MATERIALS AND METHODS
Materials and methods

4. MATERIALS AND METHODS

4.1 PHARMACOGNOSTICAL ANALYSIS

Identification and Collection of the Plant
Whole plant material of *T. purpurea* was collected from Gujarat (India) during April-May at the end of flowering season. The collection was made by uprooting and thereby collecting root also. The plant was identified by comparing it morphologically and microscopically with description given in different standard texts and floras (Kirtikar and Basu, 1975). The plant was identified and authenticated by Prof. Bhaskar Punjani, Head, Botany Department, Gujarat University, Ahmedabad, India and a voucher specimen was deposited. The plant material was cleaned and dried in shade and stored at 25°C.

Macroscopic Observations
The drug was subjected to macroscopic studies which comprised of study of organoleptic characters of the drugs viz., color, odour, appearance, taste, smell, texture and fractures.

Microscopic Observations

Stem/ Root/ Leaf
For microscopical examination of drug, transverse sections of the drug were taken; the sections were treated with phloroglucinol and a drop of concentrated hydrochloric acid to stain lignified material. Lignified elements were colored pink.
Evaluation of Physical Parameters

Moisture content

Five grams of accurately weighed drug powder was heated at 105 °C in an oven to a constant weight. Weight loss after drying gave the moisture content of the material.

Determination of foreign matter

100-500 g of the drug sample to be examined was weighed accurately, and spread out in a thin layer. Foreign matter was detected by inspection with the unaided eye or by the use of lens (10 X). The foreign matter was separated and weighed and percentage foreign matter was calculated.

Determination of Ash Values

Determination of total ash

Accurately weighed 2 g of the powdered drug was taken in a tarred silica dish and it was incinerated at a temperature not exceeding 450 °C until free from carbon. The sample was cooled and weighed. If a carbon free ash cannot be obtained in this way, the charred mass was exhausted with hot water. The residue was collected on an ashless filter paper and the residue and the filter paper were incinerated the filtrate was evaporated to dryness, and ignited at a temperature not exceeding 450 °C. The percentage of ash was calculated with reference to the air-dried drug.

Determination of acid-insoluble ash

The ash obtained as described in the section above was boiled for 5 min. with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a Gooch crucible or on an ashless filter paper, washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.
Materials and methods

**Determination of water-soluble ash**

The ash obtained as described in the determination of total ash was boiled for 5 min with 25 ml of water and insoluble matter was collected in a Gooch crucible, or on an ashless filter paper, washed with hot water and ignited for 15 min. at a temperature not exceeding 450°C. Weight of the insoluble matter was subtracted from the weight of the ash. The difference in weight represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air dried drug.

**Determination of Extractive Values**

**Determination of alcohol-soluble extractive**

Five grams of the coarsely powdered drug was macerated with 100 ml of alcohol (95%) in a closed flask for twenty four hours. The flasks were shaken intermittently during six hours and allowed to stand for eighteen hours. The extract was filtered rapidly, taking precautions against loss of solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, and dried at 100°C, to a constant weight. The percentage of alcohol-soluble extractive was calculated with reference to the air dried drug.

**Determination of water-soluble extractive**

Water-soluble extractive was obtained by following the same procedure as described for alcoholic-soluble extractive using chloroform water (0.25% chloroform in water) instead of alcohol.

**4.2 PHYTOCHEMICAL ANALYSIS**

**Preparation of extracts**

**Preparation of aqueous extract**

The 50 g of coarse air-dried powder was suspended in 250 ml of water for 2 h and then heated at 60-65°C for 30 min. The extract was collected and preserved. The process was repeated with the residual powder three times and...
the extracts thus collected were pooled and passed through fine cotton cloth. The total filtrate was evaporated at 60°C to obtain dried extract. This was stored at 0-4°C until used.

Preparation of alcoholic extract

The 500 g of the powder of air dried plant of *T. pupurea* was extracted exhaustively in a round bottom flask with alcohol for 48 hours. The alcoholic extract thus obtained was filtered and solvent removed under vacuum.

Preparation of flavonoidal fraction

The 200 g of coarse air-dried powder was soxhlet-extracted with 95% ethanol. After concentration under vacuum, the extract was suspended in distilled water and partitioned into ethyl acetate. The residue obtained after evaporation was dissolved in ethanol and treated with neutral lead acetate solution. The precipitate obtained was centrifuged, resuspended in ethanol, treated with hydrogen sulfide and filtered. The filtrate was evaporated under vacuum to yield the flavonoidal fraction.

Preliminary Phytochemical Screening

All the extracts prepared as mentioned above, were screened qualitatively for the major groups of chemical constituents using standard reagents. Small quantities of all the extracts were dissolved in ethanol and were subjected to preliminary phytochemical analysis for the detection of the individual components using specific reagents.

a. Alkaloids

A few ml (2-3 ml) of ethanolic extract was evaporated in a watch glass. One ml of dilute hydrochloric acid and a few drops of Mayer’s reagent were added to the residue. White precipitate indicated the presence of alkaloids.

A drop of ethanolic extract was spotted on a small piece of precoated TLC plate. The plate was sprayed with modified Dragendorff’s reagent. Orange coloration of the spot indicated the presence of alkaloids.

b. Steroids and Terpenoids

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To one ml of ethanolic extract of drug, one ml of chloroform and 2 to 3 ml of acetic anhydride was added. To the above mixture, 1 to 2 drops of concentrated sulphuric acid was added. Dark green coloration of the solution indicated the presence of steroids and dark pink or red coloration of the solution indicated the presence of terpenoids.

c. Anthraquinones

One ml of ethanolic extract of drug was heated in water containing 10 % ferric chloride solution and 1 ml of concentrated hydrochloric acid. The aqueous layer was separated and shaken with diethyl ether. The ether extract was further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicated the presence of Anthraquinones.

d. Flavonoids

To a 2-3 ml of ethanolic extract, a piece of magnesium ribbon and 1 ml of concentrated hydrochloric acid was added. Pink red or red coloration of the solution indicated the presence of flavonoids in the drug.

e. Phenols

A drop of ethanolic extract was spotted on a filter paper and a drop of phosphomolybdic acid reagent was added on it. The spot was then exposed to ammonia vapor. Blue coloration of the spot indicated the presence of phenols.

f. Tannins

To a 2-3 ml of ethanolic extract, 10 % ethanolic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicated the presence of tannins in the drug.

Estimation of phytoconstituents

Estimation of total phenolics (Singleton and Rossi, 1965)

All the extracts were prepared in the concentration of 1 mg/ml. To 80 μl of the extract, 6.32 ml of distilled water and 0.4 ml of 2N Folin-ciocalteu reagent were added and the mixture was kept aside for 8 min. After adding 1.2 ml of 20%w/v of sodium carbonate (Na₂CO₃) solution, the final mixture was kept aside.
**Materials and methods**

for 2 hr at 20°C. The absorbance was measured at 765 nm using distilled water as blank.

The data were compared with similarly prepared set of standard substance- ellagic acid in methanol, in concentration range of 50 - 500 μg/ml.

The total phenolic content C was measured using following equation:

\[ C = A - \frac{0.009}{0.0006}, \text{where } A \text{ - Absorbance} \]

**Estimation of total flavonoids (Baharam et al, 1996)**

All the extracts were prepared in the concentration of 1 mg/ml. To 0.3 ml of extract, 0.9 ml of distilled water and 0.09 ml 5% NaNO₂ were added and the mixture was incubated for 5 min at 25°C. To it, 0.09 ml of 10% methanolic AlCl₃ was added and kept aside for 5 min after which 0.6 ml 1mM NaOH was added. The volume was adjusted to 3 ml with distilled water. The absorbance was measured at 510 nm using distilled water as blank.

The data were compared with similarly prepared set of standard substance- quercetin in methanol, in concentration range of 50 - 500 μg/ml.

The total flavonoid content C was measured using following equation:

\[ C = A - \frac{0.0026}{0.0008}, \text{where } A \text{ - Absorbance} \]

**HPTLC Finger Printing**

TLC fingerpnnt profiles of aqueous extract, alcoholic extract as well as flavonoid fraction were established using HPTLC. Suitably diluted stock solution of all the extract were spotted on pre-coated silica gel 60 F₂₅₄ TLC plates (E. Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate were developed in the following solvent systems:

For the presence of rutin- ethyl acetate : n-butanol : formic acid : water (5: 3: 1: 1 v/v)

For the presence of quercetin- toluene: ethyl acetate: formic acid (5: 4: 1 v/v)

The plates were dried at room temperature and scanned using CAMAG TLC scanner 3 at UV 254 and 366 nm and Rₐ values, absorption spectra of the resolved bands were recorded.

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Quantitative estimation of rutin in extracts of *T. purpurea*

**Preparation of standard solution**

A stock solution of rutin (100μg/ml) was prepared by dissolving an accurately weighed 10 mg of rutin standard in 100 ml of methanol in a volumetric flask.

**Preparation of sample solution**

The sample solution of the aqueous extract, alcoholic extract and the flavonoidal fraction was prepared by dissolving an accurately weighed 10 mg of the freshly prepared dried extracts in 10 ml of methanol in a volumetric flask. The extracts were filtered and the volume is adjusted to 10 ml with methanol.

**Calibration curve for rutin**

The standard solutions 2 μl, 4 μl, 6 μl, 8 μl, 10 μl, 12 μl, 14 μl and 16 μl (200 ng, 400 ng, 600 ng, 800 ng, 1000 ng, 1200 ng 1400 ng and 1600 ng per respective spot) were applied (band width: 5 mm, distance between the bands: 10 mm) on a precoated silica gel 60 F_{254} TLC plate (E. Merck, Cat. No. 1.05554.0007) (0.2 mm thick), 10 mm from the bottom edge, using a CAMAG Linomat IV Automatic Sample Spotter. The plate was developed in a solvent system (20 ml) of ethyl acetate-n-butanol-formic acid-water (5:3:1:1, v/v) in a CAMAG glass twin–through chamber (20 x 10 cm) previously saturated with the solvent for 20 min (temperature 25±2°C, relative humidity 40%). The development distance was 8 cm. After removing the plate from the chamber, it was dried in air and scanned and quantified at 254 nm using a CAMAG TLC Scanner 3 and Cats 5 software. Data of peak area were recorded. A calibration curve was obtained by plotting peak area vs concentration of rutin applied.

**Estimation of rutin from aqueous extract, alcoholic extract and flavonoidal fraction of *T. purpurea***

10 μl, 12 μl and 14 μl of all the three sample solutions of *T.purpurea* were applied on precoated silica gel 60 F_{254} HPTLC plate (E. Merck) with the CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as...
Materials and methods

described in section 4.2.4.3. The peak areas were recorded. The amount of rutin present in the samples was calculated using calibration curve for rutin.

**Quantitative estimation of quercetin in extracts of T. purpurea**

**Preparation of standard solution**

A stock solution of quercetin (100μg/ml) was prepared by dissolving an accurately weighed 10 mg of quercetin standard in 100 ml of methanol in a volumetric flask.

**Preparation of sample solution**

The sample solution of the aqueous extract, alcoholic extract and the flavonoidal fraction was prepared by dissolving an accurately weighed 10 mg of the freshly prepared dried extracts in 10 ml of methanol in a volumetric flask. The extracts were filtered and the volume is adjusted to 10 ml with methanol.

**Calibration curve for quercetin**

The standard solutions 2 μl, 3 μl, 4 μl, 5 μl, 6 μl, 7 μl, 8 μl and 9 μl (200 ng, 300 ng, 400 ng, 500 ng, 600 ng, 700 ng, 800 ng and 900 ng per respective spot) were applied (band width: 5 mm, distance between the bands. 10 mm) on a precoated silica gel 60 F254 TLC plate (E. Merck, Cat. No. 1.05554.0007) (0.2 mm thick), 10 mm from the bottom edge, using a CAMAG Linomat IV Automatic Sample Spotter. The plate was developed in a solvent system (20 ml) of toluene-ethyl acetate-formic acid (5:4:1, v/v) in a CAMAG glass twin-through chamber (20 x 10 cm) previously saturated with the solvent for 20 min (temperature 25±2°C, relative humidity 40%). The development distance was 8 cm. After removing the plate from the chamber, it was dried in air and scanned and quantified at 374 nm using a CAMAG TLC Scanner 3 and Cats 5 software. Data of peak area were recorded. A calibration curve was obtained by plotting peak area vs concentration of quercetin applied.

**Estimation of quercetin from aqueous extract, alcoholic extract and flavonoidal fraction of T. purpurea**

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12 μl, 14 μl and 16 μl of the aqueous extract and 10 μl, 12 μl and 14 μl of the alcoholic extract and flavonoidal fraction of *T. purpurea* were applied on precoated silica gel 60 F$_{254}$ HPTLC plate (E. Merck) with the CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as described in section 4.2.5.3. The peak areas were recorded. The amount of quercetin present in the samples was calculated using calibration curve for quercetin.

4.3 PHARMACOLOGICAL EVALUATION

The protocol of the experiments were approved by institutional animal ethics committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Protocol Nos: IPS/PCOL/PHD08/001, IPS/PCOL/PhD10/001, IPS/PCOL/FAC10-11/2001 and IPS/PCOL/FAC10-11/001)

**Induction of Type 1 diabetes mellitus**

Healthy Sprague Dawley rats of either sex weighing 250-300 gm were used for the study. Animals were housed in groups of 3 rats per cage under well controlled conditions of temperature and humidity and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet and filtered water *ad libitum*.

Rats were made diabetic by single intravenous tail vein injection of streptozotocine (STZ) (Sigma Ltd., USA) 45mg/kg dissolved in 0.1mol/lit citrate buffer. Control rats were injected with 0.1mol/lit citrate buffer alone. At this dose characteristic signs of diabetes mellitus such as glycosuria (>2%), polydipsia, polyphagia etc. were observed. Animals were checked for the extent of glycosuria 48h after the injection of streptozotocin using *Diastix* (Bayer Diagnostics, India). Animals showing glycosuria (>2%) were considered as diabetic. 5% glucose solution was given 2 days before and 3 days after the
streptozotocin injection to prevent the initial hypoglycemic effect of streptozotocin.

**Treatment Protocol for Type 1 diabetes**

Study of effect of various extracts of *T. purpurea* on type 1 diabetic rats involved 3 sets of experiments. The groups of animals in three different sets were as follows.

Set I: Effect of aqueous extract of *T. purpurea* on type 1 diabetic rats.

- **CON**: Control animals
- **COT3**: Control animals treated with aqueous extract of *T. purpurea* (300mg/kg/day, p.o.),
- **COT5**: Control animals treated with aqueous extract of *T. purpurea* (500mg/kg/day, p.o.),
- **DIA**: Diabetic Control animals
- **DIA3**: Diabetic animals treated with aqueous extract of *T. purpurea* (300mg/kg/day, p.o.),
- **DIAS**: Diabetic animals treated with aqueous extract of *T. purpurea* (500mg/kg/day, p.o.)

Set II: Effect of alcoholic extract of *T. purpurea* on type 1 diabetic rats.

- **CON**: Control animals
- **COT3**: Control animals treated with alcoholic extract of *T. purpurea* (300mg/kg/day, p.o.),
- **COT5**: Control animals treated with alcoholic extract of *T. purpurea* (500mg/kg/day, p.o.),
- **DIA**: Diabetic Control animals
- **DIA3**: Diabetic animals treated with alcoholic extract of *T. purpurea* (300mg/kg/day, p.o.),
- **DIAS**: Diabetic animals treated with alcoholic extract of *T. purpurea* (500mg/kg/day, p.o.)
Set III: Effect of flavonoid fraction of *T. purpurea* on type 1 diabetic rats.

CON: Control animals

COT: Control animals treated with flavonoid fraction of *T. purpurea* (40mg/kg/day, p.o.)

DIA: Diabetic Control animals

DIT: Diabetic animals treated with flavonoid fraction of *T. purpurea* (40mg/kg/day, p.o.)

The treatment was given for a period of eight weeks. Animals were maintained for 8 weeks treatment period with free access to conventional dietary feed and water *ad libitum*. All animals were monitored regularly for changes in body weight, food intake, water intake and mortality throughout the course of study.

**Induction of Selenite Cataract**

Nine days old suckling Sprague Dawley rat pups of either sex were used for the study. The pups were kept with their mother under well controlled conditions of temperature and humidity and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet and filtered water *ad libitum*. The rat pups were randomly divided into five groups as follows:

Group I: Control animals

Group II: Animals treated with sodium selenite

Group III: Animals treated with sodium selenite and quercetin

Group IV: Animals treated with sodium selenite and flavonoid fraction of *T. purpurea*

Group V: Animals treated with sodium selenite and alcoholic extract of *T. purpurea*

Group II to group V animals were administered a single subcutaneous injection of 4 mg/kg body weight of sodium selenite (Na2SeO3). The control animals were administered 0.9% saline instead of sodium selenite. Upon opening of their eyes on the 14-15th day, 80-85% of the pups had developed bilateral
nuclear cataract and the rest of the animals developed advanced nuclear to mature cataract. Eyes were dilated with 1% tropicamide and 10% phenylephrine and the animals were examined with an ophthalmoscope to observe the frequency of cataractogenesis. The cataract was categorized into following stages:

Stage 0: Normal transparent lens
Stage 1: Lens with minimal nuclear opacity
Stage 2: Lens with partial nuclear opacity
Stage 3: Lens with mature dense opacity

Quercetin was suspended in distilled water and administered orally (p.o.) at a dose of 1 mg/kg/day for 4 weeks. The alcoholic extract and flavonoid fraction of the plant was suspended in distilled water and administered orally (p.o.) at a dose of 300 mg/kg/day and 40 mg/kg/day for 4 weeks.

**Blood sample collection and serum analysis**

Blood samples were collected at the end of 8 weeks of treatment in type 1 diabetic rats in clean dry centrifuge tubes after 12h fasting from the retro orbital plexuses under light ether anesthesia and were allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 5000 rpm for 20 min and stored at -20°C until the analysis was carried out. Serum samples were analyzed for glucose, cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, creatinine, urea, Lactate De-Hydrogenase (LDH), Creatinine Kinase (CK) spectrophotometrically (Shimadzu UV, Japan) using available biochemical diagnostic kits (Accucare Diagnostics, Ltd, India). Serum insulin was estimated by radioimmunoassay technique using kits obtained from Board of Radiation and Isotope Technology, Mumbai, India in gamma counter (Packard, USA). Hemodynamic parameter viz. blood pressure, heart rate, rate of pressure development and decay were recorded by carotid artery cannulation using transducer (BP 100) and Labscribe Systems (l-worx, USA). After withdrawal of
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blood samples from retro-orbital plexus and recording hemodynamic parameters, animals were sacrificed, hearts were excised, extraneous tissues were separated and wet weight of the entire heart and left ventricle were noted down to calculate the index of cardiac hypertrophy and left ventricular hypertrophy index. Quantification of left ventricular myocardial hydroxyproline concentrations was performed according to the method of Prockop and Udenfriend (Prockop and Udenfriend 1960). After the excision of heart, the eyes of the animals was dissected by posterior approach and the lens were isolated. The lenses were used for preparing the homogenate in 50 mM phosphate buffer saline for the measurement of several oxidative stress parameters involved in development of cataract.

Estimation of biochemical parameters for Type 1 diabetes

Glucose (GOD/POD method):

Principle:

In the single reagent system, glucose is oxidized by the enzyme Glucose oxidase (GOD) to give D- gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of enzyme Peroxidase (POD) oxidizes phenol, which combine with 4-Aminoantipyrine to produce a red colored quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration in the sample and is measured at 505nm. The final colour is stable for 2 hrs.

\[
\begin{align*}
\text{D - glucose} + \text{H}_2\text{O} + \text{O}_2 & \rightarrow \text{D - gluconic acid} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{- Aminoantipyrine} + \text{Phenol} & \rightarrow \text{Red quinoneimine dye} + 2\text{H}_2\text{O}
\end{align*}
\]

Preparation of working enzyme reagent:

One buffer/enzyme/chromogen tablet was gently dissolved in 20 ml of distilled water in a clean beaker, with continuous stirring.
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Procedure:
The readymade Glucose Standard was provided in the kit having concentration of 100 mg %. The tubes were arranged and the serum, standard, distilled water and the working enzyme reagent were added as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Enzyme Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Glucose Standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Serum sample</td>
<td></td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The content of tubes were mixed by vortexing and incubated at 37 °C. for 15 minutes. Absorbance of Test (T) and Standard (S) were measured against Blank (B) at 505 nm using UV – VISILE Spectrophotometer (UV-1601 Shimadzu, Japan).

Calculations:

Serum Glucose (mg %) = \[
\frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times 100
\]

Glycosylated Hemoglobin:
Whole blood was collected with heparin.

Principle:
Whole blood was mixed with lysing reagent to prepare a hemolysate. This was then mixed with a weakly binding cation exchange resin. The non-Glycosylated Hemoglobin binds to the resin leaving glycosylated hemoglobin (GHb) free in the supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb.
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Procedure:

Step I - Hemolysate preparation
1. 0.25 ml of lysing reagent (2) was pipette out in a test tube.
2. To it, 0.05 ml of well mixed whole Blood/control was added.
3. It was mixed well and allowed to stand at room temperature for 5 minutes.

Step II - GHb separation and assay
1. Resin Tube (1) was brought to assay temperature by incubating the tube in a water bath.
2. To it, 0.1 ml of hemolysate (from step1) was added.
3. A Resin Separator in the tube was positioned so that the rubber sleeve was approximately 3 cm above the resin level.
4. The contents were mixed on vortex mixer continuously for 5 minutes.
5. The resin was allowed to settle at assay temperature for 5 minutes, the Resin separator was push down in the tube until the Resin was firmly packed.
6. The supernatant was poured directly into a cuvette and the absorbance was measured against deionized water at 415 nm.

Step III - Total Hemoglobin (THb) assay
1. 5.0 ml of deionized water was pipetted into a test tube.
2. To it, 0.02 ml of hemolysate (from step 1) was added.
3. It was mixed and absorbance was read against deionized water at 415 nm.

Calculations:

\[
\text{GHb\%} = \frac{A \text{ of GHb}}{A \text{ of THb}} \times 7.2 \times \text{temp. factor (Tf)}
\]

For assay at 23°C Tf = 1.0; at 30°C Tf = 0.9.
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**Insulin (Radioimmunoassay method):**

**Principle:**
When serum containing insulin is added to a tube containing a fixed amount of antibody and a fixed amount of the radio labeled Insulin (I$^{125}$ labeled), insulin present in serum and the radiolabelled Insulin competes for the antibody. The amount of radiolabelled Insulin bound to the antibody is inversely proportional to the amount of Insulin in serum. A standard curve with known amounts of the test substance can thus be constructed and the amount in the unknown samples can be calculated.

**Procedure:**
All reagents were allowed to reach room temperature and mixed thoroughly before the use.

1. Tubes were arranged and labeled as total counts, non-specific binding (NSB), standards controls and unknowns.
2. 0.3 ml Assay buffer was added to unknown serum samples while 0.2 ml of assay buffer was added to Insulin standard control tubes and 0.4 ml of assay buffer was added to the NSB. Assay buffer was not added to the total count tubes.
3. Insulin standard controls and unknown (Serum samples) were added (100 µl) to the appropriate tubes.
4. 100 µl of insulin free serum was added to NSB and insulin standard control tubes. It was not added to unknown serum samples and total count tubes.
5. 100 µl insulin antiserum was added to all tubes except NSB and total count tubes.
6. All tubes were vortexed.
7. All tubes were incubated at 2°C to 8°C for 16 hours.
8. 100 µl of Insulin (Labeled I$^{125}$) reagent was added to each tube.
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9. Again all tubes were vortexed and incubated at room temperature for 3 hours.
10. 100 μl of second antibody was added to all tubes except total count tubes.
11. One ml of precipitating reagent was added to all tubes except total count tubes.
12. Again all tubes were vortexed and incubated at room temperature for 10 to 15 minutes.
13. All tubes except the total count tubes were centrifuged for 15 to 20 minutes at 4500 RPM.
14. All tubes were decanted and Radioactivity was measured with the help of I\textsuperscript{125} Gamma Counter, keeping each tube for one minute for the reading.

Calculations:

1. The background and the blank count were subtracted from all the other counts to give corrected counts.
   
   Corrected count of sample of standard

2. \[
   \text{\% B / Bo} = \frac{\text{Corrected count of zero standard}}{\text{Corrected count of sample of standard}} \times 100
   \]

3. The standard curve was prepared as \text{\% B / Bo} on the logit and μl/ml of insulin on the logarithmic scale of logit – log graph paper.

   The concentration of insulin in sample was obtained from the standard curve by extrapolation.

Serum Cholesterol:

Principle:

The estimation of cholesterol involves the following enzymatic reaction:

\[\text{Cholesterol esterase}\]

\[\text{Cholesterol Ester} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Fatty acids}\]
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**Cholesterol oxidase**

\[
\text{Cholesterol} + \text{O}_2 \longrightarrow \text{Dehydroacetone phosphate} + \text{H}_2\text{O}_2
\]

**Peroxidase**

\[
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \longrightarrow \text{Red quinone} + 4\text{H}_2\text{O}
\]

The intensity of the red complex (red quinone) formed during the reaction is directly proportional to the cholesterol concentration in the sample and is measured at 505nm.

**Procedure:**

Reagents were reconstituted as described in the leaflet supplied along with the kit. 10 µl of serum samples, distilled water serving as control and standard triglycerides (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent i.e. enzyme/chromogen mixture. They were incubated at 37°C for 10 min and absorbance was read against blank at 505nm within 60 min. The tubes were arranged and the reagents and the serum samples were added as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>10 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Working Standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Serum Sample</td>
<td></td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

**Calculations:**

\[
\text{Serum Cholesterol} = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times 200
\]
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Serum LDL-Cholesterol:

Principle:
Direct determination of serum LDL-C (low density lipoprotein cholesterol) levels was done without the need for any pre-treatment of centrifugation steps. The assay takes place in two steps.

- 1° elimination of lipoprotein non-LDL

\[
\text{Cholesterol esters} \quad \xrightarrow{\text{CHE}} \quad \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol + O}_2 \quad \xrightarrow{\text{CHOD}} \quad \text{4-Cholestenone + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 \quad \xrightarrow{\text{Catalase}} \quad 2\text{H}_2\text{O} + \text{O}_2
\]

- 2° measurement of LDL-C

\[
\text{Cholesterol esters} \quad \xrightarrow{} \quad \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol + O}_2 \quad \xrightarrow{} \quad \text{4-Cholestenone + H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{TOOS + 4-AA} \quad \xrightarrow{} \quad 2\text{H}_2\text{O} + \text{O}_2
\]

The intensity of color formed is proportional to the LDL-C concentration in the sample.

Procedure:
Reagents and samples were pipetted out as shown below:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Serum sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (μl)</td>
<td>375</td>
<td>375</td>
<td>375</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Sample (μl)</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>

Mixed and incubated for 5 mins at 37°C

| R2 (μl) | 125 | 125 | 125 |

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The contents were mixed and incubated for 30 min. at R.T. and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 546 nm.

Calculations:

LDL-C (mg/dl) = Abs. of sample / Abs. of Calibrator X Calibrator conc.

Serum HDL-Cholesterol:

Principle:

Low and Very Low-Density (VLDL) are precipitated by a solution containing PEG 6000, leaving behind the High-Density-Lipoproteins in solution. HDL Cholesterol is estimated in the supernatant by a series of enzymatic reactions which are initiated by the oxidation of Cholesterol to Cholestenone by Cholesterol oxidase, accompanied by the formation of hydrogen peroxide. In a second reaction catalyzed by peroxidase, 4-aminoantipyrine and phenol react with hydrogen peroxide to form red coloured quinoneimine. Absorbance at 505 nm is directly proportional to HDL Cholesterol concentration.

Procedure:

Reagents were reconstituted as described in the leaflet supplied along with the kit. 0.2 ml of serum sample was mixed well with 0.2 ml of precipitating reagent (Reagent 2) and kept at room temperature for 10 min. and then centrifuged at 2000 rpm for 15 min. to obtain clear supernatant.

Step A – HDL-Cholesterol Separation :-

<table>
<thead>
<tr>
<th>Pipette into centrifuge tube</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Sample</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Precipitating Reagent</td>
<td>0.2 ml</td>
</tr>
</tbody>
</table>

100 µl of supernatant and 1.0 ml of cholesterol reagent were mixed. In case of blank, 1.0 ml cholesterol reagent was taken. For standard, 100 µl of HDL-cholesterol Standard was mixed with 1.0 ml of Cholesterol reagent.

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**Step B – Color Development**

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>HDL-Cholesterol Standard</td>
<td>100 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant from Step A</td>
<td></td>
<td>100 µl</td>
<td></td>
</tr>
</tbody>
</table>

After mixing, the tubes were incubated at 37°C for 10 min. Absorbance was read against reagent blank at 505 nm within 60 minutes.

**Calculations:**

\[
\text{Serum HDL-Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50 \times 2
\]

**Serum Triglycerides:**

**Principle:**

It is based on Enzymatic Colorimetric Test (GPO – PAP). The triglycerides are enzymatically hydrolyzed to glycerol according to the following reactions:

1. Triglycerides → Glycerol + Free fatty acids
2. Glycerol + ATP → Glycerol-3-Phosphate + ADP
3. Glycerol-3-Phosphate + O₂ → Dihydroxyacetone phosphate + H₂O₂
4. H₂O₂ + 4-Aminoantipyrine + p-chlorophenol → 4H₂O + Quinoneimine dye

Following enzymes catalyze the reactions:

- Reaction no. 1 is catalyzed by Lipoprotein Lipase.
- Reaction no. 2 is catalyzed by Glycerol kinase.
- Reaction no. 3 is catalyzed by Glycerol-3-Phosphate Oxidase.
- Reaction no. 4 is catalyzed by Peroxidase (POD).
Materials and methods

Procedure:
The tubes were arranged and the reagents and the serum samples were added as follows in the blank, standard and test as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Triglyceride Standard</td>
<td></td>
<td>10 μl</td>
<td></td>
</tr>
<tr>
<td>Serum Sample</td>
<td></td>
<td></td>
<td>10 μl</td>
</tr>
</tbody>
</table>

The contents of the tubes were mixed well by vortexing, incubated at 37°C for 10 minutes and the absorbance was measured against reagent blank at 505 nm wavelength within 60 min on spectrophotometer.

Calculations:

\[
\text{Serum Triglycerides} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 200
\]

Serum VLDL-Cholesterol:

VLDL Cholesterol was estimated using Friedewald’s equation:

\[
\text{VLDL Cholesterol} = \frac{\text{Triglyceride}}{5}
\]

Serum Creatinine:

Principle:

Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured colorimetrically at 520nm.

Procedure:

0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.
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2.0 ml of the supernatant from the above step is mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively. All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520nm.

Serum Urea:

Principle:
The Berthelot reaction has been used for the measurement of urea and ammonia. Urea is converted to ammonium by the use of urease. Ammonium ion then reacts with a mixture of salicylate, sodium nitroprusside and hypochlorite to yield a blue-green chromophore. The intensity of the color formed is proportional to the urea concentration in the sample.

Procedure:
The tubes were arranged and the reagents and the serum samples were added as follows in the blank, standard and test as follows :-

<table>
<thead>
<tr>
<th></th>
<th>Blank (B)</th>
<th>Standard (S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Reagent II</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Urea Standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Serum Sample</td>
<td></td>
<td></td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Mixed and incubated for 5 mins at 37°C

Reagent III

|                  | 1.0 ml    | 1.0 ml       | 1.0 ml   |

The contents were mixed and incubated for 5 mins at 37°C and absorbance of sample (AT) and standard (AS) against reagent blank was measured at 578 nm.
Materials and methods

Calculations:

\[
\text{Serum Urea} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 50
\]

Serum Lactate Dehydrogenase (LDH):

**Principle:**
Lactate Dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate and NADH to NAD. LDH activity in serum/plasma is directly proportional to the rate of decrease in the absorbance of NADH at 340 nm.

**Preparation of reagents**
Reagent 1: Buffer reagent
Reagent II. Enzyme reagent

3 ml of reagent 1A was added into one bottle of reagent 1 containing solid coenzyme powder. It was mixed by gentle swirling till completely dissolved and waited for 5 minutes before used.

**Procedure:**
25 μL of sample were taken in the test tubes and 1 ml of reconstituted reagent was added in each test tube. The contents were mixed and read absorbance at 340 nm on spectrophotometer immediately and after 1 minute interval for 2 minutes.

**Calculations:**

\[
\text{Serum LDH (U/L)} = \frac{\text{Average difference in absorbance (ΔA)/min}}{2} \times 6592
\]

Serum Creatinine Kinase (CK):

**Principle:**
This procedure involves measurement of Creatine kinase (CK) activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the
activity of CK-MM and half of CKMB while not affecting the B subunit of CK-MB and CK-BB. Than the CK method is used to quantitatively determine CK-BB activity. CK catalyses the reaction between creatine phosphate and ADP, giving creatine and ATP. ATP and glucose in the presence of G6PDH oxidises, and reduces NAD to NADH. The rate of NADH formation is determined photometrically at 340 nm & is directly proportional to CK-BB activity. The CK-MB activity is calculated by multiplying CKBB x 2.

**Preparation of reagents**

Reagent I: Enzyme reagent I
Reagent II: Enzyme reagent II

4 ml of Enzyme reagent I was mixed with 1 ml of enzyme reagent II. The working reagent thus prepared was stable for 30 days at 2-8°C.

**Procedure:**

50 µL of sample were taken in the test tubes and 1 ml of working reagent was added in each test tubes. The contents were mixed and incubated for 10 min at 37°C. The absorbance was read at 340 nm on spectro photometer after 1 minute interval for 5 minutes.

**Calculations:**

Serum CK (U/L) = Average difference in absorbance (AΔ)/minute × 8095

**Estimation of cardiovascular parameters in Type 1 diabetes**

Hemodynamic parameters:

The animals were anaesthetized by Ketamine (100 mg/kg, i.p.) + Xylazine (7 mg/kg, i.m.). The body temperature was maintained at 37 ± 1°C during the experiment. The carotid artery behind the trachea was exposed and cannulated for the measurement of hemodynamic parameters using a transducer (BP 100) and Labscribe Systems (I-worx-118, USA). The hemodynamic parameters
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observed were systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP), rate of pressure development \( (\frac{dp}{dt_{max}}) \) and rate of pressure decay \( (\frac{dp}{dt_{min}}) \). All the data were analyzed using Labscribe software (Version 2.0.0)

Hypertrophic parameters:
After measurement of hemodynamic parameters, animals were sacrificed. The skin was quickly incised at the midline over the sternum and the hearts were exposed by cutting the pericardium. Hearts were isolated from the body, blotted with filter paper to remove excess of blood, remaining extraneous tissues were removed and weight of the heart, left ventricular weight, right ventricular weight and femur length was noted down. Index of cardiac hypertrophy was calculated as wet heart weight to femur length ratio and left ventricular hypertrophy index was calculated as wet left ventricle weight to wet heart weight ratio. Also left ventricular weight to right ventricular weight ratio was estimated. Left ventricular wall thickness was measured using screw gauge micrometer.

Left Ventricular Collagen content:
The tissue for collagen measurement was hydrolyzed with 6 N Hydrochloric acid at 110° C for 12 hrs and after 12 hrs, hydrolysate obtained was used for estimation.
Quantification of left ventricular myocardial hydroxyproline concentrations was performed according to the method of Prockop and Udenfriend (1960) Left ventricular myocardial specimens of approximately 100 mg weight were dried and hydrolyzed in 6N HCl at 110°C for 12 hours. The hydrolyzed material was dried and reconstituted in 5 mL H2O. 200 μL of Hydrolysate was mixed with 200 μL ethanol and 200 μL chloramine T solution (1.4% in citrate buffer) and allowed to oxidize for 20 minutes at room temperature. 400 μL of Ehrlich's reagent was added. After 15 min of incubation at 60°C, extinction at 573 nm was measured. Because hydroxyproline is incorporated only into collagen and assuming that collagen contains 14% hydroxyproline, the total collagen content per dry weight
Materials and methods

was calculated. Different concentrations of hydroxyproline (CDH Ltd., New Delhi) were prepared and mixed with above mentioned reagents and estimated spectrophotometrically at 573nm for standard curve.

Estimation of left ventricular pro-oxidant and antioxidant activity:

Preparation of tissue homogenate

Animals were sacrificed after measuring hemodynamic parameters by carotid artery cannulation, left ventricles were dissected out, rinsed with ice cold distilled water followed by washing with sucrose solution (0.25 M). Left ventricles were again rinsed with distilled water and immediately stored at -20°C till further biochemical analysis. 100 mg left ventricular tissue was homogenized in 2 ml ice cold tris-hydrochloride buffer (pH 7.2). Homogenate was centrifuged at 800g for 10 min followed by centrifugation of the supernatant at 12,000g for 15 min. The supernatant obtained was used for the following estimations.

Estimation of tissue protein levels (Lowry et al, 1951)

Preparation of reagents:

2% Na₂CO₃ in 0.1N NaOH solution was labeled as Reagent A, 0.5% CuSO₄ 5H₂O in 1% sodium potassium tartrate was labeled Reagent B and alkaline copper sulphate solution (mixture of 50ml of Reagent A and 1ml of Reagent B) was labeled Reagent C. Dilute Folin Phenol Reagent (1N) was labeled Reagent D. The Folin Phenol Reagent was titrated with NaOH solution to a phenolphthalein end point. On the basis of this titration, the Folin Phenol Reagent was diluted (about 2 fold) to make it 1N in acid.

Procedure:

To 0.2 ml supernatant in a test tube, 1 ml of reagent C was added. It was mixed well and allowed to stand for 10 min or longer at room temperature. About 0.1 ml of reagent D was added very rapidly and mixed within a second or two. After 30 min or longer the samples were read in a spectrophotometer at 750 nm. Final calculation was made from the standard curve.
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Measurement of Lipid peroxidation levels (Ohkawa et al, 1979)

**Principle:**
The method estimates Malondialdehyde (MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 532 nm.

**Procedure:**
The whole homogenate (0.2 ml) was mixed with 0.2 ml of 8% w/v sodium dodecyl sulfate, 1.5 ml of 20% acetic acid in 0.27 M hydrochloric acid, 1.5 ml freshly prepared of thiobarbituric acid (TBA) (1% w/v in Tris-buffer, pH-7) and 0.6 ml of distilled water. The mixture was heated in a water bath at 95 °C for 45 minute, cooled and 5 ml of the mixture was mixed with 5 ml of mixture of n-butanol:pydine (15:1). All the reagents were mixed well and pink colour developed in upper organic layer, the absorbance of which was read against blank at 532 nm. Malondialdehyde level was calculated using molar extinction coefficient of malondialdehyde $1.56 \times 10^5 \text{ M}^{-1} \text{ Cm}^{-1}$ and reported as nmoles of MDA/mg protein.

**Measurement of Reduced glutathione (GSH) levels:**
Reduced GSH levels in tissue homogenates were estimated as per the method described by Beutler et al (1963). The supernatant (2 ml) was mixed with 10% chilled trichloroacetic acid. The mixture was kept in ice bath for 30 min and centrifuged at 1000g for 10 min at 4°C. Supernatant (0.5 ml) was mixed with 2.0 ml 0.3 M disodium hydrogen phosphate and 0.25 ml 5, 5'-dithiobis-2-nitrobenzoic acid (40 mg/100ml in 1% sodium citrate) was added just before measuring the absorbance at 412 nm. Standard curve for GSH was prepared using glutathione. Results were expressed as µmole of GSH/g tissue.

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Measurement of Superoxide dismutase (SOD) levels:

SOD was estimated as per the method described by Misra and Fridovich (1984). Supernatant (0.1ml) of sample was mixed with 0.1 ml EDTA (1x10^{-4} M), 0.5 ml of carbonate buffer (pH 9.7) and 1 ml of Epinephrine (3x 10^{-3}M). The optical density of formed adrenochrome was read at 480 nm for 3 min at an interval of 30 sec. the enzyme activity has been expressed in terms of U/min/mg protein. One unit of enzyme activity is defined as the concentration required for the inhibition of the chromogen production by 50% in one minute under the defined assay conditions.

Estimation of biochemical parameters for Cataract

Preparation of erythrocyte homogenate
At the end of four weeks, blood was collected from retro-orbital plexus and centrifuged for 15 min at 4000 g in refrigerated centrifuge. After removal of plasma and buffy coats, the red cells were washed twice with two volumes of phosphate buffered saline (PBS) of pH 7.00. Hemolysates were prepared by addition of two volumes of cold distilled deionized water to erythrocytes. Cellular debris was removed by centrifugation at 4000 g for 30 min.

Preparation of lens homogenate
After the animals were sacrificed, the eye lenses were dissected by posterior approach. The lenses of both the eyes were rinsed with ice-cold distilled water, blotted, weighed and stored at -20 °C for further analysis. Known weights of lens were homogenized in phosphate buffer saline using a glass motor-pestle to prepare 10% homogenate and centrifuged at 4000 g in the refrigerated centrifuge for 30 minutes. The supernatant were taken as aliquots for the estimation of the biochemical parameters.
Materials and methods

Estimation of protein levels

The dissected lenses were homogenized in 5% trichloroacetic acid for total protein estimation. The homogenate prepared was centrifuged and the precipitated protein was dissolved in Sodium hydroxide solution and used for the estimation of total proteins. Soluble and insoluble fractions of the protein were estimated by preparing homogenate in double distilled water. The water soluble supernatant was used for estimation of soluble protein and the residue was dissolved in sodium hydroxide and used for the estimation of insoluble protein. The protein was estimated by the method described by Lowry et al (1951).

Estimation of protein sulfhydryl content of lens protein

Protein sulfhydryl content of lens proteins was determined using the Ellman’s procedure modified by Grattagliano et al. (1996). 0.2 ml of lens homogenate prepared in phosphate buffer saline was precipitated with equal volume of 4% sulfosalicylic acid (SSA) and the pellets obtained after centrifugation were washed with 1 ml of 2% SSA to remove free thiols. The washed pellets were dissolved in 0.2 ml of 6 M guanidine (pH 6.0) and read spectrophotometrically at 412 nm and 530 nm before and after 30 min incubation in the dark with 50 μl of 10 mM DTNB. Content of protein sulfhydryls was calculated using a calibration curve prepared with reduced glutathione.

Estimation of pro-oxidant levels

Measurement of Lipid peroxidation levels

The whole lens homogenate (0.5 ml) as well as erythrocyte homogenate (0.5 ml) was used to determine MDA levels as described by Ohkawa et al (1979).

Measurement of Nitrite levels

The nitrite levels in lens were determined according to the method described by Giustarini et al (2008). 0.5 ml of lens homogenate was mixed with 0.5 ml of

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Griess reagent (1% sulfanilamide and 0.1% napthylene diamine hydrochloride in 2% phosphoric acid) and incubated for 20 min at 2-5 °C. The contents were mixed well and read spectrophotometrically against blank at 550 nm. The nitrite level was calculated using standard curve which was plotted using sodium nitrite solution.

Estimation of anti-oxidant activity

Measurement of free radical scavenging activity by DPPH ((2, 2-diphenyl -2-picryl –hydrazyl)

Principle:

DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule do not dimerise. The delocalization also gives rise to the deep violet colour. The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. DPPH is a stable free radical & accepts an electron or hydrogen radical to become 2, 2, -diphenyl – l- picryl-hydrazyl stable diamagnetic molecule (Molyneux, 2004)

The reduction capability of DPPH radical is determined by decrease in its absorbance at 520 nm induced by antioxidant. The absorption maximum of a stable DPPH radical in methanol is at of 520nm. The decrease in absorbance of DPPH radical caused by antioxidants, due to the reaction between antioxidant molecules and the radical, result in the scavenging of radical by hydrogen donation. The change in the colour from violet to yellow is visually noticeable (Sharma and Gupta, 2008).

Procedure:

1ml of 0.1mM solution of DPPH in methanol was added in 3ml of test solution of alcoholic extract (10 μg, 20 μg, 50 μg, 100 μg, 200 μg 300 μg, 400 μg and 500μg/ml) and flavonoid fraction (10 μg, 20 μg, 50 μg, 80 μg and 100 μg/ml).
Materials and methods

Solutions were kept in dark for 30 min to protect from the light. The absorbance was measured spectrophotometrically at 517 nm.

Calculations:

\[
\text{% reduction in DPPH radical} = \frac{(A_{\text{control}} - A_{\text{test}})}{A_{\text{control}}} \times 100
\]

Measurement of Reduced glutathione (GSH) levels:

Reduced glutathione was measured by the method described by Beutler et al (1963). Instead of 0.5 ml, 0.3 ml of lens supernatant and 0.5 ml of erythrocyte homogenate was used for the estimation.

Measurement of Superoxide dismutase (SOD) levels:

Superoxide dismutase levels were estimated according to the method described in section 4.3.6.4.

Estimation of calcium levels

Principle

Calcium ions form a violet complex with O-cresolphthalein complexone in an alkaline medium.

Preparation of reagents:

Reagent I : AMP Buffer reagent
Reagent II : OCPC Color reagent

Equal volume of both the reagents was mixed as working reagent which was stable for 6 hr at room temperature.

Procedure:

25 µL of homogenate was taken in the test tubes and 1 ml of working reagent was added in each test tubes. The contents were mixed and incubated for 5 min
Materials and methods

at 37° C. The absorbance was read at 570 nm on spectrophotometer against reagent blank.

Calculations:

\[
\text{Calcium levels (mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 10
\]

mg/dl was then converted into μmoles/g tissue homogenate

Estimation of calcium ATP-ase levels

Ca-ATPase activity in the lens sample was measure by the method of Hjerten & Pan (1983). 0.1 ml of 125mM Tris–HCl buffer, 0.1 ml of 50mM calcium chloride, 0.1 ml 10mM ATP and 0.1 ml distilled water was taken in two sets of test tubes. 0.1 ml of lens homogenate was added in one set and in control set was added 0.1 ml of distilled water. The contents were incubated at 37° C for 15 min, after which the reaction was stopped by the addition of 0.5 ml ice-cold 10% Trichloroacetic acid. All the tubes were then centrifuged at 3500 rpm for 10 min and the supernatant was collected. The protein free supernatant was analyzed for inorganic phosphate liberated. For that, 200 μl of the supernatant was treated with 925 μl of distilled water, 125 μl of freshly prepared ammonium molybdate (2.5% w/v) and 50 μl of freshly prepared 1-amino-2-napthol-4-sulphonic acid (ANSA). The mixture was incubated for 10 min at 37 °C. The color developed was read at 640 nm against reagent blank. The phosphorus liberated was calculated using standard curve that was plotted using standard potassium dihydrogen phosphate. Results were expressed as inorganic phosphorus liberated/min/mg protein.
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Estimation of in-vitro Lens Aldose Reductase (AR) Activity

Preparation of lens homogenate
Eyes of normal Wistar rats were removed immediately after sacrifice. The lenses were enucleated through posterior approach, washed with saline and their fresh weights were recorded. Twelve non-cataractous lenses were pooled and a 10% homogenate was prepared in 0.1M phosphate buffer saline (pH 7.4). After centrifugation at 5000 × g for 10 min in a refrigerated centrifuge, the supernatant was collected and kept at -20 °C for the determination of AR activity and protein content.

Protein estimation
Protein content in the supernatant of the lens homogenate was determined by the method of Lowry et al. (1951).

Determination of AR activity
Lens AR activity was measured according to the method of Hayman and Kinoshita (1965). A sample cuvette containing 0.7 ml of phosphate buffer (0.067 M), 0.1 ml of NADPH (25 × 10⁻⁵ M), 0.1 ml of lens supernatant, 0.1 ml of D-xylose (substrate) (10 mM) to a final volume of 1ml was read against a reference cuvette containing all components but the substrate, D-xylose. The final pH of the reaction mixture was 6.2. The enzymatic reaction was started by the addition of the substrate and the absorbance (OD) was recorded spectrophotometrically at 340 nm for 3 min at 30 second interval. AR activity was expressed as ΔOD/min/mg protein.

Lens AR activity and plant extract
The alcoholic extract and flavonoid fraction were prepared for stock solutions. To determine their AR inhibiting activity, 0.1 ml of the each plant extract from various stock solutions (final concentrations: 60, 80, 100, 200, 300 and 500 µg/ml for...
Materials and methods

Alcoholic extract and 10-100 µg/ml for flavonoid fraction was added to both the reference and standard cuvettes. The reaction was initiated by the addition of 0.1 ml D-xylose. ΔOD/min/mg protein was calculated for each sample. Quercetin (10⁻⁴ M) was used as positive control and 5% DMSO in PBS was used as negative control. Percent inhibition of AR activity was then calculated considering 100% inhibition by the positive control. IC₅₀ value for each extract was obtained from a dose response curve (DRC) calculated by plotting concentration in µg/ml versus percent inhibition.