MATERIAL AND METHODS

Glucose induced cataract was chosen as a model for the present study. A total of 210 fresh goat lenses were analyzed.

Sample Collection:

Goat eyeballs were obtained from the slaughter house and were transported to the laboratory in an ice-box. Lenses were removed from the eyeballs by intracapsular lens extraction method. The weight of the lenses was recorded and lenses were then carefully placed on sterile petri dishes with a dark colored nylon net. The lenses were incubated in tissue culture medium (TC-199) by "Lens Organ Culture Technique" for 72 hours.

Preparation of Tissue Culture Medium:

Tissue Culture medium -199 (Medium 199- with Earles salt and without sodium carbonate) was supplied in powder form by *Hi Media*. The powder was reconstituted to one litre with distilled water. Penicillin and mycostatin were added to the medium to prevent bacterial and fungal contamination. The pH of the medium was adjusted and maintained at 7.2 to 7.4.

All materials and solutions required for lens organ culture were autoclaved under standard conditions for the elimination of microorganisms including bacterial spores.

The lenses were categorized under the following groups:

Sr. No	**Group**	**No. of lenses**
1	**Normal lenses:** lenses incubated in TC-199 for 72 hours	30
2	**Experimental diabetic cataract:** lenses incubated in TC-199 + 110mM Dextrose for 72 hours	30
3	**Experimental cataract with plant extracts**: i) Lenses incubated in TC-199 + 110mM Dextrose + 0.25% S.cumini (Jambhul) aqueous extract for 72 hours	30
	ii) Lenses incubated in TC-199 + 110mM Dextrose + 0.25% A.marmelos (Bael) aqueous extract for 72 hours	30
	iii) Lenses incubated in TC-199 + 110mM Dextrose +0.25% E.officinalis (Amla) aqueous extract for 72 hours	30
	iv) Lenses incubated in TC-199 + 110mM Dextrose + 0.25% A.sativum (Garlic) aqueous extract for 72 hours	30
4	**Experimental cataract with Vitamin C:** Lenses incubated in TC-199 + 110mM Dextrose + 0.25% Vit.C for 72 hours	30

The lenses were placed on a grid/net and changes in lens transparency were observed by noting the number and characteristics of the squares of the grid/net seen through the lens. Generalized haziness or opacity, intumescence, swelling, disruption and other morphological changes in the lens were also noted.

At the end of 72 hours, the lenses were removed from the culture media and gently rolled on filter paper to remove adherent water. Each lens was then homogenized in 0.1 M Sodium phosphate buffer, pH 7.4 and w/v was adjusted to 10gm %.

The homogenate was centrifuged at 10,000 x g for 30 min at – 4^0C in a refrigerated centrifuge. The supernatant was collected and stored at –20^0C until further use. The supernatant was subjected to the estimation of -

1) Lens proteins (total soluble protein concentration) –

Lowry et al (1951) [274]

2) MDA levels as an index of lipid peroxidation-

Kei Satoh (1978) [275]

3) Specific activity of antioxidant enzymes

-*****Superoxide dismutase - ***Marklund and Marklund (1974)*** [276]

- ***Glutathione peroxidase – Randox **kit (***Paglia & Valentine - 1967)*** [277]

- ***Glutathione reductase – **Randox kit (***Goldberg& Spooner - 1983)*** [278]

4) *****Specific activity of enzyme Aldose reductase - ***Kinoshita et al (1965)***[279]

* *Methods were slightly modified to obtain maximum activity of these enzymes in lens homogenate*

Methods

1) Estimation of Total Soluble Proteins (Lowry et al, 1951)

Principle:

This method gives the protein concentration of the sample directly without protein digestion. When Folin-Ciocalteu phenol reagent is added to copper coated protein at pH 10, the reagent is reduced to give color development which is measured at 660nm. The reaction proceeds in two steps:-

a) Biuret reaction (with alkaline copper) and

b) Reduction of phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan in the treated protein

Reagents:

1) Folin-Ciocalteu phenol reagent : (use 1:3 dilution)
2) Lowry's reagent A: 2% Na_2CO_3 in 0.1 N NaOH
3) Lowry's reagent B_1: 0.5 % $CuSO_4$ + 2 drops H_2SO_4
4) Lowry's reagent B_2: 1 % Sodium citrate
5) Lowry's reagent C: 1 ml Lowry's B_1 + 1 ml Lowry's B_2 in 100 ml Lowry's A (freshly prepared)
6) Stock Albumin standard: 1mg/ml
7) Working standard: 100µg/ml

Procedure:

Lens Homogenate	0.1 ml
Distilled water	2.4 ml
Lowry's reagent C	3.0 ml

Mix and wait for 15 minutes

Folin-Ciocalteu phenol reagent	0.5 ml

Mix, wait for 45 minutes, absorbance was read at 660 nm. The same procedure was performed for standard.

Calculations:

$$\text{Concentration of Protein in mg\%} = \frac{\text{O.D of Test}}{\text{O.D of Std}} \mathbf{X} \text{ Concentration of Std}$$

2) Estimation of MDA level as an index of lipid peroxidation(*Kei Satoh, 1978)*

Principle:

Auto oxidation of unsaturated fatty acids involves the formation of semi stable peroxides which then undergo a series of reactions to form short chain aldehydes like malondialdehyde (MDA). One molecule of MDA reacts with two molecules of TBA with the elimination of two molecules of water to form pink colored pigment with maximum absorbance at 532 – 535 nm.

Reagents:

1) Trichloroacetic acid 20 % w/v : 20 gms TCA dissolved in 100 ml D/W
2) Thiobarbituric acid (0.67%) : 670 mg of TBA dissolved in 100 ml D/W

Preparation of Standard: Malondialdehyde-bis-diethyl acetal (1,1,3,3,tetraethoxy propane) was used as standard for MDA.

Standard curve was plotted taking concentration of MDA against optical density.

Procedure:

1) Homogenate 1 ml
2) 20 % TCA 1 ml
3) 0.67 % TBA 2 ml

The mixture was mixed well and heated in a boiling water bath for 10 minutes, cooled and then centrifuged. The absorbance of the solution was read at 530 nm.

Calculations:

$$\text{Concentration of MDA in n moles/ gm of lens} = \frac{\text{O.D of Test}}{1.56} \times 400$$

3) Estimation of Superoxide Dismutase Activity *(Marklund and Marklund 1974)*

Principle:

Pyrogallol (1,2,3, benzenetriol) auto-oxidizes rapidly especially in alkaline solution. The auto-oxidation primary involves a free radical chain reaction involving superoxide as a chain mediating species. SOD dismutates the Superoxide radical to O_2 and H_2O_2. The degree of inhibition of autoxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity.

Reagents:

1) 50 mMTris-HCl buffer containing 1mM EDTA (pH 8.2)
2) Pyrogallol – 5mM

Procedure:

1) Control –

   3ml Tris-HCl buffer + 0.3 ml pyrogallol.

   The auto oxidation at 420 nm was determined for 5 min.

2) Experimental–

   2.9 ml Tris-HCl buffer + 0.3 ml pyrogallol + 0.1 ml lens homogenate, readings were taken at 420 nm for 5 min.

Calculations:

A = Absorbance at 3 ½ min – absorbance at 1 ½ min for control

B = Absorbance at 3 ½ min – absorbance at 1 ½ min for experimental

$$\textbf{SOD (units / mg protein)} = \frac{A - B}{A \times 50} \times 100$$

One unit of SOD is described as the amount of enzyme required to cause 50 % inhibition of pyrogallol per 3.0 ml of assay mixture.

Note:

i) The lag of 1 ½ min was allowed for steady state of auto oxidation of pyrogallol to be attained.

ii) Concentration and amount of pyrogallolwas adjusted and fixed so that the absorbance reading after 1 ½ min was between 0.020 – 0.023 / min.

4) Estimation of Glutathione Peroxidase Activity *(Paglia and Valentine 1967)*

Kit used: Randox (Ransel)

Principle:

GlutathionePeroxidase (GPX) catalyses the oxidation of Glutathione (GSH) by CumeneHydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH, the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

$$2\ GSH + ROOH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

Reagents:

1) Reagent 1a (R1a): Glutathione, Glutathione Reductase, NADPH

   Reagent 1b (R1b): Phosphate buffer, EDTA

   One vial of R1a was reconstituted with 10 ml of buffer R1b.

2) Reagent 2 (R2): CumeneHydroperoxide

   10 µl of cumene hydroperoxide was freshly diluted with 10 ml saline by shaking vigorously. (cumene hydroperoxide is difficult to dissolve).

Procedure:

Homogenate	10µl
Reagent R1	500 µl
Cumene R2	20 µl

Mix sample, R1 and R2. The initial absorbance of sample and reagent blank after 1 minute and thereafter after 2 and 3 min was recorded. Reagent blank value was subtracted from that of test.

Calculation:

Glutathione Peroxidase (U/l homogenate) = 8412 **X** △ A 340 nm/min

5) Estimation of Glutathione Reductase Activity *(Goldberg and Spooner 1983)*

Kit used: Randox

Principle:

Glutathione Reductase GR catalyses the reduction of Glutathione (GSSG) in the presence of NADPH, which in turn is oxidised to $NADP^+$. The decrease in absorbance at 340 nm is measured.

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$ (reduced glutathione)

Reagents:

1) Reagent 1a (R1a): Phosphate buffer, EDTA
2) Reagent 1b (R1b): Substrate (GSSG)

   One vial of substrate R1b was reconstituted with 5 ml of buffer R1a.

3) Reagent 2 (R2): NADPH

   One vial of NADPH R2 was reconstituted with 3 ml of redistilled water.

Procedure:

Homogenate	100µl	
Substrate R1	500 µl ,	Mix well
NADPH R2	200 µl	

Mix. The initial absorbance of sample and reagent blank after 1 minute and thereafter after 2, 3, 4 and 5 min was recorded. Reagent blank value was subtracted from that of test.

Calculation:

Glutathione Reductase (U/l homogenate) = △A 340 nm/min **X** 1286

6) Estimation of Aldose Reductase activity (*Kinoshita et al* 1965)

Principle:

Aldose reductase is an NADPH – dependent oxidoreductase that catalyses the reduction of a variety of aldehydes and carbonyls.

In this method glyceraldehyde was used as substrate and the Aldose reductase activity was measured spectrophotometrically by the determination of decrease in absorbance at 340 nm due to utilization of NADPH.

Reagents:

1) Sodium Phosphate buffer (0.064M, pH 6.2)
2) NADPH (0.104 mM)
3) DL-glyceraldehyde (10mM)

Procedure:

Sodium Phosphate buffer:	2.4 ml
NADPH:	100 µl
Homogenate:	300 µl
DL-glyceraldehyde	100 µl

Glyceraldehyde was dedemerised for 10 minutes at 85^{o}C before assay.

Decrease in absorbance at 340nm was measured for 3 minutes and △Absorbance was noted.

Aldose reductase activity was expressed in terms of nmol NADPH oxidized per minute per mg protein.

Specific activity = △ A **X** 3225

All methods for enzyme assays were standardised and standard curves were plotted.

Preparation of medicinal plant water extracts:

The guidelines mentioned in the Ayurved Pharmacopeia of India were followed for preparing the plant water extracts.

Dry powders of S.cumini seeds, Aegle marmelos (Bael leaf), Embilica officinalis (Amla)respectively were taken and 25% w/v water extract was prepared .

25 gm fresh peeled garlic cloves (Allium sativum) were ground in 100 ml distilled water to prepare the extract.

All extracts were analysed for their purity and Total Dissolved Solids at the 'Indian Drugs Research Association & Laboratory', Pune.

0.25% solution of each extract was used for the study.