MATERIALS & METHODS
Animals:

Albino male Wistar rats of 7 – 8 weeks old, weighing 150 – 200gm, were used for the present study. The animals are housed in a polypropylene cage. The animals were maintained at standard conditions of 12h light & 12h dark cycles at 25 - 27ºc and 35 – 60% humidity. The rats were fed on pellet diet (Hindustan Lever Limited, Mumbai, India) and water ad libitum. The rats used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and the study approved by the ethical committee.

Albino male Wistar rats were taken and randomized into 5 groups of control and experimental animals (six rats in each group).

Group 1: Non Diabetic control. Received only saline.

Group 2: Alloxan induced Diabetic control. Received only saline

Group 3: Alloxan induced Diabetic rats given Metformin (80mg/kg body weight)

Group 4: Alloxan induced Diabetic rats given (10mg/kg body weight) aqueous extracts of Phyllantus amarus and Piper nigrum.

Group 5: Alloxan induced Diabetic rats given (10mg/kg body weight) aqueous extract of Phyllantus amarus only.
Diabetes mellitus was induced in Wistar rats by single intraperitoneal injection of Alloxan monohydrate (150mg/kg body weight) by dissolving in physiological saline (0.9%) after overnight fast for 12hrs.

**Collection of blood:**

![Fig 3: Anesthetizing with ether](image_url)

Factors to consider in choosing the blood withdrawal technique appropriate for the purpose at hand include [Arowan,1989].

- The species to be bled.
- The size of the animal to be bled.
- The type of the sample required (e.g. serum, whole cells, etc.).
- The quality of the sample required
- The quantity of blood required.
- The frequency of sampling.
• The quantity of blood required.
• The frequency of sampling.
• Health status of the animal being bled.
• The training and experience of the phlebotomist

**Survival sampling sites:**

A) Retro-orbital
B) Mandibular
C) Saphenous- from the hind limb
D) Tail vein
E) Jugular- from the neck

**Retro-orbital Sampling:**

• Can be used in both rats and mice (though usually not a method of choice in the rat) by penetrating the retro-orbital plexus/sinus with a glass capillary.
• The NIH ARAC has determined that in the hands of a skilled operator retro-orbital bleeding is a humane procedure that produces minimal and transient pain/distress.
• Rapid – large number can be bled within a short period of time.
• Obtainable volume: medium to large.
• Good sample quality.
• The presence of a plexus in the rat can lead to greater orbital damage.
Fig 4: Retro-orbital bleeding

- Retro-orbital bleeding can be conducted in awaken mice. A topical ophthalmic anesthetic should be applied prior to the procedure.
EXPERIMENTAL PROTOCOL

Rats were made overnight fast. Initial body weight, haemoglobin concentration, fasting blood glucose, lipid profile, triacylglycerols, glycated haemoglobin were assessed.

Alloxan was injected intraperitoneally to all groups except control. Blood glucose levels are assessed after 24 hrs. After diabetic state was assessed the treatment was started.

In test groups herbal extracts (10mg/kg b.w) & Metformin (80mg/kg b.w) were given orally twice a day for 6 weeks. In control groups saline was given instead of drug. Fasting glucose was assessed at the end of every week.

At the end of experimental period, body weight was measured and the rats were deprived of food overnight and blood was collected in a tube containing potassium oxalate and sodium fluoride for the estimation of blood glucose, hemoglobin and glycosylated hemoglobin, lipid profile and triacyl glycerols.
ANALYTICAL PROCEDURES

ESTIMATION OF HAEMOGLOBIN

Haemoglobin was measured by the method of Drabkin and Austin (Drabkin, 1932).

Reagents:

Drabkins reagent: Dissolved 200 mg of potassium ferricyanide, 50mg potassium

Cyanide and 1.0mg sodium Bicarbonate in water and made up to a litre. The reagent had a pale yellow color and a pH of 9.6.

Cyanmethaemoglobin standard: 16g/100ml.

Procedure:

0.02ml of blood was diluted with 5.0ml of the Drabkin’s reagent. The diluted blood was mixed well and allowed to stand for 10 minutes, to ensure the completion of the reaction. The solution was read at 540nm together with the standard solution of cyanmethaemoglobin.

Blood hemoglobin levels were expressed as g/dl
ESTIMATION OF GLYCOSYLATED HAEMOGLOBIN (HBA1C)

Glycosylated haemoglobin (GHb) was estimated by the method of Sudhakar Nayak and Pattibiraman with modifications according to Bannaon [Sudhakar, 1981].

Reagents:

- 0.1M (PH 6.5)
- 0.1M Oxalate in 2M Hydrochloric acid
- Phenol-80%
- Concentrated sulphuric acid
- Fructose stock standard-1 mg/ml
- Fructose Citrate buffer working standard-100μg/ml
- 40% Trichloroacetic acid

Procedure

5 ml of blood was collected with EDTA and plasma was separated. To 0.5ml of packed cell, 5ml of citrate buffer was added, mixed and incubated at 37°C for 15 minutes, centrifuged and the supernatant was discarded. Then 0.5 ml of saline was added, mixed and processed for the estimation.

To an aliquot, 4.0 ml of oxalate in hydrochloric acid solution was added, mixed and heated at 100°C for 4 hours, cooled and precipitated
with 2.0 ml of 40% TCA. The mixture was centrifuged, and to an aliquot 0.05 ml of 80% phenol and 3.0 ml of Conc. H₂SO₄ was added. A set of standards (10-50 mg) was also treated in the similar manner. The color developed was read at after 30 minutes.

The values were expressed as HbA₁c%.

DETERMINATION OF BLOOD GLUCOSE

(Asatoor and King Method)[ Harold, 1988].

PRINCIPLE: Glucose reduces the alkaline copper solution forming cuprous ions. The amount of cuprous formed is estimated calorimetrically by reacting with phosphomolybdic acid. The cuprous ions will be oxidized again to cupric and the molybdic acid will be reduced to molybdenum blue. The color intensity being proportional to the amount of glucose in the sample, optical density gives the concentration.

Glucose + cupric → cuprous + gluconic acid

Cuprous + phosphomolybdic acid → cupric + molybdenum blue

Reagents:

1. Isotonic sodium sulfate-Copper sulfate solution:

   30gms of Na₂SO₄.10H₂O & 6gm of cuSO₄.5H₂O per liter.
2. Sodium tungstate (Na₂WO₄.2H₂O): 10% in water

3. Alkaline tartarate:
   
   12 gm sodium potassium tartarate
   
   20 gm sodium carbonate (anhydrous)
   
   25gm of sodium bicarbonate
   
   18gm of potassium oxalate

4. Phosphomolybdic acid reagent

5. Standard Glucose Solution: Stock: 10mg of glucose in 100ml of isotonic sodium sulfate- copper sulfate solution.

PROCEDURE:

0.1 ml of sodium tungstate, 3.8 ml of isotonic Na₂SO₄/CuSO₄ solution, 0.1 ml serum were added, mixed well and centrifuged. Clear supernatant is transferred in to a clean and dry test tube using Pasteur pipette.

Test—1ml of supernatant+1ml of alkaline tartarate

Control—1ml of isotonic Na₂SO₄ cuso₄ solution +1ml of alkaline tartarate

Mixed well and closed with cotton wool, boiled for 10min and cooled under tap water. 3ml of phosphomolybdic reagent was added to each tube, after shaking well, 3ml of distilled water is added to each
tube, mixed well to remove all air bubbles. The absorbance of Test and Standard against Blank measured at 680nm.

The values were expressed as mg/dl.

**Estimation of Total Cholesterol:**

CHOD-PAP METHOD [Liquid Gold kit, span diagnostics]

**For Total Cholesterol**

Cholesterol esters are hydrolyzed by Cholesterol Esterase (CE) to give free Cholesterol and Fatty acids. In subsequent reaction, Cholesterol Oxidase (CHOD) oxidizes the 3-OH group of free Cholesterol to liberate Cholest-4-en-3-one and Hydrogen Peroxide. In presence of Peroxides (POD), hydrogen peroxide couples with 4-Aminoantipyrine (4-APP) and phenol to produce Red Quinoneimine dye. Absorbance of colored dye is measured at 505nm and is proportional to amount of Total Cholesterol concentration in the sample

\[
\begin{align*}
\text{Cholesterol esters} & \xrightarrow{\text{CE}} \text{Cholesterol + Fatty acids} \\
\text{CHOD} & \\
\text{Cholesterol + O}_2 & \rightarrow \text{Cholest-4-en-3-one + H}_2\text{O} \\
\text{POD} & \\
2\text{H}_2\text{O}\text{Phenol + 4-AAP} & \rightarrow \text{Quinoneimine dye + H}_2\text{O}
\end{align*}
\]
Low density Lipoprotein (LDL) Cholesterol, Very Low Density Lipoproteins (VLDL) Cholesterol and Chylomicron fractions are precipitated by addition of polyethylene Glycol 6000 (PEG). After centrifugation, the high density lipoprotein (HDL) fraction remains in the supernatant and is determined with CHOD-PAP method.

**Estimation of HDL cholesterol**

By PEG -CHOD-PAP METHOD

**Estimation of LDL cholesterol**

\[
\text{Total Cholesterol - Triglycerides - HDL cholesterol} \\
\text{LDL cholesterol} = \frac{\text{---}}{5}
\]

**Estimation of VLDL cholesterol**

Total cholesterol = HDL + LDL + VLDL

VLDL = Triglycerides x 0.20

\[
\text{Triglycerides} = \frac{\text{VLDL}}{0.20}
\]
Estimation of Serum Triglycerides

100 µl of sample was shaken with a mixture of 1 N sulfuric acid and chloroform.

The protein precipitates on centrifugation at the interface of the two solvents, the triglycerides dissolving in the chloroform. Phospholipids are removed with silica gel. An aliquot of the chloroform is evaporated.

Hydrolysis is carried out in the presence of a mixture of KOH and Ba (OH)₂ solution. The barium hydroxide is added so that on acidification with dilute sulfuric acid, the fatty acids will co-precipitate with the barium sulfate, permitting their easy removal. The glycerol formed is then determined by oxidation to formaldehyde and reaction with chromotrope acid. The procedure is readily applied in the routine laboratory and is reproducible [Peter, 2005].

The values were expressed as mg/dl.

Statistical analysis

Statistical analysis of the data was followed by student's t-test. The difference was considered significant when P<0.05. Values are shown as Mean ± SEM.