116 ml of AMP to 600 ml of distilled water. Mixed and adjusted the pH to 10.5 with 6 M HCl and then made up to 1 litre with distilled water.
2. Magnesium chloride (1.5 mmol/1): Dissolved 300 mg of magnesium chloride hexahydrate in distilled water and made up to 1 litre.
3. Substrate: Dissolved 83.5 mg of disodium paranitrophenyl phosphate in 1.0ml magnesium chloride solution.

4. **Sodium hydroxide 0.25 M: Dissolved 10 g of NaOH in about 800 ml of distilled water and then made up to 1 litre with distilled water.**
5. **Stock paranitrophenol (PNP) 10.8 mmol/l: Weighed out 150 mg of PNP and dissolved in about 80ml of NaOH (0.25M) and then made up to 100 ml with the same NaOH solution.**
6. **Working PNP 54 mmol/l: Pipetted 0.5 ml of the PNP stock solution into a 100ml volumetric flask and made up to the mark with NaOH solution (0.25 M). Prepared fresh before use.**

Procedure

Enzyme measurement in test sample

Pipetted out 1.4 ml of the AMP buffer in to each tube appropriately labelled as Control and Test. Incubated at 37° C for 5 minutes. Then 0.1 ml of the substrate was added to each of the tubes. 0.05 ml of plasma or serum was added to the 'Test' test tube. Mixed and incubated at 37° C for 15 minutes. 4.0 ml of NaOH was added to both the tubes and 0.05 ml of serum/ plasma was now added to the 'control' test tube. Mixed and cooled the tubes to room temperature. Measured the absorbance of test at 410nm /violet filter, setting the spectrophotometer /filter photometer to zero with the blank.

Pipetted 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ml of working PNP solution into the test tubes labelled S_1, S_2, S_3, S_4, S_5 and S_6 respectively. The final volume in each test tube was adjusted to 5.0 ml using NaOH solution. To the test tube labelled as blank 5.0 ml of NaOH was added and mixed well. The spectrophotometer / filter photometer was adjusted to zero absorbance at 410 nm / violet filter against 0.25M NaOH and measured the absorbance of the above standards.

Construction of calibration curve

The working PNP concentration is 54 m mol/L. Standard S1 contains 0.5ml PNP

$$Concentration \text{ of PNP in } S_1 = \frac{54}{1000} x0.5 = 0.027 \text{ mmol}$$

ALP activity in U/L = Liberation of 1 mmol of PNP per minute at 37^0C incubation per liter serum.

In the assay protocol, 0.05 ml serum is mixed with reagent and incubated for 15 minutes and the total volume is made up to 5.55ml. But the total volume in the case of each standard (S_1 to S_6) is 5.0 ml.

$$\text{ALP activity in U/L in the test sample} = \frac{\text{Test absorbance} \times (0.027) \times (5.55) \times 1000}{\text{Std. absorbance} \times (15) \times (5.0) \times 0.05}$$

$$\text{ALP activity in U/L in the test sample} = \frac{\text{Test absorbance}}{\text{Std. absorbance}} \times 40$$

Estimation of Plasma Creatinine

The plasma creatinine was measured by the Jaffe's Method (Slot, 1965).

Creatinine present in serum or plasma directly reacts with alkaline picrate resulting in the formation of a red colour, the intensity of which is measured at 505nm/green filter. Protein interference is eliminated using sodium lauryl sulphate. A second absorbance reading after acidifying with 30% acetic acid corrects for non-specific chromogens in the samples.

Reagents

1. Reagent A: Into 400ml of distilled water taken in a 500 ml beaker added 4.4g of NaOH. Mixed to dissolve, then added 9.5g trisodium phosphate [$Na_3PO_4.12H_2O$], dissolved and then added 9.5g of sodium tetraborate [$Na_2B_4O_7.10H_2O$]. After dissolving checked that the pH is above 10, adjusted if necessary by the drop wise addition of 1M NaOH. Transferred to a 500 ml volume flask and made up to 500ml with distilled water. Mixed well.
2. Reagent B: Dissolved 20g sodium lauryl sulfate in a final volume of 500ml distilled water.
3. Reagent C: Picric acid supplied commercially contains 50% by weight of water to ensure safety in transit. Therefore the amount of picric acid weighed out should be proportionally more than the amount of the required anhydrous picric acid. For reagent C, 4.6g of anhydrous picric acid is required. Therefore weighed approximately 7.0g but not less than 6.0g moist picric acid and added to 500ml of distilled water taken in a volumetric flask, mixed and left

overnight at 37^0 C. Then filtered and stored in brown glass bottle at room temperature (25-35^0C).

4. Working reagent: At the time of analysis freshly mixed equal volumes of the above three reagents.
5. Stock creatinine standard 100mg/dl: Dissolved 100 mg of pure creatinine in 0.1 M HCl and made up to 100 ml with 0.1 M HCl in a volumetric flask.
6. Working creatinine standard: Diluted 2, 4, 6 and 8 ml of stock creatinine standard each to 100 ml with 0.1 M HCl to get creatinine concentrations of 2, 4, 6 and 8 mg/dl, respectively. Stable for 6 months at 2-8 °C.
7. 30% (v/v) Acetic acid: Diluted 30ml of glacial acetic acid to 100ml with distilled water.

Procedure

Pipetted 3.0 ml of working reagent into each of the test tubes appropriately labelled as blank, S_1, S_2, S_3, S_4 and Test. Added 0.2 ml of distilled water to the blank. Added 0.2 ml of the respective standard to respective tube (i.e. Standards: S_1=2mg/dl, S_2=4mg/dl, S_3=6mg/dl & S_4=8mg/dl). Added 0.2 ml of the plasma to the 'test" in a test tube. Mixed well and left at room temperature for 30 minutes. The spectrophotometer/ filter photometer was adjusted to zero with blank at 505 nm/green filter and measured the absorbance of the other tubes. After measuring the absorbance poured the solutions back into the respective tubes. Then added 0.2 ml of 30% acetic acid to the 'Test' test tube, mixed well and left at room temperature (25-35^0C) for 5 minutes. Again set the spectrophotometer/filter photometer to zero with blank at 505 nm/green filter and measured the absorbance of test.

Construction of calibration curve

Subtracted the second absorbance values of test from the first set of values. Drew a calibration graph by plotting the absorbance values of standards against their respective concentrations. Plotted the corrected absorbance of test & read off the values of creatinine.

Estimation of Serum urea

Estimation of serum urea was done by the Diacetyl monoxime method (Wybenga *et al.*, (1971).

Urea reacts directly with diacetyl monoxime under strong acidic conditions to give a yellow condensation product. The reaction is intensified by the presence of ferric ions and thiosemicarbazide. The intense red colour formed is measured at 540nm/ yellow green filter.

Reagents

1. Stock acid reagent: Dissolved 1.0g of ferric chloride hexahydrate in 30 ml of distilled water. Added 20 ml orthophosphoric acid and mixed.
2. Mixed acid reagent: Added slowly 100 ml of Conc. H_2S04 to 400 ml distilled water taken in a 1-litre flat-bottom conical flask kept in an ice cold water bath. Mixed well and added 0.3ml of stock acid reagent. Mixed well.
3. Stock colour reagent – A: Dissolved 2g diacetyl monoxime in distilled water and make the volume up to 100 ml in a volumetric flask.
4. Stock colour reagent – B: Dissolved 0.5 g thiosemicarbazide in distilled water and made up to 100 ml in a volumetric flask.
5. Mixed colour reagent: Mixed 35 ml of stock colour reagent A with 35 ml of stock colour reagent B and made up to 500 ml with distilled water.
6. Colour reagent: The colour reagent is prepared fresh at the time of analysis by mixing distilled water, mixed acid reagent and mixed colour reagent in the ratio 1:1:1.
7. Stock urea standard: Weighed 1.0g of analytical-grade urea and dissolved in 100ml of benzoic acid (1g/dl).
8. Working standard 50mg/dl: Diluted 5.0ml of stock urea standard to 100 ml with benzoic acid.

Procedure

Four tubes were labeled appropriately as S_1, S_2, S_3, and Test. 0.1, 0.2 and 0.3 ml of urea solution was added to S_1, S_2 and S_3 respectively. Serum 0.1 ml of serum was added to the 'Test' test tube. The final volume in each test tube was adjusted to 2.0 ml with distilled water & Mixed well.

Another five test tubes were appropriately labeled as blank, S_1, S_2, S_3, and Test. 3.1 ml of the colour reagent was added to the blank whereas to all the remaining tubes 3.0 ml of the colour reagent was added. 0.1 ml from each of the first set of S_1, S_2, S_3, and 'Test' test tubes was transferred to each of the second set of S_1, S_2, S_3, and 'Test' test tubes respectively. All the tubes were mixed well. Kept them in a boiling water bath for 15 minutes. Removed from water bath and cooled the tubes for 5 minutes. The spectrophometer/filter photometer was adjusted to zero with blank at 540nm/yellow green filter and measured the absorbance of the other tubes.

Construction of calibration curve

The absorbance values of standards were plotted against their respective concentrations.

Statistical Analysis

The comparison of means before and after treatment has been carried out using paired *t*-test. And in the rest of the chapters the significance of the difference between the 6 groups has been tested using one way ANOVA. The calculated F-value and its significance are shown in the same table along with the p-value. It is observed that in all the cases the observed F-ratio is found to be significant with p-value less than 0.05. This recommends a further investigation into such groups whose means do not differ significantly.

Duncan'sMultipleRange Test (DMRT) test is one method used to compare the means of experimental groups with the means of control group. The procedure identifies the homogeneous groups and gives a list of such means which do not differ significantly. These groups are indicated with a common letter. The complete analysis has been carried out using MS excel 2007 and SPSS 16.

***In silico* studies**

Homolog modeling

Sur1 receptor is huge complex structure which possesses 12 trans-membrane helices which are aligned in the double membrane and two ATP binding domains were located at the cytoplasmic region. In the present study, ATP binding was evaluated for docking simulation. The protein sequence of domain II (ABC

transporter) (Uniprot ID: Q09428) consists of 242 residues (1344-1578) of Human was retrieved from SWISSPROT database. Template structure was obtained on the basis of sequences identity with high score, less e-value, highest resolution and R-factor by performing the search against PDB (Protein Data Bank). The coordinates for the query structure were assigned from template structure by using pair wise sequence alignment using ClustalX (Thompson *et al.*, 1997). The 3D models of AcrB efflux pump was built by using MODELLER$_{9.14}$ (Sali & Blundell, 1993). The least modeller objective (low DOPE Score) was obtained and loop modeling was performed by using MODLOOP Server (Fiser & Sali, 2003). In order to obtain stable conformation model energy minimization was carried out with GROMOS96 force field using SPDBV software.

In order to obtain stable conformation of Sur1 receptor, we performed Molecular dynamics (MD) simulations by means of GROMACS 5.0.2 (Berendsen *et al.*, 1995) using HP Workstations available at Sri Venkateswara University. The protein was subjected to 10 ns MD at 310 K in near physiological conditions (pH 7.0, 1atm pressure and 0.1 M ionic strength). AMBER99SB-ILDN force field was employed for all MD simulations carried out in the study (Lindorff-Larsen *et al.*, 2010). The structure of the proteins were used to prepare their conformations at pH 7.0 by PDB2PQR ver1.8 Server (Dolinsky *et al.*, 2004) and the resultant structures were used in all the MD simulations of the present study. In each MD, the protein molecule was placed at the center of a dodecahedron box and minimum distance of 1.0 nm between the wall and any part of the protein was set at initial stages of the simulations.

Molecular Dynamics simulations were performed under aqueous environment of 0.1 M ionic strength by treating the protein molecules with SPC water model (Berendsen, 1987) and addition of Na^+ (sodium) and Cl^- (chloride) ions. Initially, the solvated system was subjected to energy minimization using steepest descent algorithm (Vrahatis *et al.*, 2000), down to a maximum gradient of 1000 kJ/mol/nm to remove steric clashes and irregular geometry such as unrealistic bond distances, bond angles and torsion angles. The minimized system was then heated to desired temperatures for 1 ns under isothermal ensemble by soft coupling with Berendsen thermostat (NVT) (Berendsen *et al.*, 1984). The Vander Waals cutoff was set to 14 Å. The integration time step was 1 femto second (fs), with the neighbor list being

updated every fifth step by using the grid option and a cutoff distance of 12 Å. Periodic boundary condition was used with constant number of particles in the systems, constant pressure, and constant temperature simulation criteria (NPT). In this simulation, the systems were coupled with Parrinello-Rahman barostat (Parrinello & Rahman, 1981) to equilibrate at 1 bar pressure for 1 ns. Production simulations for 10 ns were performed.

Molecular docking

All the ligand molecules were uploaded into the Open Babel module of AUTODOCKVINA$_{4.0}$ (Trott *et al.*, 2010) with PyRx (Wolf, 2009) interface and energy minimized with UFF force field using conjugate-gradient algorithm with 200 run iterations and converted into pdbqt format. Protein was uploaded and converted into pdbqt format. Docking was carried out with all the compounds against Sur1 receptor by using Lamarkian genetic algorithm (Solis, 1981). Docking parameters were set as 150 Number of individual population, 25000 Max no. of energy evaluation, 27000 Max no of generation, Top individuals to survive to next generation is 1, Gene mutation rate of 0.02, Crossover rate of 0.8, Cauchy beta of 1.0 and GA window size is 10.0. The grid was set to whole protein due to the multi binding pocket at center x=-65.76, center y=-4.72, center z=25.59, size x= 98.20, size y=52.22, size z=89.10 and exhaustiveness 8. The best docked ligand conformations were saved and analyzed the bond angle, lengths, hydrogen bonding interactions etc., using PyMol (www.pymol.com).