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IJPBS |Volume 2| Issue 4 |OCT-DEC |2012|320-331

Research Article
Biological Sciences

IMPACT OF HYDROALCOHOLIC EXTRACT OF *CYPERUS ROTOUNDUS* ON GLUCOSE INDUCED CATARACT- AN *INVITRO* STUDY

*P.Ramya, S.Seema and K.Vijayalakshmi**

Department of Biochemistry, Bharathi Women's College, North Chennai 600-108, Tamil Nadu, India.

**Corresponding Author Email: viji42research@yahoo.co.in, ramya0288@gmail.com*

ABSTRACT

Cataract is a clouding that developes in the crystalline lens of the eye or in its envelope, varying in degree from slight to complete opacity and obstructing the passage of light. Cataract was induced by incubating the chick lenses in 55mM glucose for 72 hours at 37°C. Impact of hydroalcoholic extract of various concentrations was studied on the levels of trace elements, electrolytes, total proteins, ATPases activity and protein cross linkage during cataract. The weight of lens decreased during cataract formation and upon treatment with Cyperus rotundus, the weight increased. The levels of zinc decreased during cataract formation and on treatment with Cyperus rotundus, the zinc level was found to increase significantly. The levels of iron, copper, calcium, sodium and potassium was found to increase during cataract and upon treatment with Cyperus rotundus their levels decreased and was brought to near normal. The activities of total ATPase, Na+ - K+ ATPase and calcium ATPase decreased during cataract formation and upon treatment with Cyperus rotundus their activities were brought to near normal. SDS-PAGE of lens protein showed aggregation of protein during cataract. On treatment with Cyperus rotundus the aggregation gradually disappeared, indicating the protective role of Cyperus rotundus in preventing cataract.

KEYWORDS

Cataract, Cyperus rotundus, ATPases, SDS-PAGE, electrolytes, trace elements.

INTRODUCTION

Cataract is the clouding that develops in the lens of the eye or in its envelope obstructing the passage of light. Cataract progress slowly to cause vision loss if untreated. Cataract is a multifactorial disease with many associated risk factors. Cataract involves numerous changes in the lens. These include glycation of lens proteins increased hydration, defects in amino acid transport metabolism, failure of membrane pumps, lowering of ATP content and swelling.

During hyperglycemia the excess glucose levels via polyol pathway results in sorbitol formation. Sorbitol accumulates in lens leading to swelling, production of free radicals leading to oxidative stress. [1]

Cataracts develops for a variety of reasons, including long-term exposure to ultraviolet light, exposure to radiation, secondary effects of diseases such as diabetes, hypertension and advanced age, or trauma (possibly much earlier). They are usually a result of denaturation of lens protein. Genetic factors are often a cause of congenital cataracts and positive family history may also play a role in predisposing someone to cataracts at an earlier age, a phenomenon of "anticipation" in pre senile cataracts.[2] Exposure to microwave radiation can cause cataracts. Atopic or allergic conditions are also known to quicken the progression of cataracts, especially in children.[3] Cataracts can also be caused by iodine deficiency.[4]

In recent years, a great emphasis has been laid on exploring the possibility of using our natural resources to delay the onset and progression of cataract. A great number of medicinal plants and their formulations are reported to possess antioxidant properties and offer protection against cataract.

Cyperus rotundus is a perennial plant, reaching a height of up to 40 cm. The names "nut grass" and "nut sedge" (shared with the related species *Cyperus esculentus*) are derived from its tubers, that somewhat resemble nuts. The root system of a young plant initially forms white, fleshy rhizomes.

Cyperus rotundus plant is mentioned in the ancient Indian ayurvedic medicine Charaka Samhita (ca. 100 A.D.). Modern ayurvedic medicine uses the plant for treating fevers, digestive system disorders, dysmenorrheal and other maladies. Arabs of the Levant traditionally use roasted tubers, while they are still hot or hot ashes from burned tubers, to treat wounds, bruises and carbuncles.

Cataract is very complicated process in which there are important metabolic changes in lens. These changes may be influenced by trace elements acting as a catalysts in enzymatic processes and with the aid of adenosine triphosphate affects the transfer of energy and there upon the nutrition of the eye lens.[5] Extracts of *Cyperus rotundus* are prepared and their effiency tested with cataract induced lens in ***in vitro condition***. The objective of the study was to note if there is any corelation between the phytochemicals present in the hydroalcoholic extract of *Cyperus rotundus* and the activities of ATPases and the levels of trace elements during cataractogenesis.

MATERIALS AND METHODS

Collection of plant material and extraction

Rhizome of *Cyperus rotundus* was obtained from the local herbal vendor without any external defects. The collected rhizomes were identified and authenticated by Botanist Prof. P. Jayaraman, Director, Plant Anatomy Research Center, Chennai – 45. They were shade dried at room temperature and made into coarse powder. 10 g coarse powder of rhizomes of *Cyperus rotundus* was homogenized in 50ml of absolute alcohol and 50ml distilled water separately using waring blender. The extract was refrigerated for 72 hours and filtered through 4 layers of muslin cloth and the residue was re-extracted under the same condition with 50ml of absolute alcohol and 50ml distilled water and the organic layer was allowed to evaporate and the dry residue was dissolved in alcohol. These hydroethanolic extracts were used for the further analysis.

Isolation of lens

Healthy chicks were obtained from the local market. Chick eyes were enucleated without any delay. The lenses were carefully dissected out from a posterior approach to avoid any damage. The lenses were incubated initially in saline for a period of 2 hours to discard any lens that had opacified due to damage during dissection procedure.

Induction of cataract

Cataract was induced by incubating the chick lenses in 55mM Glucose for 72 hours at 37°C. To study the anticataract activity of hydroalcoholic extracts of *Cyperus rotundus*, the cataract induced lenses were incubated with different concentrations (0.5mg, 1.5mg, 2.5mg) for 72 hours. After the stipulated period the lenses were removed and washed with saline. The weights of the lenses were noted and they were homogenized in 50mM Phosphate buffer (pH 7.8) containing 0.1mM EDTA. Aliquots were

taken for the analysis of trace elements and ATPases.

LEVELS OF TRACE ELEMENTS:

Iron analysis:

Iron content were estimated by the method of **Ramasay(1958).** To 0.5ml of homogenate added 1.0ml of sodium sulphite and 1ml of dipyridyl reagent were added. The solution was heated in a boiling water bath for 5 minutes and cooled. The pink colour developed was read at 520nm. Standard and blank were treated similarly. The iron content was expressed as µg/mg protein.

Estimation of zinc:

Determination of Zinc was carried out in the varian techtron AA/475 Atomic Absorption spectrophotometer. The homogenate was injected after five fold dilution (1 ml homogenate and 4 ml deionized water) with water. The absorption measurements were carried out at the wavelength 213.9 nm, emitted by a Zinc hollow cathode lamp.

The zinc content was expressed as µg/mg protein.

Estimation of electrolytes:

Esimation of sodium and potassium by flame photometry

Sodium and potassium content of lens were determined on a diluted aliquot of sample solution by flame photometry. Known concentrations of sodium and potassium solution were used to set the absorbance at zero. The flame is simultaneously monitored for both sodium and potassium channels. Dilution correction was made for the sample. The levels of sodium and potassium were expressed as milliequavalent/mg of lens.

Calcium analysis:

Calcium was determined by the method of **Sendroy J. (1944).** To 1ml of homogenate, 1ml of distilled water and 0.5ml of 4% ammonium oxalate solution were added, mixed well and allowed to stand for half an hour and centrifuged, supernatant was decanted. 3.0ml of 2% ammonia solution was added and mixed vigorously and was allowed to centrifuge again and the supernatant was decanted. 2ml of concentrated sulphuric acid was added, mixed well, and heated at 70 - 75°C then it was titrated with 0.01N permanganate to obtain a faint pink colour which persists for about a minute. As blank 2ml of sulphuric acid was titrated to the same end point. The difference between this titration gives the volume of 0.01N permanaganate.

The value of calcium was expressed as µg/mg protein.

Chloride analysis:

Chloride content of lens was estimated by the method **Vanslkye,D.D.[8]** The test samples and a blank was prepared simultaneously comprising 3ml of silvernitrate solution, 2ml of con. HNO_3 and 6ml of ferric alum was taken and titrated with 0.02N ammonium thiocyanate. The end point was the appearance of reddish brown colour. The titration was repeated to get concordant value.

The values of chloride were expressed as millimoles/litre.

Copper estimation:

The copper content was estimated by sodium diethyl dithio carbamate method of **Ventura and King (1951).** 3ml of homogenate was taken and 1ml of 100mM hydrochloric acid was added and warmed in a boiling water bath and stirred until the mixture begins to cloud. It was allowed to cool and 1.5ml of 6N HCl was added and allowed it to stand for 10 minutes. Then 3ml of 20% TCA was added and centrifuged. The supernatant was decanted and to this 1ml of sodium pyrophosphate and 2ml of ammonia solution were added. Then 1ml of sodium diethyl dithio carbamate was added and shaked well for 2 minutes with 5ml of amyl – alcohol ether mixture to extract copper.

The organic layer was removed and dried by shaking it with little powdered anhydrous sodium sulphate. The absorbance was read at 490 nm. The standard was also treated in the same way as homogenate.

The copper content of the sample was expressed as µg/mg of protein.

Determination of total protein:

Total proteins was estimated by the method of **Lowry OH et.al, (1951).** Different volumes of standard solution ranging from 0.2 – 1.0ml containing 20 - 100µg of protein respectively were taken in different test tubes. The volume in each test tube was made up to 1ml with distilled water and 1ml of water was taken as blank. 5ml of alkaline copper reagent was added to each tube and mixed thoroughly. The test tubes were allowed to stand at room temperature for 10 minutes. 0.5ml of folins ciocalteau reagent was added to each tube rapidly and mixed thoroughly. After incubation at room temperature the colour developed was read against blank at 680nm and 0.1ml of the sample was treated similarly.

The level of total protein was expressed as mg/g of wet tissue.

Assay of total ATPase (Total ATPase E.C.3.6.1.3)

Total ATPase was assayed by the method of **Evans (1969).** To 1.5ml of buffer, 0.1ml of each NaCl, KC1, $MgCl_2$, $CaCl_2$, and ATP solutions were added. Then 0.1ml of homogenate was added. The tubes were incubated at 37°C for 20 minutes. The reaction was arrested by the addition of 1ml of 10% TCA. The tubes were centrifuged and the phosphorous content in the supernatant was determined by **Fiske and Subbarow method (1925).** The supernatant and aliquots of standards were made upto 5.0ml with water. To these tubes, 1.0ml ammonium molybdate followed by 0.5ml ANSA reagent were added and mixed. The amount of phosphorous liberated was read at 620nm after 20 minutes against a reagent blank in a colorimeter.

The activities of ATPases were expressed as µmoles of phosphorous liberated/hr/mg protein.

Assay of sodium potassium dependant ATPase (Na^+ - K^+ ATPase E.C.3.6.1.3)

Na+ - K+ ATPase was assayed according to the method of **Bonting (1970).** To 1ml of tris buffer, 0.2ml of each of $MgSO_4$, KC1, NaCl, EDTA were added and equilibrated at 37°C for 10 minutes and the enzyme reaction was initiated by the addition of 0.1ml of homogenate. The assay medium was then incubated for one hour at 37°C. The reaction was arrested by the addition of 1ml of 10% TCA. Then 0.1ml of homogenate was added to the control tubes. The tubes were centrifuged and the phosphorous content in the supernatant was estimated according to the method of **Fiske and Subbarow(1925)**.

The activity of Na+ - K+ ATPases were expressed as µmoles of phosphorous liberated/hr/mg protein.

Assay of calcium dependant ATPase (Ca^{2+} ATPase E.C. 3.6.1.3):

Ca^{2+} ATPase was assayed by the method of **Hjerten and Pan (1983).** The incubation mixture contained 0.1 ml each buffer, $CaCl_2$ATP and water. After equilibrating the tubes at 37°C the reaction was initiated by the addition of 0.1ml of homogenate. The contents were incubated at 37°C for half an hour. The reaction was arrested by the addition of 1ml of cold 10% TCA. Then 0.1ml of enzyme was added to the control tubes. The tubes were centrifuged and phosphorous content in the supernatant was estimated by **Fiske and subbarow method (1925).**

The activities of Ca2+ ATPases were expressed as µmoles of phosphorous liberated/hr/mg protein.

SDS-PAGE

SDS-PAGE is a method widely used for the separation and characterization of proteins.

Analysis and comparison of proteins in a large number of samples is easily made on SDS-PAGE. Polyacrylamide gels are formed by polymerizing acrylamide with a cross-linker, bisacrylamide in the presence of detergent SDS, free radical APS and TEMED. The polymerization is initiated when APS undergoes homolytic cleavage in the presence of water, giving rise to persulphate free radicals (unpaired electrons). SDS is an anionic detergent which binds strongly to, and denatures proteins. Thereby, all the proteins get a net, uniform negative charge and hence more towards the anode.

STATISTICAL ANALYSIS

The values are expressed as mean ± SD. Statistical analysis was done by students't' test and 'p' value was arrived at to assess the statistical significance of changes observed. 'p' < 0.02 was considered significant.

RESULTS AND DISCUSSION

Table 1: Shows the weight of lens from the control, cataract induced and *Cyperus rotundus* treated groups. It was observed from the table that, upon cataract induction the weight of the lens decreased (P<0.01). *Cyperus rotundus* treated lens showed an increase in weight. 0.5 And 1.5mg concentrations of the extract did not significantly increase the weight of the lens. However, 2.5mg concentration of the extract increased (P<0.01) the weight of the lens. Agarwal *et.al.,*[15] reported that weight of the crystalline lens gradually increase with increase in age.

Table 2: Shows the levels of zinc, iron and copper in control, cataract induced and treated lens. It was observed from the table that upon cataract induction there was a drastic (P<0.001) decrease in the level of zinc. Various concentrations of *Cyperus rotundus* extract restored the zinc levels. Zinc acts as a cofactor for several antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase as reported by Ulmer.[16]

It was observed from the table that upon cataract induction there is a drastic (P<0.001) increase in the levels of iron in the cataract lenses. Upon treatment with *Cyperus rotundus* the levels of iron decreased (P<0.001) and were brought to near normal levels. It has been reported by Wada and Ou [17] that these phytochemicals protect the cells from oxidative damage caused by free radicals.

The levels of copper in control, cataract induced and treated lens are shown in table 2. From the table it is observed that there is much significant (P<0.001) increase in the level copper, in cataract induced lenses. Upon treatment with hydroalcoholic extract of *Cyperus rotundus*, it was observed that the level of copper decreased (P<0.001) with increasing concentrations of extract. It has been reported by Fong *et.al.,*[18] that there is elevated levels of copper during cataract maturity. It is observed from this study that there is an increase in the level of copper during cataract and upon treatment with *Cyperus rotundus* the levels were significantly decreased.

Table 3: Shows the levels of calcium, sodium and potassium in control, cataract induced and treated lens. It was observed that upon cataract induction there was an (P<0.001) increase in the levels of calcium. Upon treatment with *Cyperus rotundus* the levels of calcium decreased (P<0.001) and were brought to near normal levels. Recent data from the selenite model of nuclear cataract suggested that increased lens calcium may be a cause rather than an effect of cataract.[19]

It was observed from the table 3 that there is a significant (P<0.001) increase in cataract induced lenses. Upon treatment with various concentrations of *Cyperus rotundus* it was noticed that the level of sodium decreased

(P<0.002). There was a significant decrease in the level of sodium, with 2.5mg of *Cyperus rotundus* extract.

The impact of hydroalcoholic extract of *Cyperus rotundus* on the levels of potassium in control, cataract induced and treated lens shows that there is a drastic (P<0.001) increase in the potassium levels during cataract formation. Treatment with *Cyperus rotundus* significantly reduced the level and brought it to near normal. Potassium is a principal cation mediating osmotic balance with external medium and it is accumulated within the cells with the expulsion of sodium. About 50 enzymes are known to be activated by alkali metals as reported by Chock P.B and Titus E.U.[20]

Table 4: Shows the levels of chloride and total protein in control, cataract induced and treated lens. It was observed from the table that there is a significant (P<0.01) increase in the level of chloride in the cataract induced lenses. Upon treatment with various concentrations of *Cyperus rotundus* it was noticed that the chloride level decreased with increasing concentration of *Cyperus rotundus* extract. Elevated levels of chloride in the lens tissue may block the chloride channels resulting in swelling and opacification as reported by JJ. Zhang and T.Jacob.[21]

The impact of hydroalcholic extract of *Cyperus rotundus* on protein levels in control and cataract induced lens is shown in table 4. It was noticed from the table that during cataract formation there is a drastic decrease (P<0.001) in the level of protein. Upon treatment with *Cyperus rotundus* the levels of protein increased (P<0.001). At 2.5mg concentration of *Cyperus rotundus,* the increase in protein was highly significant. It has been reported by Eman & Aly and Eman Elabrak [22] that free radicals could damage the lens membrane, leading to protein release.

Table 5: Shows the levels of total ATPase, Na^+-K^+ ATPase and calcium dependent ATPase in control, cataract induced and treated lens. It was observed that upon cataract induction there was a drastic (P<0.001) decrease in the activity of total ATPase. Various concentrations of *Cyperus rotundus* restored the total ATPase activity.

It was observed that upon cataract induction there was a drastic (P<0.01) decrease in the levels of Na^+-K^+ ATPase. Various concentrations of *Cyperus rotundus* restored the Na^+-K^+ ATPase activity. It has been reported by Schachter [23] that the decrease in membrane fluidity is associated with decrease in Na^+-K^+ ATPase activity.

It was observed from the table 5 that during cataract induction there is a decrease (P<0.001) in the activity of calcium dependent ATPase. Upon treatment with *Cyperus rotundus* extract it was observed that the activity was restored to near normal levels. Therefore, free radical damage to the lens membrane could reduce the level of SH groups as reported by Seema *et.al.,* [24], leading to decreased activities of ATPases.

PLATE 1: Shows the lens of control (1a) and cataract induced lens (1b). From the photograph it is observed that in cataract induced system there was opacity of lens. Upon treatment with the extracts (1c) the level of opacity decreases and appeared to be transparent as that of the control lens.

PLATE 2: Shows the protein profile of lens isolated from control, cataract induced and *Cyperus rotundus* treated. Since, significant changes were noted in the levels of trace elements like zinc, calcium, iron and electrolytes such as sodium and potassium, it was planned to carry out SDS-PAGE (Polyacryl amide gel electrophoresis) of lens proteins. It was observed that (plate 2) during cataract the bandwidth of proteins decreased and addition of *Cyperus*

rotundus extract showed an increase in protein levels as visualized by densitometry.

TABLE – 1 SHOWS THE WET WEIGHT OF (mg/lens) LENSES OF CONTROL, CATARACT INDUCED AND TREATED LENS

EXTRACT(mg)	CONTROL	CATARACT INDUCED	*Cyperus rotundus* TREATED
0.5	60.2±2	◊ ** 52±3.1	# NS 54.5±3.7
1.5			# NS 56.1±2.5
2.5			# ** 58±1.7

Values are expressed as mean ± SD for six different preparations.
** p<0.01, NS – Non significant.
◊ Comparison between control and cataract induced system.
Comparison between cataract induced and hydroalcoholic extract of *Cyperus rotundus*.

TABLE – 2 IMPACT OF HYDROALCOHOLIC EXTRACT OF *Cyperus rotundus* ON ZINC, IRON AND COPPER LEVELS (µg/mg of protein) OF CONTROL AND CATARACT INDUCED AND TREATED LENS

COMPONENTS	CONTROL	CATARACT INDUCED	EXTRACT TREATED(mg)		
			0.5	1.5	2.5
ZINC	35.1±1.2	◊ **** 23.5±1.6	# NS 25.8±0.9	# *** 28.7±1.2	# **** 31.2±1.9
IRON	5.71±0.1	◊ **** 10.5±0.7	# NS 9.2±0.3	# **** 7.9±0.4	# **** 6.1±0.2
COPPER	0.91±0.01	◊ **** 1.78±0.1	# NS 1.6±0.15	# **** 1.3±0.05	# **** 1.0±0.03

Values are expressed as mean ± SD for six different preparations.
*** p<0.002, **** p<0.001, NS – Non significant.
◊ Comparison between control and cataract induced system.
Comparison between cataract induced and hydroalcoholic extract of *Cyperus rotundus*.

TABLE –3 IMPACT OF HYDROALCOHOLIC EXTRACT OF *Cyperus rotundus* ON CALCIUM (µg/mg ofprotein), SODIUM (millimoles/mg of lens), AND POTASSIUM (millimoles/mg of lens) IN CONTROL AND CATARACT INDUCED AND TREATED LENS

COMPONENTS	CONTROL	CATARACT INDUCED	EXTRACT TREATED(mg)		
			0.5	1.5	2.5
CALCIUM	1.7±0.06	◊ **** 2.5±0.09	# ** 2.2±0.1	# **** 2.0±0.08	# **** 1.65±0.04
SODIUM	18±1.1	◊ **** 25±1.7	# NS 23±1.5	# ** 21.5±1.1	# *** 19.5±1.0
POTASSIUM	120±3	◊ **** 140.7±5	# NS 136±2	# ** 130±4	# *** 125±6

Values are expressed as mean ± SD for six different preparations.
** p<0.01, *** p<0.002, **** p<0.001, NS – Non significant.
◊ Comparison between control and cataract induced system.
Comparison between cataract induced and hydroalcoholic extract of *Cyperus rotundus*.

TABLE – 4 IMPACT OF HYDROALCOHOLIC EXTRACT OF *Cyperus rotundus* ON CHLORIDE (millimoles/litre) AND PROTEIN LEVELS (µg/mg of wet weight of lens tissue) IN CONTROL AND CATARACT INDUCED AND TREATED LENS

COMPONENTS	CONTROL	CATARACT INDUCED	EXTRACT TREATED(mg)		
			0.5	1.5	2.5
CHLORIDE	13.7±1.0	◊ ** 17.3±1.2	# NS 16.2±0.8	# ** 14.1±0.7	# ** 13.9±0.9
PROTEIN	5.74±0.2	◊ **** 1.30±0.07	# NS 1.9±0.05	# **** 3.5±0.09	# **** 5.2±0.3

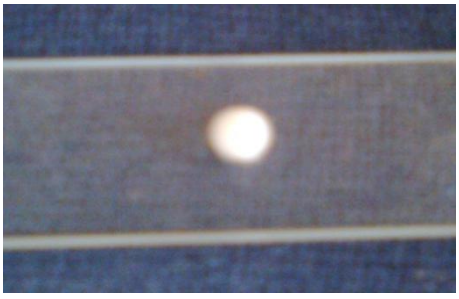
Values are expressed as mean ± SD for six different preparations.
** p<0.01, **** p<0.001, NS – Non significant.
◊ Comparison between control and cataract induced system.
Comparison between cataract induced and hydroalcoholic extract of *Cyperus rotundus*.

TABLE – 5 IMPACT OF HYDROALCOHOLIC EXTRACT OF *Cyperus rotundus* ON ACTIVITY OF TOTAL ATPases, Na^+-K^+ ATPases AND Ca^{++} ATPases (µmoles of phosphorus liberated/hr/mg of protein) IN CONTROL AND CATARACT INDUCED AND TREATED LENS

COMPONENTS	CONTROL	CATARACT INDUCED	EXTRACT TREATED(mg)		
			0.5	1.5	2.5
TOTAL ATP'ases	170±13	◊ **** 102±8	# NS 108±6	# ** 127±10	# **** 148±11
Na^+-K^+ ATP'ases	110±14	◊ ** 75±5	# NS 82±4	# ** 94±7	# **** 107±8
Ca^{++} ATP'ases	80±5	◊ **** 57±3	# NS 63±2	# ** 71±5	# **** 77±2

Values are expressed as mean ± SD for six different preparations.
** p<0.01, **** p<0.001, NS – Non significant.
◊ Comparison between control and cataract induced system.
Comparison between cataract induced and hydroalcoholic extract of *Cyperus rotundus*.

PLATE – 1



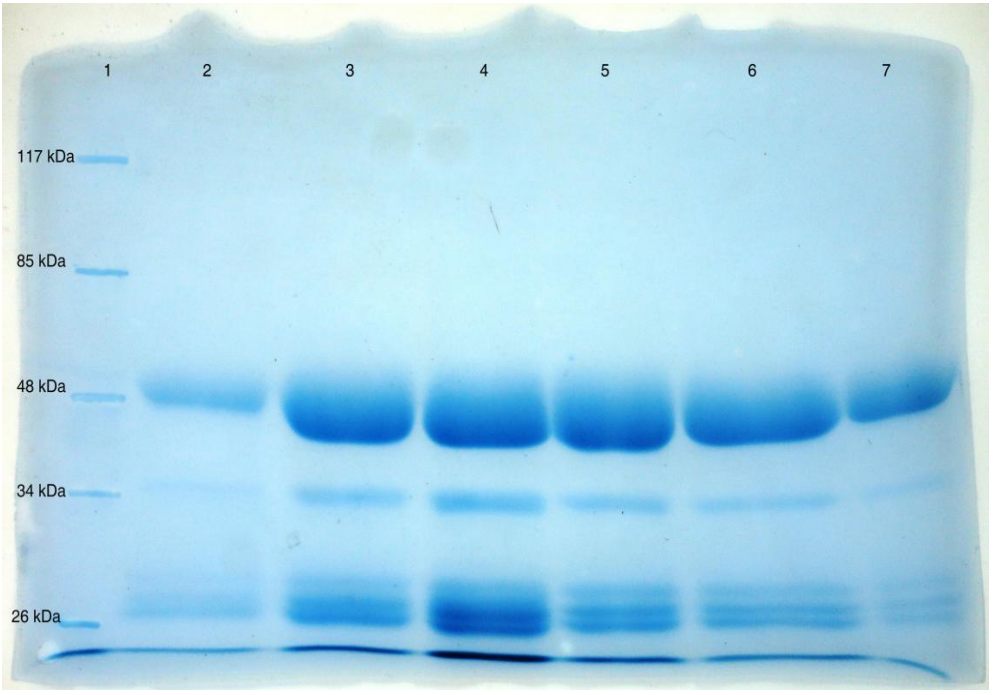
1.a

1.b

1.c

1.a - Control eye lens
1.b - Shows the opacity lens in cataract induced *in vitro* system
1.c - Shows the impact of *Cyperus rotundus* on cataract induced lens

PLATE – 2:



1- Protein Marker
2- Sample C_1 (CATARACT)
3- Sample C (CONTROL)
4- Sample T_3 (2.5mg EXTRACT TREATED)
5- Sample T_2 (1.5mg EXTRACT TREATED)
6- Sample T_1 (0.5mg EXTRACT TREATED)
7- Sample T_1D (0.5mg EXTRACT TREATED)

CONCLUSION

Our previous results [24] clearly indicated the promosing role of *Cyperus rotundus* in preventing cataract. Hence, it was planned to further investigate on the levels of trace elements and electrolytes and to study the protein cross–linking during cataract and the impact of *Cyperus rotundus* extract. Our results indicate that *Cyperus rotundus* lowered the levels of copper and iron which are culprits in inducing oxidative stress. *Cyperus rotundus* exerts its effect by maintaining the electrolyte balance and osmotic pressure by elevating the activity of ATPases. The electrochemical gradient is maintained by maintaining the calcium levels. The mechanism of action of *Cyperus rotundus* could be by increasing the activity of ATPase there by maintaining the electrolyte levels. The level of Iron and Copper are reduced and Zinc is elevated. These changes prevent the free radical formation and protein aggregation.

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***Corresponding Author:**
Dr.K.Vijayalakshmi
Associate professor in biochemistry
Bharathi women's college, Chennai – 600108