MATERIALS AND METHODS

Experimental Animals: Albino Wistar strain either sex rats weighing 100±5grams were procured from Central Animal House, Kasturba Medical College, Mangalore. All animals were fed in an animal facility with a 12 to 12 hour light-dark cycle and were given the standard rat chow and water ad libitum(246).

Study site: Department of Biochemistry, Centre for Basic Sciences, Kasturba Medical College, Mangalore.

Diabetes induction: Diabetes was induced in rats by administering Nicotinamide (NA) and Streptozotocin (STZ). NA & STZ was tried in different doses to induce diabetes without mortality incidents. Dose was finally decided to NA- 25mg/kg & STZ- 50mg/kg bodyweight. Animals received intraperitoneal administrations of Nicotinamide - 25 mg/kg dissolved in normal saline 15 minutes before an administration of STZ- 50 mg/kg. 0.1M citrate buffer was used to dissolve STZ (pH 4.5)(134). Only rats having a blood glucose above 250 mg/dL was chosen and divided into groups.

Plant Material: *T. terrestris* dry fruit aqueous extract was bought from Himalaya Health Care, Bangalore. It was dissolved in distilled water and administered to animals. Heartwood of *P. marsupium* was purchased from Alva's herbal pharmacy and its identity was ascertained by a reputed Botanist. Aqueous extract of *P. marsupium* was prepared in Biochemistry research lab.

Preparation of plant Extract: One part of dry coarse powder of *P. marsupium* heartwood was boiled in sixteen parts of water for 15 minutes at 50° C. It was filtered through the muslin cloth and filtrate was kept for flash evaporation in rotary vacuum flash evaporator at 5 rpm (75˚C) for seven hours. Remaining residue was collected from round bottom flask and dried in heating mantle for one hour to obtain semi-solid form of extract(220). Extraction was done in different set of phases and the average yield was 5.9 grams. Extract was stored at -4ºC in a sealed container to prevent contamination/growth.

Ethical Approval: In accordance to CPCSEA guidelines, 6-8 rats were planned per group. This study was approved by Institutional animal ethics committee, KMC, Mangalore with 6 animals in each group (Annexure-1). However in the event of death of

the animals in any of the groups, such animals were replaced & study protocol was repeated to ensure that for statistical analysis each group had six animals at the end of the study period.

Acute toxicity studies: Rats were divided into 4 groups of 6 animals each. Dose of plant extracts were calculated according to body weight of animal. Plant extract was administered by oral route using oral feeding tube.

T. terristris aqueous extract was given at doses of 300, 600, 1200 and 2400 mg/kg body weight. Animals were closely observed for 24 hours. No mortality was seen with higher dose but animals were paranormally active after 4 hours of drug administration.

P. marsupium aqueous extract was given at doses of 250, 500, 1000 and 2000 mg/kg. Animals were observed for 24 hours. No mortality was seen but animals were lethargic and drowsy. It was found that even higher dose (4gm/kg) of *P.marsupium* was non-lethal to animals. Results of the study strongly support the previous toxicity study reports of *T.terrestris* and *P. marsupium*.(190)(234)

Selection of dosages of the plant extracts: Based on the present acute toxicity study results and literature search, the following doses was chosen for the plant extracts.(186)(247)

The plant extract dosage is as follows:

Plant	Dose I (mg/kg)	Dose II (mg/kg)
T. terrestris	150	300
P. marsupium	250	500

Study period:

The duration of the study was for 3 years. The study was divided into two phases.

In phase-1, immediately after induction of diabetes treatment was started and continued for 16 weeks

In phase-2, treatment was started 30 days after induction of diabetes and continued for another 30 days.

Study Design:

Phase-1 study: To determine the efficacy of plant extracts in prevention of diabetic complications the rats were divided into 11 groups and treatment was started on 7th day after STZ-NA injection and treatment was continued for 16 weeks.

Groups (n = 6)		Plant Extract/Drug	Dose (mg/kg) Route – P.O.
I	Normal controls	Normal saline	-
II	Diabetic controls	Normal saline	-
III	Diabetic rats	Glibenclamide	500 µg/kg
IV	Diabetic rats	*T. terrestris*	150
V	Non-diabetic rats	*T. terrestris*	150
VI	Diabetic rats	*T. terrestris*	300
VII	Non-diabetic rats	*T. terrestris*	300
VIII	Diabetic rats	*P. marsupium*	250
IX	Non-diabetic rats	*P. marsupium*	250
X	Diabetic rats	*P. marsupium*	500
XI	Non-diabetic rats	*P. marsupium*	500

Euthanasia and carcass disposal:

Animals were sacrificed at the end of the study by cervical dislocation. Procedure was carried out quickly and painlessly in an atmosphere free from fear or anxiety according to CPCSEA guidelines. Blood was collected by cardiac puncture. All other organs to be studied were dissected out for various biochemical estimations & histopathological studies. Tissues were fixed using 10% formalin for histology. Carcass was wrapped in yellow polythene and it was further disposed through the bio medical waste management firm engaged by the institution.

Phase-2 study: To determine the efficacy of plant extracts in reverting the diabetic complications rats were divided into 7 groups and treatment was started 30 days after development of diabetes & treatment was continued for next 30 days.

Groups (n = 8)		Plant Extract/Drug	Dose (mg/kg) Route – P.O.
I	Normal controls	Normal saline	-
II	Diabetic controls	Normal saline	-
III	Diabetic rats	Glibenclamide	500µg/kg
IV	Diabetic rats	*T. terrestris*	150
V	Diabetic rats	*P. marsupium*	250
VI	Diabetic rats	Glibenclamide + *T. terrestris*	500+150
VII	Diabetic rats	Glibenclamide + *P. marsupium*	500+250

Dose selection for IV, V, VI & VII groups was made based on the results of phase I study.

Euthanasia and carcass disposal:

Animals were sacrificed at the end of the study by cervical dislocation. Procedure was carried out quickly and painlessly in an atmosphere free from fear or anxiety according to CPCSEA guidelines. Blood was collected by cardiac puncture. All other organs to be studied were dissected out for various biochemical estimations & histopathological studies. Tissues were fixed using 10% formalin for histology. Carcass was wrapped in yellow polythene and it was further disposed through the bio medical waste management firm engaged by the institution.

Investigation Details:

Test done in blood:

Estimation of blood glucose: The test was done using ACCU-CHEK Active blood glucose monitor using disposable strips from Roche Diagnostics. The strips contain glucose dye oxidoreductase. Addition of 1-2µl of blood to the test pad results in the chemical reaction and the glucose values were read in 5-10 seconds.

Test principle: Glucose oxidase catalyse the oxidation of Beta D- glucose present in the plasma to D glucono -1 ,5 - lactone with the formation of hydrogen peroxide; the lactone is then slowly hydrolysed to D-gluconic acid. The hydrogen peroxide produced is then broken down to oxygen and water by a peroxidase enzyme. Oxygen then react with an oxygen acceptor such as ortho toluidine which itself converted to a coloured compound, the amount of which was measured glucometer.(248)

Test done in plasma:

Estimation of glycated hemoglobin (HbA1c): At the end of the study period, by turbidimetric inhibition immunoassay (TINIA) on hemolysed whole blood.

Test principle:

HbA1c in the sample reacts with anti-HbA1c antibody to form soluble antigen-antibody complexes. Since the specific HbA1c antibody site is present only once on the HbA1c molecule, complex formation does not take place. The polyhaptens react with excess anti-HbA1c antibodies to form an insoluble antibody-polyhapten complex which can be determined turbidimetrically.(249)

Test done in serum:

Estimation of Insulin: Insulin was quantitated in serum collected at the end of the study period, by Sandwich ELISA technique using anti-rat insulin antibodies from Genexbio pvt.ltd as per the instructions given by the manufacturer.

Test principle:

The sequence of reactions is as follows:

a) Capture of insulin molecules from samples to the wells of a microtitre plate coated by pre-titered amount of a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to the captured insulin
b) Wash away of unbound materials from samples
c) Binding of horseradish peroxidase to the immobilized biotinylated antibodies
d) Wash away of free enzyme conjugates
e) Quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3', 5,5'-tetramethylbenzidine.

Interleukin-6 and Interleukin-10 levels were quantitated by ELISA technique (ELX 800 ELISA reader). Rat IL-6 & IL-10 kits were bought from Genexbio Company.

Test principle is same as insulin estimation.

Following estimations were carried out in serum at the end of the study period in on the Autoanalyser using Roche kits as per the instructions given by the company.(250)

Fasting Lipid Profile:

a. Total Cholesterol (TC): The estimation of Total cholesterol is based on CHOD-PAP method. Test principle: The colorimetric indicator is quinonieimine which generates 4- aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase. The colour intensity produced is measured at 505 nm.

b. HDL Cholesterol (HDL-C): The estimation of HDL-C is based on CHOD-PAP method.

Test principle: Chylomicrons, VLDL, LDL present in serum are precipitated by polyions. HDL-C remains in the supernatant. Cholesterol esters in the supernatant are hydrolysed by cholesterol esterase to free cholesterol and fatty acid. Free and liberated cholesterol are oxidised by CHOD to Chol-4-ene-3one and hydrogen peroxide is liberates. The hydrogen peroxide produced couples with 4-aminoantipyrine and phenol in the presence of peroxidase to form a coloured complex. The intensity is measured at 505 nm.

c. Triglycerides (TG): The estimation of triglycerides is based on GPO-PAP **(**glycerol-3-phosphate oxidase – phenol + aminophenazone) method.

Test principle: The colorimetric indicator is quinonieimine which is generated 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase. The colour intensity produced was measured at 505 nm.

Urea:

Urea was estimated by Urease/GLDH method.

Test principle: Urea is first hydrolysed by urease to give ammonia and carbon dioxide. The ammonia produced reacts with α ketoglutarate and NADH in the presence of glutamate dehydrogenase (GLDH) to yield glutamate and NAD. The oxidation of NADH causes a decrease in absorbance. The rate of decrease in absorbance is proportional to urea concentration which was measured at 340 nm.

Creatinine:

Creatinine was estimated by Jaffe's method.

Test principle: With alkaline picrate solution, creatinine forms a reddish yellow colour complex of creatinine picrate. The intensity of the reddish yellow colour was read at 500nm.

Aspartate aminotransaminase (AST):

The concentration of AST estimated by determining the decrease in the concentration of NADH in the presence of malate dehydrogenase.

Test principle: The rate of decrease of NADH is proportional to the rate of formation of oxaloacetate and thus AST activity. AST was measured by the oxaloacetate formed from the reaction with α ketoglutarate and aspartate. Unstable oxaloacetate gradually converted to pyruvate with loss of carbon dioxide.

Alanine aminotransaminase (ALT):

The concentration of ALT estimated by determining the decrease in the concentration of NADH in the presence of lactate dehydrogenase.

Test principle: The rate of decrease of NADH proportional to the rate of formation of pyruvate and thus ALT activity. The activity of this enzyme was measured by pyruvate from the reaction with α ketoglutarate and alanine.

Gamma – glutamyltransferase (GGT):

The concentration of GGT estimated by enzymatic colorimetric assay.

Test principle: The amount of 5- amino-2-nitrobenzoate liberated was proportional to the GGT activity & determined photometrically.

Test done in urine:

Microalbumin:

Microalbumin was estimated by Turbidimetric immunoassay method

Test principle: Addition of anti-albumin antibodies to the sample results in the start of reaction. Anti-albumin antibodies react with the antigen in the sample to form antigen/antibody complexes which, following agglutination, was measured turbidimetrically.

Following investigations were performed in various tissue homogenates after sacrificing the rats at the end of the study. The experiments were conducted in the Research laboratory, Department of Biochemistry, KMC, Mangalore, using UV-Visible Spectrophotometer.

Estimation of liver glycogen content:

By the method of Lo *et al.*, The liver tissue was digested by alkali and glycogen precipitates, which was made to react with phenol and sulphuric acid and formed orange colored complex was measured at 495nm.(251)

Reagents required:

1. 30%KOH saturated with Na_2SO_4.
2. Ethanol- 95%.
3. 98% H_2SO_4.
4. Phenol reagent- 5%.
5. Stock Standard glycogen 5mg/ml in distilled water.
6. Working standard: 1,2,3,4 and 5mg/ml.

Procedure: Liver tissue piece of 50mg was cut and transferred to a glass tube with a screw cap and kept deep frozen till further process. 0.5ml KOH was added to the tubes and ensured a proper immersion of the liver tissue in this solution. Then the

tubes with the screw cap on were kept in boiling water bath for 25min until a homogenous solution was obtained. The tubes were cooled in ice, glycogen from the alkaline digest was precipitated by adding 1.1 volume of ethanol. The tubes were kept in ice for 30min, centrifuged at 3500rpm for 30min. supernatant was aspirated and discarded. The glycogen precipitate was dissolved in 3ml water. 1ml from this solution was transferred to a test tube, 1ml phenol reagent was added and 5ml sulphuric acid was added rapidly directly to the surface of the solution for proper mixing. The tube was allowed to stand for 10min, shaken to mix the contents, placed in water bath at 25-30^{0}C for 15 min, and reading was taken at 495nm. A blank with 1ml distilled water and standards were also treated simultaneously.

Glycogen (g/100g liver) = A/k X V/v X 10^{-4}/W

Where, A = absorbance, k= slope of the standard curve, V = total volume of glycogen solution, v= volume of aliquot used in the colour reaction, W= weight of the tissue sample in grams

Reduced glutathione (GSH) in lens homogenate:

The concentration of reduced glutathione was estimated by the reaction with 5,5′ - dithionitrobenzoic acid (252). This method was based upon the development of a yellow colour when 5,5'- dithiobis-2-nitro benzoic acid (DTNB) is added to the test solution containing sulphydryl compound like glutathione. The yellow colour was measured at 412nm. A standard having known concentration of GSH and a blank was treated simultaneously.

Reagents required:

1. Standard glutathione (GSH): 5mg/10ml in distilled water.
2. Precipitating solution: (a) Glacial metaphosphoric acid – 1.67g
   (b) Ethylene diamine tetra acetic acid disodium or dipotassium salt – 0.2g
   (c) Sodium chloride – 30g.
   Add (a), (b) and (c) to a 100ml volumetric flask, dissolve and made up to the mark in distilled water.
3. Phosphate solution: 0.3M Na_2HPO_4 in distilled water.

4. DTNB reagent: 5,5' dithio-bis 2-nitro benzoic acid – 40mg/ 100ml in 1.1% sodium citrate.

Test procedure: The lens was weighed and homogenate was prepared in 0.9% saline in a final volume of 10 times the weight of the lens. It was centrifuged at a speed of 3000rpm for 10 minutes and supernatant was takes as test.

2ml of the centrifugate was taken and 3ml precipitating solution was added to it. Mixed well and allowed to stand for 5 minutes. Centrifuged and 1ml supernatant was taken, 4ml phosphate solution and 0.5ml DTNB reagent were added. Allowed to stand for 10 minutes at room temperature and readings were taken at 412nm.
For the standard, 0.2ml of standard GSH was made to 2ml with 0.9% saline. This was treated similar to the test.
For blank, 2ml of distilled water was mixed with 3ml of precipitating solution. 1ml of this was treated similarly.

The amount of GSH in the test sample was calculated graphically and expressed in µmol/g tissue.

Aldose reductase activity in lens & Sciatic nerve homogenate:

Assayed spectrophotometrically by determining NADPH consumption. Method described by Karasu *et al.,* was used for assay(253). AR activity was studied using glyceraldehyde as substrate and NADPH as coenzyme. The decrease in the absorbance of the solution at 340nm gave a measure of decrease in the concentration of NADPH which acted as the coenzyme for the action of AR on glyceraldehyde.

A (absorbance) = € X C X L ,

Where, € - molar extinction coefficient of NADPH = 6.22×10^3 $mol^{-1}cm^{-1}$.

C – Concentration of the substance of interest

L – Length of the solution or thickness of the cuvette = 1cm

So, $C = \frac{A}{€ \times L}$ or $C\ (mol/L) = \frac{A \times Vt}{€ \times L \times Vs}$

Where, Vt = volume of solution = 2.1ml.

Vs = volume of sample = 0.2ml.

$$\text{Thus, } C = \frac{\Delta A/\text{min} \times 2.1}{6.22 \times 10^3 \times 1 \times 0.2}$$

$$C\ (\mu\text{mol/L}) = \frac{\Delta A/\text{min} \times 2.1 \times 10^6}{6.22 \times 10^3 \times 1 \times 0.2}$$

$$= \text{O.D} \times 1688.1$$

The activity of AR was to be expressed as Units of activity per mg of protein in the lens homogenate.

The activity of AR was expressed in µmol/dL which was considered as one mU – expressed as X. Total proteins in the lens homogenate was estimated by Lowry's method and the value was expressed in mg/dL – Y. X/Y gave the AR activity in mU/mg protein.

Test procedure: The lens was weighed and a homogenate was prepared using 12 volumes of 135mM Na^+ - K^+ phosphate buffer of pH-7.0 containing 0.5mM para methyl phenyl sulphonic acid and 10mM 2-mercaptoethanol. The homogenate was centrifuged at 4^0C at 5000 rpm for 30 minutes.

A test was prepared where 1.6ml 135mM phosphate buffer of pH 7.0, 0.1ml lithium sulphate (1.2796g/50ml in 135mM phosphate buffer of pH 7.0), 0.1ml DL-glyceraldehyde (3.6mg/50ml in 135mM phosphate buffer of pH 7.0), 0.1ml freshly prepared NADPH (2mg/ml in 135mM phosphate buffer of pH 7.0) and 0.2ml of the centrifugate were mixed and the absorbance was measured at 340nm exactly at the 30^{th}, 60^{th},90^{th},120^{th},150^{th} and 180^{th} seconds of mixing. A blank was also run with all the contents except for the substrate, glyceraldehyde. Thus 1.7ml of phosphate buffer was added here instead of 1.6ml in the test.

The decrease in the absorbance in one minute was calculated by subtracting the absorbance of 30^{th} second from that of 90^{th} second, 60^{th} from 120^{th}, 90^{th} from 150^{th} and 120^{th} from 180^{th} second. The mean of these four values was taken for further calculations. Similar calculations were done for the blank as

well. The decrease in the absorbance of the blank was subtracted from that of the test. This value was multiplied by 1688.1 to get the concentration of AR in µmols/L.

Estimation of total proteins by Lowry's method:(254)

Here the final colour is as a result of biuret reaction of the protein with copper ions in alkaline medium and reduction of phosphotungstic acid by tyrosine and tryptophan present in the treated protein.

Reagents required:

1. Reagent A: 2% Na_2CO_3 in 0.1N NaOH.
2. Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartarate.
3. Reagent C: Alkaline copper solution. Prepared fresh by mixing 50ml of reagent A with 1ml of reagent B.
4. Reagent D: Folin ciocalteu reagent.
5. Protein standard: 500mg of bovine albumin/100ml of water.
6. Working standard: Dilute 5ml of the stock standard to 100ml with water.
7. Test: Take 0.1ml of serum and dilute it to 5ml. 0.2ml is taken in the test tube named as T.

   A test tube containing 4ml distilled water was 'B', a series of standards with increasing concentrations of protein, and a test tube with 0.2ml serum and 3.8ml distilled water as 'T'. 5.5ml of alkaline copper reagent was added to all the tubes, kept at 37^0C for 10 minutes, then added 0.5ml reagent D. Waited for 30minutes at 37^0C and readings were taken at 650nm.

The concentration of proteins present in 100ml serum =

$$\frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D. of S} - \text{O.D. of B}} \times \frac{\text{Amount of S}}{\text{Volume of undiluted T}} \times 100$$

Histopathological sections: Liver, pancreas and kidney were dissected out and collected in normal saline. The parts of all the above tissues were cut and fixed in 10% formalin. After fixing for 3-4 days, the tissue was cleared and embedded to paraffin wax (60-62^{0}C). Uniform sections of 5µm thickness was cut and stained with haematoxylin and eosin by routine procedure. The stained sections was examined for pathological changes at various levels of magnification.

In vitro estimations with both plant extracts:

***In vitro* α-glucosidase inhibitory activity**: (255)

α- glucosidase inhibitory activity of the extracts was estimated by adding the extracts to commercially available α- glucosidase (Sigma Aldrich pvt.ltd.) as per the instructions given by the company. Estimation done by the method using Ueda *et al.,* The amount of glucose liberated at the end was measured by glucose oxidase method using Agappe Diagnostics kit.

Test Principle: α- glucosidase acts on the substrate and releases glucose as product. The glucose concentration was directly proportional to the action of α- glucosidase. The plant extracts acts as inhibitors of the enzyme. α-glucosidase inhibitory action of the extracts are directly proportional to the decrease in glucose release.

Estimation of glucose was done by enzymatic colorimetric method where glucose oxidase was made to act on glucose. The released H_2O_2 reacted with phenol and 4 – aminoantipyrine in the presence of peroxidase to give a red quinonimine which was measured spectrophotometrically at 505nm.

Reagents required:

1. HEPES-Na i.e, 2-[4-(2-hydroxy ethyl)-1- piperazinyl]- ethanesulphonic acid sodium salt buffer substance – 0.15M, pH-6.8. From Merck Chemicals.
2. α– glucosidase – 5Units/ml in HEPES buffer 0.15M. From Sigma Chemical Company.
3. Substrate – Maltose 0.1M in 0.15M HEPES buffer.
4. Glucose oxidase kit from Agappe Diagnostics.

Test procedure: A sample of *P.marsupium* and *T.terrestris* was prepared by dissolving it in DH_2O to get a final concentration of 50µg in 0.1ml and 30µg in 0.1 ml respectively was taken for glucose estimation.

0.1ml of substrate, 0.1ml of the plant extract and 0.1ml of α-glucosidase was taken in the tube marked as test whereas 0.1ml of substrate and 0.1ml of α- glucosidase was taken in the control. The tubes were kept for incubation at 37ºC for 30 minutes. The reaction was stopped by keeping the tubes in boiling water for 20 seconds. Contents of these tubes were taken as sample for glucose estimation.

Glucose estimation was done by taking 1ml of the working reagent each in tubes marked as blank, test and standard and adding 10µl of sample and standard in the tubes test and standard respectively. The tubes were incubated at 37ºC for 10 minutes. The absorbance of the test and standard were measured against reagent blank.

$$\text{Glucose concentration (mg/dl)} = \frac{\text{O.D. of Test} - \text{O.D. of Blank}}{\text{O.D. of Std} - \text{O.D. of Blank}} \times 100$$

$$\text{α- glucosidase inhibitory effect (\%)} = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \times 100$$

Ac = glucose production by control.

As = glucose production by standard/test.

Total phenolic content estimation:

The concentration of phenolics in plant extracts was determined using spectrophotometric method of Singleton *et al*., Gallic acid was used as a reference standard for plotting calibration curve.(256)

Test principle: Phenolic compounds undergo redox reaction with phosphomolydbic acid and phosphotungstic acid present in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex. 0.5 mL of the plant extract (100µg/mL) was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature (30±2ºC) for 30 min with intermittent

shaking for the blue color development. The absorbance was measured at 765 nm using double beam UV-VIS spectrophotometer. Phenolic contents was determined from the standard curve of gallic acid. Phenols expressed as milligrams/gram gallic acid equivalent (GAE) of dry extract.

Free radical scavenging property:

The DPPH free radical scavenging activity of the both extracts was determined by the modified method of Bhuiyan *et al*., (257)

Test principle: Antioxidants react with the stable DPPH radical and convert into 1, 1-diphenyl-2-picryl hydrazine. Ability to scavenge the DPPH radical is measured by a decrease in the absorbance. Aliquots containing various concentrations (2–100µg/ml) of *P. marsupium* & *T. terrestris* in the final volume of 1 ml were mixed with 1 ml of 0.05 mM DPPH, equal amount of methanol served as control. Ascorbic acid (2-100µg/ml) was used as standard. Reaction mixtures were incubated at 37°C for 20 min& the absorbance was recorded at 517 nm.

The capability of DPPH free radical scavenging activity by the plant extracts were calculated using the following formula:

DPPH scavenging effect (%) = $[(A_0-A_1)/A_0) \times 100]$.

A_0= absorbance of the control and A_1= absorbance of test/standard.

Effect of plant extract on diabetes induced cognitive dysfunction in animal models:

Morris Water Maze model:

Cognitive function testing was started on 31stday after treatment & continued for 9 days. Learning, memory and cognitive flexibility test was performed by the method described by Papadopoulos *et al.,*. Rats were trained to escape onto a platform located in a circular pool (1.4 m diameter) filled with opaque water (25±2°C). The pool was subdivided into four quadrants numbered in a clockwise order.

There were two platform locations: (1) the visible platform (original quadrant) for a three-day training session, and (2) the hidden platform (target quadrant) submerged ~1cm below the surface of the water for a five-day training session. Rats were given three trials daily with a maximum duration of 60s/trial and 90s/trial for the visible and hidden platform training sessions respectively. On the first day of each session, rats that failed to locate the platform in the allotted time were guided to and allowed to stay on it for 10sec. Spatial memory tested during the probe trial (platform removed) 24h after the last hidden platform trial. Rats were kept warm with a heating lamp and testing begin at the same time every day. Escape latencies recorded manually and probe trial was recorded by using video camera.(258)

STATISTICAL ANALYSIS

All results are expressed as the mean ± SD for six animals in each group. The data which was following normal distribution was analysed by parametric test: Comparison of various parameters between the groups was done using one way ANOVA. The pre and post comparison of parameters was done using paired samples test. "p" value of < 0.05 was considered statistically significant.

The data which was not following normal distribution was analysed by using non-parametric tests: To find the p value between various groups was done by Wilcoxon signed rank test and two group comparison was made by Mann Whitney-U test.

For analysing Morris water maze parameters, one way ANOVA was used for between the group analysis and Repeated measure ANOVA was used to analyse the repeated time intervals.

All grouped data were evaluated with SPSS 20 software (SPSS, Chicago, IL, USA)