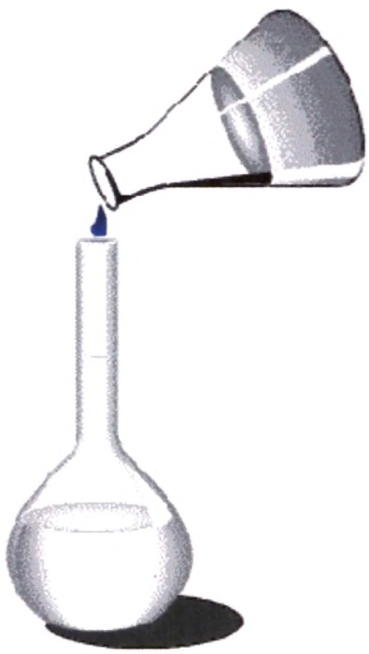
CHAPTER – II

MATERIALS AND METHODS



Chapter 2

Materials and Methods

The plant materials were collected from different localities of Baroda. The voucher specimens of these plants are deposited in BARO, the herbarium of Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat. Plant materials collected were washed, shade dried and later completely dried by keeping in an oven at 60°.The dried materials were powdered and stored in airtight plastic bags. This powder was used for the analysis of all the chemical constituents.

A. Pharmacognostic studies:

Micromorphology and Anatomy:

Micromorphological studies were carried out on fresh materials. Fresh leaves were washed and small fragments of leaves were taken from the middle region of the mature leaves. Washed leaf fragments were first boiled in 90% alcohol for about 3-5minutes to remove chlorophyll, then washed 2-3 times in water, then again boiled with 10% KOH solution for 2-3 minutes and washed 4-5 times in water and kept in clean water to remove all traces of the clearing agent. Both the epidermal layers were stripped off gently from the mesophyll tissue with the help of pointed needle and forceps. The epidermal peels were washed in water, stained with Toluidine blue (0.5%) and mounted in 50% glycerin; the margins of the cover slips were sealed with DPX. T. S. of leaf were taken by free hand and were stained in Toluidine blue (0.5%) and mounted in 50% glycerin. The slides were examined under the microscope and Camera Lucida sketches were drawn at 400x magnification and the size were measured using an ocular micrometer. The quantitative data are based on the average of 20 readings. Leaf constants such as stomatal index, stomatal frequency, size of guard cells, vein islet number, vein termination number and palisade ratio were calculated. Stomatal index (SI) was calculated as defined by Salisbury (1932) viz,

$$S.I = S / E+S \times 100$$

Where 'S' is the number of stomata per unit area, and 'E' is the number of epidermal cells in the same area. Stomatal frequency and stomatal index have been calculated out of an average of 20 readings.

Palisade ratio (PR), is calculated as the average of palisade cells (P) beneath each epidermal cell (E) as defined by Zoring and Weiss (1925).

$$PR = P / E$$

Small areas of the green tissue outlined by the veinlets are termed as vein-islets or areoles. The vein-islet number is defined as the number of vein-islet per sq.mm of the leaf surface midway between the midrib and the margins. Vein termination number is defined as the number of veinlet terminations per sq.mm of the leaf surface midway between the midrib and margin. A vein termination is the ultimate free termination of veinlet.

CLASS	SUBCLASS	TANGENTIAL DIAMETER (μM).
SMALL	1. Extremely small	< 25
	2.Very small	25 - 50
	3. Moderately small	50 - 100
MEDIUM	4. Medium	100 - 200
LARGE	5. Moderately large	200 - 300
	6. Very large	300 - 400
	7. Extremely large	> - 400

Powder study:

The finely powdered drug was scanned under 400x magnification for recording the cell elements.

B. Phytochemical Studies

1. Flavonoids:

Flavonoids are the widely distributed polyphenols and include all the C_6—C_3-C_6 compounds related to a flavone skeleton, consisting of a C_6-C_3 fragment (phenyl propane unit) that contain the B-ring and a C_6 fragment the A- ring, both being of different biosynthetic origin. They are subdivided on the oxidation levels of C_3 fragment of the phenyl propane unit as flavones, flavonols, chalcones, aurones etc (Geissman, 1962). Flavonoids occur mostly as water soluble glycosodes, though the lipophilic aglycones are less abundant. Out of the 2000 and odd flavonoids known (Harborne, 1988), about 500 are reported to be present in the Free State in lipid fractions, while the glycosides are located in the cell sap. Flavonoids containing up to five hydroxyl groups are sparingly soluble in water, but those with more methoxyl substituents become more lipophilic. Derivatives of the usual flavonoids with two and more methyl groups can be assumed to occur in most cases, without sugar attachment in lipid soluble fractions; where as monomethyl esters like isorhamnetin are often present in glycosidic combination.

The flavonoids are extracted in 70% ethanol or methanol and their glycosides remain in the aqueous layer, following partition of this extract with solvent ether. Due to the phenolic nature of flavonoids when treated with bases, (especially ammonia), their colours get changed and thus are easily detected in chromatograms or in solutions. Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in UV and in the visible regions of the spectrum. A single flavonoid aglycone may occur in a plant in several glycosidic combinations (Harborne, 1984) and for this reason, it is considered better to examine the aglycones present in hydrolysed plant extracts.

Fourty grams of the plant material was extracted in Soxhlet's extractor with methanol for 48hrs, till the plant material became colourless.The methanolic extract was concentrated to dryness in a water bath.100ml of water was added to the dry residue and the water soluble phenolic glycosides were filtered out.The filtrate was

hydrolysed in a water bath for 1hr using 7% HCl. The hydrolysate was extracted with ethylacetate whereby the aglycones separated in to the ethyl acetate fraction (fraction A).The remaining aqueous fraction was further hydrolysed for 10hrs to ensure the hydrolysis of all the O-glycosides. Aglycones were once again extracted into ethyl acetate (fraction B) and the residual aqueous fraction was neutralised and evaporated for the analysis of glycoflavones. Ethylacetate fractions A & B were combined and analysed for aglycones using standard procedures (Harborne, 1984; Mabry *et al.*,1970; Daniel 1991). The combined concentrated extract was banded on Whatman no.1 paper and chromatographed.The solvent system employed were 30% or 15% glacial acetic acid. The developed chromatograms were then sprayed with 10% aqueous sodium carbonate or 1% ferric chloride and the colour changes were recorded. The unsprayed chromatograms were then viewed in UV light (360 nm) and the flourescent regions marked with pencil. These bands of the compounds were cut out from chromatograms and were eluted with spectroscopic grade methanol. The UV absorption spectra of the compounds were recorded in methanol using 'Schimadzu UV 240' recorder spectrometer. The bathochromic and hypsochromic shifts induced by the addition of various reagents were studied. The reagents used and their preparation are given below (Mabry *et al* ., 1970).

Sodium methoxide (NaOMe): Freshly cut sodium metal (2.5gms) was added cautiously in small portions of spectroscopic methanol (100ml). The solution was stored in a tightly closed glass bottle.

Aluminium chloride ($AlCl_3$): Five grams of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow-green and reacted violently when mixed with water) were added continously to spectroscopic methanol (100ml).

Hydrochloric acid (HCl): Concentrated AR grade HCl (50ml) was mixed with distilled water (100ml) and the solution was stored in glass stoppered bottle.

Sodium acetate (NaOAc): Anhydrous powdered AR grade NaOAc was used.

Boric acid (H_3BO_3): Anhydrous powdered Argrade H_3BO_3 was used.

The concentrations of the sample solution prepared by eluting chromatogram strips were adjusted so that the optical density (OD) fell in the region of 0.6 to 0.8. The methanol spectrum was taken using 2-3ml of this stock solution. A reference solution was prepared by extracting a piece of blank chromatographic paper from the same chromatogram with spectroscopic methanol. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the flavonoidal solution used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of $AlCl_3$. Stock solution to 2-3ml of fresh stock solution of the flavonoids. $AlCl_3$ / HCl spectrum was recorded next, after the addition of three drops of the HCl stock solution to the cuvette containing $AlCl_3$.The solution was then discarded. For NaOAC spectrum, NaOAc was added by shaking the cuvette containing 2-3 ml of fresh solution of the flavonoids, till about a 2mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was recorded two minutes after the addition of NaOAc. NaOAc / H_3BO_3 spectrum was taken after sufficient H_3BO_3 was added to give a saturated solution. The solution was discarded after recording the spectrum.

The structure of a flavonoid was established by its absorption maxima (λ max) shape of the curves, shifts (both bathochromic and hypsochromic) with different reagents and colour reactions and R_f values the identifications were confirmed by co-chromatography with authentic samples.

Normally, a flavonoiod is linked to a sugar by O-glycosidic bonds, which are easily hydrolysed by mineral acids. But there is another type of bonding in which, sugars are linked to aglycone by C-C bonds. The latter group of compounds, known as C-glycosides (glycoflavones), are generally observed among flavones. They are resistant to normal methods of hydrolysis and will remain in the aqueous layer when hydrolysed extract was extracted with ether to remove aglycones. The procedures followed for isolating glycoflavones are described below.

The aqueous fraction remaining after the separation of aglycones was neutralized by the addition of anhydrous sodium carbonate / barium carbonate and later concentrated to dryness.

When barium carbonate was used barium chloride got precipitated and this is filtered. To this dried residue, ethanol was added to dissolve the glycoflavones. The alcoholic filtrate was concentrated, and banded on Whatman No.1 paper and the chromatogram was developed with water as the solvent system. Glycoflavones were visualized by their colour in UV and with 10% aqueous sodium carbonate spray. Further analysis and identification were done by measuring the λ max and spectral shifts and co-chromatography with authentic samples.

2. Phenolic acids:

Phenolic acids are phenols having a carboxylic group and varying number of hydroxyl groups. Acid hydrolysis of plant tissue releases a number of ether soluble phenolic acids, some of which are universal in distribution. These acids occur either associated with lignin or bound to the glycosides. They are also seen as depsides or as esters in hydrolysable tannin. Phenolic acids can be broadly divided into two groups: benzoic acids and cinnamic acids: Cinnamic acids (C_6-C_3) are the compounds derived from the aromatic amino acids. Some of the examples of cinnamic acids are caffeic, snapic acid and ferulic acid. Benzoic acids (C_6-C_1) are formed by side chain degradative removal of an acetate unit from corresponding cinnamic acids. The common benzoic acids are vanillic, syringic, gentisic, gallic and *p*-OH Benzoic acids. The phenolic acids are extracted in ether/ethyl acetate along with the flavonoid aglycones from the hydrolysed extract (Fraction A) of plant materials. Analysis of phenolic acids was carried out by two dimesional ascending paper chromatography. Solvent system used were Benzene: Acetic acid: Water (6 : 7 : 3, upper organic layer) in the first direction and Sodium formate: Formic acid: Water (10 : 1: 200) in the second direction. The spray reagents used to locate the compounds on the chromatograms were diazotized *p*- nitraniline and sulphanilic acid with 10% aqueous sodium carbonate as over spray (Ibrahim and Towers, 1960).

Preparation of *p*- nitraniline / sulphanilic acid:

0.7gms of *p*-nitraniline / sulphanilic acid was dissolved in 9ml of HCl and the volume made up to 100ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature was below 4°C. The diazotized sprays were prepared by adding 4ml of *p*-nitraniline / sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made up to 100ml with cold distilled water. The various phenolic acids were identified based on colour reactions they produce against the azo dyes, the relative R_f values and co-chromatography with authentic samples.

3. Tannins:

Tannins are polyphenols of high molecular weight which can combine with protein, forming water insoluble and non-putrescible leather. There are two main types of tannins recognised based on this reaction with mineral acid viz:- the condensed tannins and the hydrolysable tannins. Condensed tannins which are universally present in ferns, gymnosperms and woody angiosperms, get easily polymerized on hydrolysis to phlobaphenes and anthocyanidins. They are mostly insoluble in water and are dimers containing hydroxy flavans such as catechins and flavan 3, 4- diols. Since the condensed tannins liberate anthocyanidins on hydrolysis, they are known as leucoanthocyanidins or proanthocyanidins. Hydrolysable tannins are mostly gallotannins and ellagitannins depending on whether gallic acid or ellagic acid is present esterified within glucose.They yield the corresponding phenolic acids and glucose on hydrolysis. Hydrolysable tannins are limited to dicotyledonous plants.Tannins are extracted in water and are tested by treating them with protein solution.

Five grams of plant material was boiled in 10ml of distilled water and filtered. To this, freshly prepared 2% gelatin solution was added. The formation of a white (milky) precipitate indicated the presence of tannins (Hungund and Pathak, 1971).

4. Steroids:

They are derived triterpenoids having a cyclopentanoperhydrophenanthrene skeleton. The plant steroids generally occur free or in a form conjugated with one or more sugar residues including glucose, galactose, xylose, rhamnose etc. The various

sterols found in plants differ in the number of carbon atoms in the side chain attached to C_{17}, the degree and position of unsaturation in the rings and in the side chain substitution at C_3 and in the steriochemistry at asymmetric centres. The names of the plant sterols are often perplexing because the most common sterols are refered by their trivial names. The sterol most often isolated from plants is β-sitosterol. The ether fraction of the plant extract which contained the steroids was analyzed by thin layer chromatography, using Chloroform: Carbon tetrachloride: Acetone (2: 2: 1) as the solvent system. The plates was sprayed with Libermann Burchard reagent or 50% sulphuric acid and heated at 80-90°C for 15 minutes. (The Libermann Burchard reagent was prepared by mixing 1ml of concentrated sulphuric acid, 12ml of acetic anhydride and 50ml of chloroform). Steroids gave characteristic colours.

5. Saponins:

They are steroidal or triterpenoid glycosides which form emulsions with water due to their hydrophobic and hydrophylic ends and possess marked haemolytic properties. The steroidal saponins are common in monocots, while the triterpenoid saponins are found in dicots. In saponins, the sugars occur as oligosaccharides combined with the aglycone (sapogenin). Some of the saponins exhibit biological activities in higher plants like promotion of seed germination or inhibition of root growth.

For testing saponins, about 5gms of the powdered plant material was boiled with 50ml water for half an hour. The extract was filtered; the filtrate was taken in a test tube after cooling and shaken vigorously for a minute or two. The formation of a persistant froth of 1cm height indicated the presence of saponins (Hungund and Pathak, 1971). Foam formation takes place even during extraction with aqueous solvents if the concentrations of the saponins are more in the plant materials (Harborne, 1984; Daniel, 1991).

6. Proanthocyanidins:

Proanthocyanidins are condensed tannins and as the name indicates, they yield anthocyanidins on hydrolysis. Since proanthocyanidins are colourless polymers, their

presence was confirmed by hydrolysing the plant material and subsequent extraction and identification of the anthocyanidins.

For testing the presence of proanthocyanidins, five grams of finely chopped fresh plant material or two grams of dry powdered material was taken in a test tube and treated with 2N HCl. The test tube was kept in boiling water bath for 30 minutes. The extract was denoted after cooling and shaken with amyl alcohol. The presence of a red or near carmine colour in the upper alcohol layer indicated the presence of proanthocyanidins in the plant material. An olive yellow colour represented a negative reaction (Gibbs., 1974).

7. Iridoids:

Iridoids are bitter monoterpenoid glycosides having a cyclopentanopyran/ furan ring, and are mostly confined to the advanced dicots. For the analysis of iridoids, one gram of fresh plant material was taken in a test tube and was covered with 5ml of 1% aqueous HCl. The test tube was kept for 3-6 hours and filtered. 0.1ml of filtrate was added to another test tube containing 1ml of Trim- Hill reagent (1ml of 0.2%$CuSO_4.5H_2O$ in water and 0.5ml con.HCl.). When the test tube was heated for a short while on a flame, a characteristic colour was produced, if iridoids were present. Asperulosides and aucubins and monoterpene give blue colour and herpagide, a red violet colour (Harborne, 1984).

8. Quinones:

Quinones are aromatic di-ketones and form the largest class of natural colouring matters. They are generally known from higher plants and fungi. In plants, they are present in the bark or underground parts. In the leaves, their colour is masked by other pigments. It is assumed that they play some role in oxidation- reduction processes. Quinones are classified into benzo, naphtha and anthraquinones, depending on the mono, bi or tricyclic ring system they contain.

For extraction of quinones, approximately 10 grams of dried, powdered plant material was exhaustively extracted with hot benzene for 36 hours. The extract was filtered and the filtrate was then concentrated to a dry residue. The residue was dissolved in solvent ether and segregated into acidic and neutral fractions by repeatedly shaking with 2N Na_2CO_3. The Na_2CO_3 soluble fraction was acidified with ice-cold 2N HCl added drop wise, till the precipitate formed, settled down. The acidified solution, in turn, was extracted from diethyl ether.

The ether fraction is chromatographed over T. L. C (Silica gel) plates using petroleum ether: benzene (9:1) as the solvent system. The neutral fraction was also chromatographed over silica gel T L C plates using the same solvent system. The various quinones (anthra, benzo and naphthaquinones) were visualised by their colours in visible / UV light, colour reactions after spraying with 2% magnesium acetate or 10% aqueous sodium hydroxide. The quinones gave purple/ pink/ orange/ yellow colours and characteristic absorption spectra in UV and visible light.

9. Alkaloids:

They comprise the largest class of plant products. Though almost all the plant bases are included in this group, a typical alkaloid is a basic plant product possessing nitrogen containing heterocyclic ring and marked pharmacological activity. The alkaline nature of alkaloids is due to nitrogen, the number of which may vary from one (atropine) to five (ergotamine). Alkaloids are normally extracted from plants into weak acids (1M HCl or 10% acetic acid) or acidic alcoholic solvents and then precipitated with concentrated ammonia. They are also extracted into any organic solvent after treating plant materials with a base. The bases free the alkaloids and make them soluble in organic solvents. From the organic solvents, the alkaloids are extracted into acidic solutions and tested with specific reagents. For testing the presence of alkaloids, five grams of powdered plant material was extracted with 50ml of 5% ammoniacal ethanol for 48 hrs. The extract was concentrated by distillation and the residue was treated with 10 ml of 0.1N H_2SO_4. The acid soluble fraction was tested with Mayer's reagent. Formation of a white precipitate denoted the presence of

alkaloids. (Amarsingham *et al.*, 1964). Other reagents used were Wagner's and Dragendorff,s reagents (Peach and Tracey, 1955).

Mayer's reagent (Potassium mercuric iodide): 1.36 grams of mercuric chloride was dissolved in 60ml of water. The two solutions were mixed and diluted to 100ml with distilled water.

Wagner's reagent (Potassium iodide): 1.27 grams of iodine and 2 grams of potassium iodide were dissolved in 5ml of water and the solutions diluted to 100ml.

Dragendorff's reagent (Potassium bismuth iodide):

0.85 gram of bismuth subnitrite dissolved in 40ml water and 10ml acetic acid 8 grams of potassium iodide in 20ml of water. The two solutions were mixed and allowed to stand, when potassium nitrate crystallised out. The supernatent was decanted and made up to 100ml with distilled water.

C. Pharmacological Studies

In this part of study clinical evaluation of antidiabetic efficacy, antioxidant activity, hypolipidemic effect and renal toxicity of the selected plants, in diabetes induced rats along with that evaluation of optimum dose determination and its efficacy in improving glucose tolerance and their safety profile in normal rats were also tested.

The Chemicals used were procured from standard chemical companies. Streptozotocin (STZ), were procured from Sigma Chemical Co., U.S.A., Alloxan monohydrate and all other chemicals were of AR grade and purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Qualigens Fine Chemicals, Mumbai, India and kits for serum total cholesterol, triglycerides, HDL-cholesterol and GOD-POD kits for blood glucose estimation were purchased from Reckon Diagnostic Pvt. Ltd., Baroda, India. Kit for determination of plasma urea, uric acid, and creatinine were procured from Randox, Laboratories Ltd., Ardmore, United Kingdom.

Preparation of Aqueous extract:

Five hundred grams of fresh leaves was boiled in water for 2 h. The extract was then filtered and the process of boiling was repeated three times with the residue, each time collecting the extract. The collected extract was pooled and passed through a fine muslin cloth. The filtrate upon evaporation at 40°C yielded 14.5% semi solid extract.

Herbal Formulations:

The plants used in formulation were collected fresh, dried in shade and crushed. The plants were taken in the ratio mentioned and the aqueous extract was prepared as mentioned above, the filtrates were combined and concentrated to get the concentration as 1gm dry plant equivalent extract/ml.

Animals:

Male Wister and Sprague Dawley rats (Weighing 150-200g) were used in the experiment under standard environmental conditions, and the animals were fed on standard pellet diet and allowed water *ad libitum.*

To induce diabetes Alloxan as well as Streptozotocin were used, which are diabetogens specifically destroys β-cells by generating free radicals and produces diabetes like condition in rats. Alloxan [(150 mg/kg body weight) dissolved in Phosphate buffer saline (0.1M, pH 7.4)] and Streptozotocin [(50 mg/ kg body weight) dissolved in citrate buffer (0.1 M, pH 4.5)]. The route of injection to induce diabetes was intraperitoneal. Diabetes was confirmed in rats by measuring the fasting blood glucose concentration, 96 h after the injection. Rats with blood glucose level above 250 mg/dl were considered to be diabetic and were used in the experiment. After the induction of diabetes the rats were divided in to different groups and treated with plant extract for different time period. After the treatment, the rats were fasted for 12 hours then sacrificed by cervical dislocation. Blood samples were collected in tubes containing anticoagulant oxalate: fluoride (1:3). Plasma samples were obtained by centrifugation and stored at -20^0C till measurement. After plasma separation, the buffy boat was removed and packed cells were washed thrice with physiological

saline (0.89% NaCl). Aliquots of packed cells were kept at 4°C overnight. Known volume of RBC was lysed with water. The hemolysate was separated by centrifugation at 10,000 X g for 15 minutes at 20°C. Aliquots were immediately used for enzyme assay. Weighed kidney, liver, brain samples were homogenized on ice in the appropriate buffer (1/10 parts (w/v)). The homogenate was centrifuged at 10,000 X g for 15 min at 4°C to discard any cell debris. The supernatant was immediately used for enzyme assay.

Statistical analysis:

All quantitative measurements were expressed as Mean ± SD for control and experimental animals. Statistical analysis was carried out by using SPSS* / PC ie., statistical package for social science (Duncan's multiple Range Test [DMRT]). A probability level of $P < 0.05$ was chosen as the criterion of statistical significance.

Experimental Designs:

SR.	PLANTS	EXTRACTS	ANIMAL MODEL	CHEMICALS USED TO INDUCE DIABETES	ROUTE OF INJECTION
1.	*Cassia alata*	Alcoholic extract	Male wister Rats	Streptozotocin	Intraperitoneal
		Aqueous extract of dry leaf			
		Aqueous extract of fresh leaf	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
2.	*Artocarpus heterophyllus*	Aqueous extract of fresh leaf	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
3.	*Bauhinia purpurea*	Aqueous extract of fresh leaf	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
4.	*Costus pictus*	Aqueous extract of fresh leaf	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
5.	Herbal formulation A	Aqueous extract	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
6.	Herbal formulation B	Aqueous extract	Male Sprague Dawley Rats	Alloxan	Intraperitoneal
7.	Herbal formulation C	Aqueous extract	Male Sprague Dawley Rats	Alloxan	Intraperitoneal

THE WEIGHT OF THE RATS WERE BETWEEN 150-200 G

To check the toxicity and the blood glucose lowering capacity of the plant extract (Hypoglycemic activity) the rats (weighing up to 150-200 g) were divided in to different groups in which each group contain six animals. In each group the first group served as control which received vehicle solution containing 2% gum acacia. Table listed below shows different doses of plant extract, Route of administration and Period of treatment.

SR.	PLANT	EXTRACT	GROUPS	DOSES/ MG BODY WEIGHT	ROUTE OF ADMINISTRATION	PERIOD OF TREATMENT
1.	*Cassia alata*	Alcoholic extract	Six	Control, 100, 200, 400, 600 and 1000	Oral	15 days
		Aqueous extract of dry leaf	Six	Control, 100, 200, 400, 600 and 1000	Oral	15 days
		Aqueous extract of fresh leaf	Six	Control, 100, 200, 400, 600 and 1000	Oral	15 days
2.	*Artocarpus heterophyllus*	Aqueous extract of fresh leaf	Seven	Control, 100, 200, 400, 600, 800 and 1000	Oral	30 days
3.	*Bauhinia purpurea*	Aqueous extract of fresh leaf	Six	Control, 100, 200, 400, 600, 800 and 1000	Oral	30 days
4.	*Costus pictus*	Aqueous extract of fresh leaf	Six	Control, 100, 200, 400, 600 and 1000	Oral	30 days
5.	Herbal extract A	Aqueous extract	Six	Control, 100, 150, 300, 400, and 500	Oral	15 days
6.	Herbal extract B	Aqueous extract	Six	Control, 100, 200, 300, 400 and 500	Oral	15 days
7.	Herbal extract C	Aqueous extract	Six	Control, 100, 200, 300, 400 and 500	Oral	15 days

* ORAL ADMINISTRATION WAS GIVEN USING AN INTRAGASTRIC TUBE

To check the antidiabetic activity of the plant extract, the diabetes induced rats (weighing up to 150-200 g) were divided in to different groups and treated for different time intervals as given in the table below. The first group served as control- normal rats which received 2% gum acacia and second group served as diabetic control which was made diabetic and not treated. Last group served as standard which received 600µg of standard drug glibenclamide dissolved in 2% gum acacia to compare the effect of the plant extract.

SR. NO	PLANT	EXTRACTS	GROUPS	DOSES/ MG BODY WEIGHT	ROUTE OF ADMINISTRATION	PERIOD OF TREATMENT
1.	*Cassia alata*	Aqueous extract of dry leaf	Seven	400 and 600	Oral	45 days
		Aqueous extract of fresh leaf	Five	400 and 600	Oral	45 days
2.	*Artocarpus heterophyllus*	Aqueous extract of fresh leaf	Five	400 and 600	Oral	60 days
3.	*Bauhinia purpurea*	Aqueous extract of fresh leaf	Five	200 and 400	Oral	60 days
4.	*Costus pictus*	Aqueous extract of fresh leaf	Five	200 and 400	Oral	60 days
5.	Herbal extract A	Aqueous extract	Five	100 and 150	Oral	30 days
6.	Herbal extract B	Aqueous extract	Five	100 and 200	Oral	40 days
7.	Herbal extract C	Aqueous extract	Five	200 and 300	Oral	40 days

*** ORAL ADMINISTRATION WAS GIVEN USING AN INTRAGASTRIC TUBE**

Biochemical estimations:

1. Determination of Glucose in Plasma. (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 aldehydes 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 in to 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:

I. Commercially available kits contain following reagents:

II. Enzymes (Glucose oxidase- peroxidase) with chromogen and phenol.

III. Glucose standard (100mg/dl) (Range of standard: 10-50 µg).

Procedure:

REAGENT	BLANK	STANDARD	TEST
ENZYME REAGENT	1.0 ml	1.0 ml	1.0 ml
GLUCOSE STANDARD	-	0.01 ml	-
SERUM	-	-	0.01 ml

All the test tubes were incubated for 15 minutes at RT or for 10 minutes at 37^0C and then absorbance were read at 505 nm against blank.

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

Units: Mg/dl

2. Determination of Oral Glucose Tolerance Test. (Whittington *et al.*, 1991)

The animals were fasted over night and given only water to drink. Fasting blood samples were drawn by sino-ocular puncture and each animal was given an oral glucose load of 2g/kg body weight. From a 30% (W/V) solution 30ml of glucose was given orally by oral syringe. Additional tail blood samples were drawn at 0, 30, 60 and 90 minutes after the glucose load. All the samples were collected in heparinized test tube and were immediately used for glucose determination by a glucose kit (Tinder et al., 1969)

OGTT curve was prepared by plotting blood glucose (mg/dl).

3. Determination of Total protein. (Lowry, 1951)

Principle:
The principle involved two steps. The carbomide groups of protein react with copper and potassium of lowery's reagent to give blue colour complex. This complex together with tyrosine and phenolic compound present in the protein reduce. The phosphomolybdate of the folin's phenol reagent to intense blue colour read at 650 nm.

Reagents:
Lowry's reagent:

Solution A:-

2% sodium carbonate in 0.1N NaoH.

Solution B:-

0.5% copper sulphate in 1% sodium potassium tartarate mix solution A to solution B before use.

Standard protein solution :-

100 mg of BSA dissolved in 100 ml of 0.9% Nacl Concentration = 1 mg/ml.

Working standard solution :-

25 ml of the stock was made up to 100ml concentration = 250 mg/ml

Folin's reagent = 1: 2

Procedure:

From the working standard 0.2-1 ml of protein solution is pipetted out in to another test tube and made up to 4ml with distilled water. The blank containing 4ml of distilled water was also maintained 0.1 ml of serum was diluted to 1 ml with distilled water from this 0.1 ml was taken and again diluted to 1 ml with 10% TCA and centrifuged. The supernatant was discarded and to the precipitate 1 ml of 0.1N NaOH was added from that 0.1 ml was taken for estimation.

To all the tubes 5.5 ml of lowry's reagent was added and allowed to stand for 10 minutes then 0.5 ml of folin's phenol reagent was added and the colour developed was read at 650 nm after 30 minutes.

The standard graph was drawn at concentration at X-axis and OD on Y-axis the amount of protein in the serum was calculated.

Values were expressed in g/dl.

4. Estimation of Tissue and plasma TBARS. (Nichans and Samuelson, 1968)

Principle:-

In this method melondialdehyde and other thiobarbituric acid reactive substances (TBARS) were measured by their reactivity with Thiobarbutric acid in the acidic condition to generate a pink coloured chromophore which was read at 535 nm.

Reagents Required:

1. Trichloro Acetic acid (TCA) = 15%

Hydrochloric acid (HCL) = 0.25%

Thibarbutric acid (TBA) = 0.375 % in hot distilled water

TCA-TBA-HCL reagent: - Mix the solutions in the ration of 1:1:1 (Freshly prepared)

Stock Standard:
4.8 mmol of stock was prepared from 1, 1', 3, 3'-Tetramethoxy propane commercially.

Working Standard: - Stock solution was diluted to get a concentration of 48 nmol/ml.

Procedure:

Reagent:

REAGENTS	BLANK	BLOOD	TISSUE
SAMPLE	-	0.5 ml	0.5 ml
D/W	2.5 ml	2 ml	2 ml
TBA-TCA-HCL	2 ml	2 ml	2 ml

The mixture was kept in a boiling water bath for 15 minutes, cooled and centrifuged at 3000 rpm for 15 minutes and read the absorbance at 532 nm.

A series of standard solution in the concentration of 2-10 nmole were treated in a similar manner

Calculation:
Calculation was done according to the slope calculated from the standard graph

The values were expressed as nmol/ml plasma or g tissue.

5. Estimation of RBC TBARS. (Donnan, 1950).

Principle:
The pink chromogen formed by the reaction of thiobarbituric with breakdown products of lipid peroxides was read at 535nm.

Reagents:
1. Stock malondialdehydes solution: 1, 1', 3, 3' Teramethoxypropane
2. 10% Trichloro acetic acid (TCA)
3. 0.67% Thiobarbituric acid (TBA)

Procedure:
The reaction mixture in total volume of 1.7 ml contained 0.2 ml of erythrocytes and 1.5 ml of 10% Trichloro acetic acid, the mixture was filtered through whatman no: 1filter paper. Thiobarbituric acid was added to the portion of filtrate (usually 0.6 ml or 0.8 ml)

in the ration of 1.2 ml to 1 ml. The mixtures cooled to room temperature and the colour developed was measured at 535 nm.

Values were expressed as nmoles / ml packed cell.

6. Determination of 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 form GSSG (by GSH reductase). And its utilization (by peroxidases, transferases, trenshydrogenases 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 (2mM GSH) (Standard range 10-100 µg)

Sample prepration – Anticoagulated whole blood or 10% tissue homogenated in phosphate buffered saline (PBS) (0.1M, pH 7.4).

Procedure:-

REAGENTS	BLANK	BLOOD	TISSUE
SAMPLE	-	0.1 ml	1.0 ml
D/W	1.0 ml	0.9 ml	-
PPTING REAGENT	1.5 ml	1.5 ml	1.5 ml

The tubes were kept for 5minutes and then centrifuged at 3000 rpm for 15 minutes

SUPERNATANT	0.5 ml	0.5 ml	0.5 ml
Na_2HPO_4 SOLUTION	2.0 ml	2.0 ml	2.0 ml
DTNB	0.25 ml	0.25 ml	0.25 ml

Absorbance was read 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.

7. Estimation of Ascorbic acid. (Omaye *et al.*, 1979)

Principle:

Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketoglutonic. These precipitates are treated with 2,4 – dinitrophenyl hydrazone, this compound in strong H_2SO_4 undergoes a rearrangement to form a precipitate with an absorbance maxima at 520nm.

Reagents:

1. TCA: - 5% and 10% in distilled H_2O
2. 2, 4:- DNPH/ Thioure/ Copper (DTC) Solution:-

0.4 g thiourea, 0.05 g $CuSO_4$ and 3.0 g 2, 4 DTNB. Were added and made up to a volume of 100 ml with 9N sulfuric acid.

3. H_2SO_4 65% solution

4. Standard :- 10 mg of /100 ml of 5% TCA.

Procedure:

1.0 ml of whole blood was added to 1.0 ml of ice cold 10% TCA mixed thoroughly and centrifuged for 20 minutes at 3,500 g to 0.5 ml of supernatant was taken and mixed 0.1 ml of DTC and incubated for 3 hours at 37^0C to form the Bis- 2, 4- dinitrophenyl hydrazine then 0.75 ml of ice cold 65% H_2SO_4 was added and mixed well and the solution were allowed to stand at room temperature for half an hour and read at 520 nm, standard were made in 5% and ranged from 0-20 µg/ml of ascorbic acid were taken and processed similarly along with a blank containing 2.0 ml of 4% acid.

Values were expressed as mg/dl.

8. Estimation of α -Tocopherol. (Baker *et al.*, 1980)

Principle:-

This method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured complex with 2, 2'-dipyridyl, absorbance of the chromophore was measured at 520nm

Reagents:-

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

Procedure:-

To 0.5 ml of plasma, 2.0 ml of petroleum ether and 1.5 ml of ethanol were added, mixed and centrifuged. To the Supernatant, 0.2 ml of 2, 2'-dipyridyl solution and 0.2 ml of ferric chloride solution were added, mixed well and kept in dark for 15 minutes. An intense red colour was developed; 4.0 ml of water was added to all the tubes and mixed well.

Standard α-Tocopherol in the range of 10-100 μg were taken and treated similarly along with a reagent blank. The colour in the aqueous layer was read at 520 nm.

Values were expressed as mg/dl.

9. Determination of Superoxide Dismutase (SOD). (Kakkar *et al.*, 1984).

SOD is present in the entire aerobic organism. 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 metallo enzyme, which catalyses dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen (O_2)

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

Two isoenzymes that is CU-Zn SOD (Cytosol) and Mn-SOD (mitochondria) are present in eukaryotic animals, which are present in eukaryotic animals, which are independently regulated according to the degree of oxidative stress experienced in the respective sub cellular compartments, but how it is communicated at molecular level is unknown.

Reagents:

1. 0.89%
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 1-2 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:

Mixture of NADH and phenazine methosulphate (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 colored formazone product, which can be measured at 560 nm.

Procedure

REAGENTS	TEST	CONTROL
SODIUM PHOSPHATE BUFFER	1.2 ml	1.2 ml
PMS	0.1 ml	0.1 ml
NBT	0.3 ml	0.3 ml
DILUTED ENZYME	0.01 ml/0.02 ml	-
D/W	1.2 ml/1.18 ml	1.2 ml
NADH	0.2 ml	0.2 ml

All the tubes were incubated for 90 seconds art 37^{0C} 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:

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

Or Tissue wt (g/dl)

Unit: one unit of SOD id defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

10. Superoxide dismutase (SOD). (Marklund and Marklund, 1974).

Principle:
The superoxide dismutase activity inhibits pyrogallol auto-oxidation under standard assay condition, the reaction being measured at 420 nm.

Reagents:
1. KPO_4 (0.2M, pH 8)

2. Pyrogallol: a pinch of pyrogallol is dissolved in 0.5N HCL, so as to achieve auto-oxidation within 0.1 to 0.120 O.D/ 180 sec (freshly prepared)

Protocol:
Pyrogallol auto-oxidation: 0.5 ml buffer = 0.450 ml D/W (auto zero) + 0.050 ml pyrogallol (start)

Auto-oxidation should be between 0.1 to 0.120 O. D. /180 seconds. The reaction proceeds as per the protocol, reaction starts by addition of pyrogallol.

REAGENTS	BLANK	TEST
ALIQUOT (ML)	-	0.03
KPO_4 (ML)	0.5	0.5
D/W (ML)	0.45	0.42
PYROGALLOL (ML)	0.05	0.05
O. D. 420		

Auto-oxidation (X) – Test (Y) = Difference (Z)

$$\% \text{ Inhibition (A)} = \frac{z \times 100}{X}$$

Now, % inhibition (A) → 30 µl

Therefore 50% inhibition →? (Say, B µl)

Again, B µl → 1 unit

There fore 1000 µl →? Unit → $\frac{1000\ \mu l}{B\ \mu l}$

Units: Enzyme required for 50% inhibition of pyrogallol auto-oxidation.

11. Assay of Catalase (CAT). (Sinha, 1972).

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 (micro bodies). Highest CAT activity is found in liver and kidney and lowest in connective tissue. In tissue it is mianly present bound to the membranes of mitochondria and peroxisomes, where as it exist in soluble state in erythrocyte (Hugo, 1987).

Principle:-

Catalase was assayed colorimetrically by the method of Sinha (1972)

$$2H_2O_2 \longrightarrow O_2 + 2H_2O$$

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic acid as an unstable intermediate. The chromium acetate is measured colorimetrically at 620 nm. The Catalase preparation is allowed to split H_2O_2 for different time. This reaction is stopped at different time interval by the addition of dichromic acetic acid mixture and the remaining H_2O_2 is determined colorimetrically.

Reagents:

1. Phosphate buffer, pH 7.0, 0.01 M

2. Dichromate acetic acid reagent:-

This reagent was prepared by mixing 5% solution of potassium dichromate ($K_2Cr_2O_7$) with glacial acetic acid in the ratio 1:3

3. H_2O_2 (0.2 M)

2.09 ml of H_2O_2 was dissolved in 100 ml of distilled water and was prepared freshly and kept in dark bottle.

Procedure:

To 100 ml of the phosphate buffer, taken in each of the test tube, 100 µl of homogenate was added. To this 100 µl of H_2O_2 was added. The reaction was stopped at 0 and 60^{th} seconds by the addition of 2.0 ml of dichromate acetic acid reagent. The tubes were heated for 10 minutes in a water bath and read at 620 nm. For standard different concentrations of H_2O_2 ranging 40 to 160 µmoles were taken in test tubes and reaction mixture was prepared as discussed above.

Activity of Catalase was expressed as µmoles of H_2O_2 utilized/minutes/mg of protein.

12. Assay of glutathione peroxidase. (Paglia and Valentine, 1976).

Principle:

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

Reagents:

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

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

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

4. Sodium azide (10mM) 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 (0.4M)

Procedure:

Ten percentage (10%) homogenate was prepared in PBS (pH 7.4) or Haemolysate as mentioned in earlier methods. Centrifuged and the supernatant (tissue) was used as enzyme source. Proceed as follows.

0.4M PHOSPHATE BUFFER	0.10 ml
2MM GSH	0.10 ml
10MM NaN_3/KCN	0.10 ml
ENZYME	0.01 ml
D/W	0.09 ml

Incubate at 37^{0}C for 10 minutes Now add, 10 mM H_2O_2 – 0.10 ml

Incubate at 37^{0}C for 3 minutes. Add precipitating reagent – 0.40 ml

Wait for 5 minutes and centrifuge. Use supernatant and proceed as follows.

SUPERNATANT	0.6 ml
0.4M Na_2HPO_4	0.6 ml
DTNB	0.03 ml

Control tubes were processed without enzymes

Blank was processed without GSH

Reading was taken at 412 nm

Units:-

Mmoles of GSH utilized/g Hb/min or mmoles of GSH utilized / mg protein / minute.

13. Determination of Glucose-6-phosphatase [E. C. 3.13.9]. (Koide and oda, 1959).

Reagents

1. 0.01 M maleic acid buffer at pH 6.5

2. Glucose-6-phosphate: - 0.01 M in distilled water

3. Ammonium molybdate solution 2.5 g of ammonium molybdate was dissolved in 100 ml of 3N sulphuric acid.

4. Amino naphthol sulphonic acid (ANSA) 500 mg of 2-amono-4-naphthol sulphonic acid was dissolved in 195 ml of 15 percent sodium bisulphate and 5.0 ml of 20 percent sodium sulphite solution was added for complete solubilisation. The solution was filtered and stored in the brown bottle

5. 10 per cent TCA.

Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.3 ml of buffer, 0.5 ml of substrate and 0.2 ml of tissue homogenate. The rotion mixture was incubated at 37^0C for one hour.

Addition of 1.0 ml and 10 percent TCA to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphrous content of the supernatant was estimated by the method of fiske and subbarow for phosphorus estimation.

The supernatant was made up to a known volume, to this, 1.0 ml of ammonium molybdate solution was added followed by 0.4 ml of ANSA. The blue colour developed after 20 minutes was read at 620 nm. A set of standard phosphorus was also treated as given above.

The enzyme activity was expressed as μmoles of phosphate liberated/minute/ml.

14. Assay of fructose-1, 6-bisphosphatase [EC 3. 1. 3. 11]. (Gancedo and Gancedo, 1971)

Reagents

1. Tris-HCL buffer: 0.1 M, pH 7.0

2. Substrate: Fructose-1, 6-bisphosphate, 0.05 M

3. Magnesium chloride: 0.1 M

4. Potassium chloride: 0.1 M

5. EDTA solution: 0.001 M

6. TCA: 10%

7. Molybdic acid: 2.5% ammonium molybdate in 3 N sulphuric acid.

8. Aminonaphthol sulphonic acid (ANSA)

9. Phosphorus stock standard: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100 ml of distilled water (80 μg/ml).

Procedure

The assay medium in a final volume of 2.0 ml contained 1.0 ml buffer, 0.4 ml of substrate, 0.1 ml each of magnesium chloride, 0.2 ml potassium chloride, 0.1 ml of EDTA and 0.2 ml of enzyme source. The incubation was carried out at 37^0C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus content of the supernatant was estimated according to the method described by fiske and subbarow.

To 1 ml of an aliquot of the supernatant, 0.3 ml of distilled water and 0.5 ml of ammonium molybdate were added. After 10 minutes, 0.2 ml of ANSA was added. The tubes were shaken well, kept aside for 20minutes and the blue colour developed was read at 620 nm.

The values are expressed as μmoles of inorganic phosphrous liberated/h/mg protein.

15. Estimation of phosphorus by the method of Fiske and Subbarow.

Aim:-

To estimate the amount of phosphorus present in the given sample

Principle

In the determination of total phosphrous, organic matter is destroyed by the digestion with H_2SO_4. The phosphrous in the sample reacts with ammonium molybdate to produce phosphomolybdate compound which was then reduced by the addition of amino naphthol sulphonic acid (ANSA) to give a blue coloured compound which was colorimetrically read at 680 nm.

Reagents required:-

1. Molybdate I reagent:

Dissolve 2.5% of ammonium molybdate in $5NH_2SO_4$

2. Molybdate III reagent:

Dissolve 2.5% of ammonium molybdate in 3N H_2SO_4

3. 0.25% ANSA:

Add 0.5g of ANSA to 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite

4. 10% TCA

5. 10N H_2SO_4

6. 15% sodium bisulphate

7. 20% sodium sulphite

8. Stock standard solution:

Dissolve 35% of pure potassium dihydrogen phosphate in 10 ml of 10N H_2SO_4 and made up to 100 ml with distilled water [rain water]

9. working standard solution:

1 in 10 dilution

Procedure:

To 0.3 ml of serum, add 4 ml of 10% TCA. Mixed well and centrifuged. To 0.5 ml of supernatant added 1 ml of molybdate III reagent. Mixed well and then added 0.4 ml of ANSA reagent and make up to 7 ml with distilled water.

For the standard curve various concentration of working standard solution ranging from 0.5-2.5 ml are taken and made up to 7 ml with distilled water (rain water) added 1 ml of molybdate I reagent and 0.4 ml of ANSA. The blank was also maintained the colour developed was read at 680 nm.

The amount of phosphrous present in the given sample is expressed as mg/dl.

16. Assay of Hexokinase. (Brand trup *et al.*, 1957)

Reagents:

1. 0.005 M glucose solution
2. 0.72 M ATP solution
3. 0.05 M magnesium chloride solution
4. 0.0125 M dipotassium hydrogen phosphate
5. 0.1 M potassium chloride solution
6. 0.5 M sodium fluoride solution
7. 0.1 M Tris-HCL buffer, pH 8.0

Procedure

The reaction mixture in a total volume of 5 ml contained the following:

1.0 ml of glucose solution, 0.5 ml of ATP solution, 0.1 ml of magnesium chloride solution, 0.4 ml of dipotassium hydrogen phosphate, 0.4 ml of potassium chloride, 0.1 ml

of sodium fluoride and 2.5 ml of Tris-HCL buffer, (pH8.0). The mixture was pre incubated at 37^0 C for 5 minutes.

The reaction was initiated by the addition of 2 ml of tissue homogenate, 1.0 ml of the reaction mixture was immediately removed to tubes containing 1.0 ml of which are considered as zero time tube.

A second aliquot was removed after 30 min of incubation at 37^0 C. the protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated by the ortho toludine method of Sasaki *et al.*, (1972). Using the following reagent with a reagent blank in each test.

1. 10% TCA
2. Ortho toludine Reagent:

12.5 gm of thiourea and 12 g, acetic acid (AR) and redistilled ortho toluidine in the ratio of 10:75 and kept in the cold over night.

3. Standard glucose solution:

20 mg of glucose solution was dissolved in 100 ml of distilled water (200 mg/ml)

Procedure:

An aliquot of the supernatant containing the residual glucose was mixed with 4.0 ml or ortho toluidine reagent and heated in a boiling water bath, for 15 minutes.

The green colour obtained was read at 600 nm. A set of glucose standard solution treated similarly was used to calibrate the standard graphs. The glucose concentrations were arrived by comparison with the standard curve.

The difference between the two values gave the amount of glucose phosphorylation. The enzyme activity was expressed as micromoles (μm) of glucose phosphorylated/ hr/mg protein.

17. Determination of Plasma urea/ BUN. (Henery *et al.*, 1968).

Principle

Urease breaks down urea into ammonia and carbon dioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxyindophenol, a coloured compound. The reaction is catalysed by sodium nitroprusside. The intensity of colour produced is measured photometrically at 578 nm (570-620 nm)

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} \text{Ammonia} + CO_2$$

$$\text{Ammonia} + \text{Salicylate} + \text{Hypochlorite} \xrightarrow{\text{Nitriprusside}} \text{2-2 Dicarboxy indophenol}$$

Reagents:

All reagents to be stored at 2-8^{0}C

- Urea A
- Urea B
- 2Urea (colour developing reagent)
- Urea standard (40 mg/dl)

Procedure:

The following mixtures were pipetted out in to a blank test tube

PROCEDURE FOR 1 ML			
Reagents	Blank	Standard	Test
STANDARD (ML)	-	0.005	-
SAMPLE (ML)	-	-	0.005
DISTILLED WATER (ML)	0.005	-	-
WORKING ENZYME REAGENT E (ML)	0.50	0.50	0.50

Mixed well and incubated for ten minutes at room temperature (25^0 - 30^{0}C) or for five minutes at 37^{0}C after that the working colour reagent was added as follows.

WORKING COLOUR REAGENT C (ML)	0.50	0.50	0.50

Mixed well and incubated for ten minutes at room temperature (25^0-30^0C) or five minutes at 37^0C. Absorbance of test and standard against reagent blank was read at 578 nm (570-620 nm or red filter).

Calculation:

Urea concentration (mg/dl) = $\frac{\text{Absorbance of test}}{\text{Absorbance of STD}}$ x 40

BUN concentration (mg/dl) = 0.467 x urea concentration (mg/dl)

To convert urea concentration (mg/dl) to mmol/lit., use the following equation.

Urea concentration (mg/dl) x 0.167 = mmol/l.

18. Determination of Uric Acid in plasma. (Barham *et al.*, 1972).

Principle :

Uric acid is converted in to uricase to allantion and hydrogen peroxide, which under the catalytic influence of peroxidase, oxidizes 3,5-Dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a red violet quinoneimine compound.

Uric Acid + O_2 + $2H_2O$ $\xrightarrow{\text{Uricase}}$ Allantoin + CO_2 + H_2O_2

$2H_2O_2$ + 3, 5- dichloro-2-hydroxybenzenesulfonic acid + 4 - aminophenazone $\xrightarrow{\text{Peroxidase}}$ N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzo-quinoneimine

Reagents:

Enzymatic reagent

1. HEPES buffer = 200 mmol/l, pH 7.55

2. 4-Aminophenazone= 0.25 mmol/l

3. 3, 5 DCHBS = 4.0 mmol/l

4. Uricase = >200 U/l

5. Peroxidase = > 1000 U/l

6. Standard = 585 µmol/l (10 mg/dl)

Procedure:
The following reagents were pipetted in to the test tubes.

PIPETTE IN TO TEST TUBE			
	Reagent Blank µl	Sample µl	Standard µl
SAMPLE	-	20	-
STANDARD	-	-	20
REAGENT	1000	1000	1000

Mixed and incubated for 15 minutes at 20-25^{0}C or for exactly 5 minutes at 37^{0}C. The absorbance of the sample were measured (A_{sample}) and the standard ($A_{standard}$) against reagent blank with in 15 minutes.

Although the absorbance may be read at any time up to 15 minutes after the specified incubation time, the time interval from sample addition to read time must be exactly the same for Standard/Control and Sample.

Calculation:

Uric acid concentration = $\frac{A_{sample}}{A_{Standard}} \times$ concentration of sample

19. Determination of Creatinine in plasma. (Bartels *et al.*, 1972).

Principle:

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Reagents:

1. Standard: - 177 µmol/l (2 mg/dl)

2. Picric acid: - 35 mmol/l

3. Sodium hydroxide: - 0.32 mol/l

Procedure:

Reaction rate and absorbance of the reaction product are very sensitive to temperature. The specified temperature must therefore be maintained.

	STANDARD		SAMPLE	
	MACRO	SEMI MICRO	MACRO	SEMI MICRO
WORKING REAGENT	2.0 ml	1.0 ml	2.0 ml	1.0 ml
STANDARD SOLUTION	0.2 ml	0.1 ml	-	-
SAMPLE	-	-	0.2 ml	0.1 ml

Mixed and after 3 seconds read the absorbance A_1 of the standard and sample. Exactly two minutes later, read absorbance A_2 of standard and sample.

Calculation:

$A_2 - A_1 = \Delta A$ sample or ΔA Standard

Concentration of creatinine in serum or plasma.

$$\frac{\Delta A_{sample}}{\Delta A_{Standard}} \times 177 = \mu mol/l \qquad \frac{\Delta A_{sample}}{\Delta A_{Standard}} \times 2 = mg/dl$$

20. Estimation of Free Fatty Acids. (Falbolt *et al.*, 1973).

Reagents

1. Phosphate buffer at pH 6.4: Weighed 4.539 gm/L potassium dihydrogen phosphate and 5.938 g/l di-sodium hydrogen phosphate dehydrate mixed two volume of (A) and one volume of (B) just prior to use.
2. Chloroform: heptanes: Methanol solvent: This was prepared by mixing chloroform-heptane and methanol in 5:5:1 ratio.
3. Copper nitrate – Triethanolamine (Cu - TEA) reagent. This was prepared by mixing 40 ml of 0.1M copper nitrate and 40 ml of 0.2 M triethanolamine, 6 ml of 1M NaOH and 3.3 g of sodium chloride. Volume was made up to 100 ml with distilled water and pH was adjusted to 8.1.
4. Colouring reagent: 1, 5 diphenylhydrazine 4 g/l
5. Standard palmitic acid solution: A solution containing 2 mg/ml of palmitic acid was prepared in chloroform. Working standard containing 200 µg/ml was prepared by diluting 1 volume of stock to 10 volumes with chloroform.

Procedure

0.1 ml of serum was added to 6 ml of chloroform-heptane-methnol solvent. And the tubes were kept aside for 30 minutes. The solution was then centrifuged and the supernatant was transferred to tubes containing 2 ml of Cu-TEA solution. Solvent was used as blank. Standards with different concentration were made up to a known volume with the solvents and the contents were mixed in a mechanical stirrer for 20 minutes. They were then centrifuged so that two layers were formed, 2 ml of the upper layer was mixed with 1 ml of the colour reagent, and the colour developed was read at 430 nm.

Units:

Free fatty acids were expressed as mg/dL serum.

21. Estimation of phospholipids. (Zilversmit and Davies , 1950)

The phosphrous organic phospholipids was converted to inorganic phosphrous which reacts with ammonium molybdate to form phosphomolybdic acid. This is reduced to a stable, coloured aminonaphthol sulfonic acid.

Reagents:

1. 20% Trichloro acetic acid (TCA) (W/V)
2. Molybdic acid Reagent: 2.5 percent ammonium molybdate in 3N sulphuric acid.
3. Amino naphthol sulphonic acid reagent (ANSA) 500 mg of ANSA was dissolved in 195 ml of 15% sodium disulphite and 5ml of 5% sodium sulphite was added for complete solubilisation. The solution was filtered and stored at 4^0C.
4. Standard phosphayte : 80 µg of phosphrous/ ml 35.1 mg of potassium dihydrogen phosphate was dissolved and made up to 100 ml with distilled water.
5. Working standard: (8.0 µg of phosphrous/ml) 1.0 ml of the stock standard was diluted to 10 ml before use.

Digestion procedure:

To 0.1 ml of plasma, 2 ml of 20% TCA was added, mixed well and centrifuged for 10 minutes. As aliquot of the lipid extract was pipetted in to a kijeldahl flask and evoprated to dryness, 1.0 ml of 5N H_2SO_4 was added and digested in a digestion rack till the light brown colour comes, then 2 to 3 drops of concentrated nitric acid was added and the digestion continued till it becomes colourless. The kjeldal flask was cooled, then added 1.0 ml of water and heated in a boiling water bath for about 5 minutes. Then 0.1 ml of ANSA was added. The volume was then made up to 10 ml with distilled water and the absorbance was measured at 660 nm with in 10 minutes.

Unit:

Plasma phospholipids was expressed as mg/dl

Lipid peroxidation levels:

Polyunsaturated fatty acids (PUFA) are vulnerable to oxidative damage. ROS generated during various biochemical reactions indicated 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 with in 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 tissue, especially the membrane lipids.

22. Estimation of Triglycerides by Foster and Dunn (1973).

Principle:
An aliquot of the lipid extract was taken, evaporated to drynesss and triglycerides were estimated by following the method.

Reagents

1. Isopropanol
2. Activated aluminum oxide (Neutral)
3. Saponification reagent: - 5.0 g of KOH was dissolved in 60 ml of distilled water and 40 ml of isopropanol was added to it.
4. Sodium metaperiodate reagent: - 77 gm of anhydrous ammonium acetate was dissolved in about 700 ml of distilled water 60 ml of glacial acetic acid was added to it, followed by 650 mg of sodium metaperiodate, the mixture was dissolved and diluted to one liter with distilled water.
5. Standard solution of Triolein: - 1.0 g of triolein was dissolved in 100 ml of isopropanol, 1 ml of stock standard was diluted to 100 ml to prepare a working standard containing 10 µg of triolein/ml. This was stable for at least 6 month in a tightly seated container.

Procedure:
To the dried lipid containing tube, 0.1 ml of methanol was added which was followed by 4 ml of isopropanol. 0.4 g of aluminia was added to all the tubes and shaken well 15 minutes, centrifuged and then 2 ml of supernatant fluid was transferred to appropriately labeled tubes, the tubes were placed in a water bath at 65^0 C for 15 minutes for saponification after adding 0.6 ml of the saponification reagent and followed by 0.5 ml of

(1 ml of metaperriodate) acetyl acetone reagent, after mixing the tubes were kept in a water bath at 65^0 C for an hour.

The content was cooled and the absorbance was read at 420 nm.

A series of standards of concentration 8 µg to 40 µg triolein was treated similarly along with blank containing only the reagent

The triglycerides content of tissue were expressed as mg/g of wet tissue.

23. Estimation of Triglycerides by Kit (Fossati *et al.*, 1982).

Principle

Lipase hydrolyses triglycerides sequentially to Di and Monoglycerides and finally to glycerol. Glycerol Kinase (GK) using ATP as PO_4 source converts Glycerol liberated to Glycetrol-3-Phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidizes G-3-Phosphate formed to dihydroxy acetone phosphate and hydrogen peroxide is formed. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidize 4-aminoantipyrine and ADPS to a purple colored complex. The absorbance of the coloured complex is measured at 546 nm (530-570 nm or yellow filter which is proportional to triglyceride concentration).

Triglycerides + H_2o $\xrightarrow{\text{Lipase}}$ Glycerol + Fatty Acids

Glycerol + ATP $\xrightarrow{\text{GK}}$ Glycerol-3-Phosphate + ADP

Glycerol-3-Phosphate + O_2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + H_2O_2

H_2O_2 + 4-Aminoantiyrine + ADPS $\xrightarrow{\text{POD}}$ Quinoneimine + H_2O

Reagents

1. Liquid triglycerides reagent

2. Triglycerides Standard (200 mg/dl)

Procedure

PIPETTE IN TO TEST TUBE	PROCEDURE FOR 1ML			PROCEDURE FOR 0.5ML		
	BLK	STD	TEST	BLK	STD	TEST

WORKING REAGENT (ML)	1.0	1.0	1.0	0.5	0.5	0.5
STANDARD (ML)	-	0.01	-	-	0.005	-
SAMPLE (ML)	-	-	0.01	-	-	0.05

Mixed and incubated at 37^0 C for 10 minutes and read absorbance of test and standard against reagent blank at 546 nm (530-570 nm or yellow filter).

Calculation:

Triglycerides (mg/dl) = $\frac{\text{Absorbance of Test}}{\text{Absorbance of Std}}$ x200

To convert (mg/dl) to mmol/lit

Mmol/lit = mg/dl x 0.0114

24. Estimation of HDL-Cholesterol (PTA- method).

Principle:

High density lipoproteins (HDL) were separated from other lipoprotein fractions by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated; cholesterol content of which is estimated by enzymatic method.

Serum + PTA Reagent → Supernatant (HDL) + precipitates (other fractions).

Reagents

1. HDL- Cholesterol (Precipitating reagent)

2. HDL-cholesterol standard (50 mg/dl.)

Procedure:

Separation of HDL fraction

PIPETTE IN TO TEST TUBES	
TEST	

SAMPLE	(ML)	0.2
3-HDL-CHOLESTROL	(ML)	0.2

Mixed well and centrifuge at 3500-4000 rpm for 10 minutes. Separate the clear supernatant immediately and determine cholesterol content as follows.

PIPETTE IN TO TEST TUBES	BLANK	STD	TEST
CHOLESTEROL REAGENT (ML)	1.0	1.0	1.0
HDL STANDARD (ML)	-	0.05	-
SUPERNATANT (ML)	-	-	0.05

Mix well and incubate for ten minutes at 37^0C and read absorbance of test and standard against reagent blank at 505nm (500-540 nm green filter).

Calculation:

Plasma HDL-C = $\frac{\text{Abs. of test}}{\text{Abs. of std}}$ x Conc. Of standard x dilution factor = $\frac{\text{Absorbance of test}}{\text{Abs. of standard}}$

25. Determination of LDL and VLDL cholesterol

Both low density lipoprotein cholesterol and very low density lipoprotein levels were calculated from the estimated total cholesterol triglycerides and high density lipoprotein cholesterol as given below.

Very low density lipoprotein

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

Low density lipoprotein

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

26. Estimation of Cholesterol. (Zak, 1953)

Principle:

Plasma was treated with ferric chloride acetic acid reagent to precipitate the protein, the protein free filtrate containing cholesterol free chloride was treated with concentrated H_2SO_4. The reaction involves the 3-OH-5-ene part of cholesterol molecule. It was first dehydrated to form cholesta-3, 5- diene and then oxidized by sulphuric acid to link two molecules together as bis cholesta-3, 5- diene, this material was sulphated by sulphuric acid to red coloured disulfonic acid in the presence of ferric ion as catalyst (Salkowski's reaction). The red colour was read at 560 nm using suitable standards and a reagent blank.

Reagents:

1. Ferric chloride-acetic acid reagent: - 1 g of $Fecl_3.6H_2O$ dissolved in 100 ml of CH_3COOH (AR, glacial acetic acid)

2. $Fecl_3$-COOH working: - 5ml of stock diluted to 100 ml with CH_3COOH.

3. Cholesterol working standard: - 20-100 µg in ferric chloride-acetic acid reagent.

Procedure:

To 0.1 ml of plasma in a glass stopper centrifuge tube, 4.9ml of standard $Fecl_3$-CH_3COOH reagent was added, mixed well, allowed to stand for 15 minutes and then centrifuged. To 25ml of centrifugate 1.5 ml of concentrated H_2SO_4 were added and the test solution were measured at 560 nm.

Different aliquots of working standard In a range of 1.5 ml, 2 ml, 2.5 ml were taken and made up to 2.5 ml with $FeCl_3$-CH_3COOH reagent, to this 1.5 ml of concentrated H_2SO_4 was added mixed well, allowed to stand for 30 minutes and read at 560 nm using a reagent blank.

Units:

Values were expressed as mg/dl plasma

27. Estimation of Cholesterol by kit method.

Principle:

The cholesterol esters were hydrolysed to free cholesterol by cholesterol esterase (CE). The free cholesterol was then oxidized by cholesterol oxidase (CO) to cholesten 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield coloured complex which was read at 505 nm.

Cholesterol esters $\xrightarrow{\textbf{CE}}$ Cholesterol + fatty acids

Cholesterol + O_2 $\xrightarrow{\textbf{CO}}$ Cholesterol-4-en-3-one + H_2O_2

$2H_2O_2$ + 4AAP + Phenolic compounds $\xrightarrow{\textbf{POD}}$ Quinoneimine dye + $4H_2O$

The intensity of the color produced was directly proportional to the concentration of total cholesterol in the sample.

Reagents:

1. Cholesterol-L (Ready to use)

2. Cholesterol-Standard (200 mg/dl).

Procedure:

PIPETTE IN TO TUBES	BLANK	STD	TEST
CHOLESTEROL REAGENT (ML.)	1.0	1.0	1.0
STANDARD (ML.)	-	0.01	-
SAMPLE (ML)	-	-	0.01

Mixed well and incubated for 10 minutes at 37^0C or 30 minutes at R. T. (25 ± 5^0C) Read absorbance of standard and test at 505 nm (505-530) (Green filter) against reagent blank.

Calculation:

Cholesterol Concentration (mg/dl) = $\frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 200$

OR : Absorbance of the Test x 547.20.