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Journal:	<i>Food & Function</i>
Manuscript ID	FO-ART-07-2015-000905.R2
Article Type:	Paper
Date Submitted by the Author:	04-Dec-2015
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Anthraquinones and flavonoids of *Cassia tora* leaves ameliorate sodium selenite induced cataractogenesis in neonatal rats

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¹Abstract

The present study was undertaken to evaluate the efficacy of *Cassia tora* leaves, an edible plant traditionally used for eye ailments; in preventing experimental cataractogenesis, as cataract is the leading cause of irreversible visual impairment worldwide characterized by the cloudiness or opacification of the lens due to the disturbance of even distribution of lens proteins and lipids. A significant number of epidemiological studies have suggested the potential role of herbal medicine in the prevention of cataract by keeping lens architecture. The study was conducted in neonatal rat pups of 8-10 days old and ethyl acetate fraction of *Cassia tora* leaves (ECT) administered by gastric intubation. After 30 days, the animals were sacrificed and various parameters such as redox status and gene expressions were evaluated in lenses. ECT administration caused a significant decrease in the onset and maturation of cataract, potentiated antioxidant defense and normalized lens crystallins expression against cataract induced animals. HPLC and ESI-MS analysis of ECT revealed the presence of flavonoids and anthraquinones. Thus, the present study indicates the therapeutic potential of *Cassia tora* leaves in preventing cataract and the effect is endorsed to the presence of antioxidants in *Cassia tora* leaves.

Key words: *Cassia tora*, Cataract, Lens, Crystallins, flavonoids, anthraquinones

Introduction

¹ ECT: Ethyl acetate fraction of *Cassia tora* leaves

Cataract is the foremost cause of blindness and accounts for 51% of total blindness worldwide.¹ It is a condition characterized by the opacification of the eye lens and associated poor visual acuity.² The lens has a unique transparent structure and is the densest tissue in the body with highest long-lived protein content and lowest water percentage with stable, tightly packed fiber cells of specialized organization. Lens functions to collect and concentrate light on to the retina. The transparency of the lens is maintained by proper packing of lens soluble proteins like crystallins (Alpha, beta and gamma) and a well-defined architecture of the lens fiber cells.³ To enable transparency, crystallins form hetero/oligomers that assemble into ordered structures; protein-protein interaction, especially between alpha and gamma crystallins is also a major factor responsible for lens transparency and maintenance of refractive index. As lens proteins do not turnover appreciably, there is ample opportunity for them to become modified. Cataract is caused by the disruption of the lens architecture resulting in the scattering of light entering the eye before reaching the retina. Development of cataract can be largely attributed to lens protein aggregation and precipitation via oxidative modification, deamidation, chemical changes, osmotic changes and phase separation, ion imbalance, apoptosis of lens epithelial cells etc. Presently the only available treatment for cataract is the replacement of the cataractous lens with an artificial intraocular lens. But it has its own complications such as posterior capsular rupture, zonular dehiscence, suprachoroidal haemorrhage, posterior capsular opacification, raised intraocular pressure, corneal decompensation, retinal detachment etc.⁴ Therefore, an alternative treatment of cataract has been a topic of great interest.

Recent abundant researches suggesting the importance of plant foods in the maintenance of human health and prevention and treatment of diseases because of the presence of polyphenols and lesser side effects and many plants have been reported to have a role in prevention and control of cataract.⁵ *Cassia tora* Linn. is an annual, under shrub, belongs to the family *Caesalpiniaceae* with ethnomedicinal importance. Leaves of *Cassia tora* have been consumed as a leafy vegetable food and traditionally, the whole plants as well as specific parts such as leaves, seeds and roots have been widely used against different diseases. The leaves have been experimentally examined for various pharmacological applications such as antimicrobial, hypolipidemic, anti-inflammatory, antiproliferative,

antidiabetic, hepatoprotective etc.⁶ The plant has been used in the Ayurvedic system for the treatment of eye disease⁷ and no reports are available till date on its anticataract effect. We have attempted to evaluate the anticataractogenic potential of polyphenol rich fraction of *Cassia tora* leaves in experimental animals and to identify the active principles in the plant; to provide an easily accessible way for the preventive protection against cataract.

Materials and methods

Chemicals

All the chemicals and biochemicals used were of analytical grade and purchased from Sigma India, SRL, Ranbaxy and Spectrochem, India.

Plant material

Cassia tora Linn. leaves were collected from Kariavattom Campus, authenticated by an expert and deposited in the herbarium of Department of Botany, University of Kerala, Thiruvananthapuram, India (Accession No: KUBH 5844). The leaves were dried under shade and extracted with 80% methanol, filtered and the solvent was evaporated under vacuum (Yield; 29.80%). The dry extract was partitioned successively using petroleum ether, ethyl acetate, butanol and water. Each fraction was concentrated again and the bulk of the antioxidant activity was showed by ethyl acetate fraction (Yield; 12.45%) of *Cassia tora* leaves (ECT). It was dissolved in phosphate buffered saline (prepared in sterile water) for the animal experimentations.

Animals

Sprague-Dawley rat pups at 8-10 days postpartum were housed along with their mother in polypropylene cages under a day/night cycle of 12 h, at $25 \pm 1^\circ\text{C}$ room temperature. The rats received laboratory chow (Hindustan Lever Ltd., India) and drinking/tap water. All ethical guidelines were followed for the conduct of animal experiments in strict compliance with the Institutional Animal Ethical Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (IAEC-KU-5/2012-13, BC. AA32b).

Experimental procedures

The rat pups were grouped into four with 6 pups in each group.

Group I	Control
Group II	ECT (5µg/g body weight)
Group III	Sodium selenite (4µg/g body weight)
Group IV	Sodium selenite (4µg/body weight) + ECT (5µg/g body weight)

Group III and Group IV were given a single subcutaneous injection of sodium selenite (4 µg/g body weight) on the 10th day⁸ while ECT was administered by gastric intubation from 8th day up to 12th day at a concentration 5µg/g body weight to Group II and IV. Cataract could be visualized from the 15th day with the help of an ophthalmoscope and later on with the naked eye. Animals were euthanized by sodium pentothal injection on the 30th day and lenses were extracted through posterior approach.

Grading and visualization of lens opacification

The lenses were visualized with the help of an ophthalmoscope or with the naked eye (15th day onwards). The gradation of opacity was assessed weekly by slit-lamp illumination. The pupils were dilated with using a topical ophthalmic solution containing tropicamide with phenylephrine. Each stage was graded and identified with the help of an expert ophthalmologist. Classification of the cataract stages was following based on a scale.⁹ Stage 0= normal clear lens; Stage 1 = initial sign of posterior sub capsular or nuclear opacity involving tiny scatters; Stage 2 = slight nuclear opacity with swollen fibers or posterior sub capsular scattering foci; Stage 3 = diffuse nuclear opacity with cortical scattering; Stage 4 = partial nuclear opacity; Stage 5 = nuclear opacity not involving lens cortex; Stage 6 = mature dense opacity involving the entire lens.

Analytical procedures

Xanthine oxidase was assayed by the method of Prajda and Weber 1975.¹⁰ Transglutaminase 2(TGase2) was assayed by the method of Pierre et al., 2000.¹¹ Thioltransferase activity was estimated by the method of Park et al., 1999.¹² The content of sulfhydryl group in lens proteins was determined using the Ellman's procedure.¹³ The

content of free carbonyl in the total lens proteins was determined by the procedure of Levine et al., 1990.¹⁴ The activity of Ca²⁺ ATPase was estimated by the method of Rorive and Kleinzeller, 1974.¹⁵ The activity of caspase-3 was measured using a colorimetric assay kit (Sigma-Aldrich Co, USA).¹⁶

Analysis of calcium in the lens

The lenses were weighed, cleaned and washed with phosphate buffered saline. Then the tissue was digested with the necessary volume of concentrated nitric acid: perchloric acid mixture (5:1). After complete digestion, the lenses were dried and diluted with 1% nitric acid. The analysis was carried by atomic absorption spectroscopy (Shimadzu) operated with a slit width of 0.5 nm, with the wavelength set at 422.7 nm.

Isolation of RNA from lens and RT-PCR study

RNA was isolated from the isolated rat lens using trizol reagent (Sigma-Aldrich, St. Louis, MO, USA).¹⁷ The RNA concentration was determined from the absorbance at 260 nm (BioPhotometer, Eppendorf AG, Hamburg, Germany). All samples had a 260/ 280 nm absorbance ratio 1.78 ± 0.06 . RT-PCR and PCR amplifications were carried out using kits from Thermo Scientific, India. Initial PCR activation for 15 min at 95 °C was followed by 3 steps of cycling process. The primer sequences used are listed in Table 1. Each cycle consists of denaturation for 1 min at 94 °C, annealing for 1 min at 65 °C, extension for 1 min at 72°C, repeated for 37 cycles and final extension for 10 min at 72 °C. The PCR products were electrophoresed in 1% agarose gels containing 0.05 µg/ml ethidium bromide. The mRNA expression was quantified using a phosphorimager and using the Image Quant software and the relative expression was compared and normalized to the expression of β actin in the same sample.

ELISA analysis

Indirect ELISA was performed using alpha B Crystallin antibodies.¹⁸ Antigen was coated on wells, washed with blocking buffer after 3 hours of incubation and incubated with primary antibodies for 3 hours at room temperature. After washing, wells were treated with HRP conjugated secondary antibody and washed again. O-dianisidine, citrate

phosphate buffer and hydrogen peroxide were added to the wells, kept in dark for 15 minutes and the reaction was stopped with HCl and absorbance was read at 405nm in an ELISA reader. Results were expressed as absorbance per milligram protein.

Column chromatography of ECT

Ethyl acetate fraction was subjected to silica gel column chromatography. The column was packed with silica gel (Kieselgel 60, mesh 70-230) and the slurry of silica gel was added into a glass column having the length and diameter of 55 cm and 1.1 cm respectively. When sufficient height of the adsorbent bed was obtained, petroleum ether (60-80⁰C) was run through the column for proper packing. The sample was prepared by adsorbing 1.5g of ethyl acetate fraction on 3g silica gel, allowed to dry and subsequently applied on top of the adsorbent layer. Elution was started with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate of increasing polarity, then by ethyl acetate and finally with ethyl acetate and methanol mixtures of increasing polarity. Fractions each of 20 ml were collected and screened by TLC on pre-coated silica gel plates 60 F 254 (Merck). Plates are examined under UV (240-360nm) and sprayed with anisaldehyde reagent and alcoholic potassium hydroxide. Similar fractions were pooled to give three main fractions. Two fractions A and B [PE: EA (60:40 and 80:20)] were selected based on the antioxidant activity and *in vitro* anticataractogenic potential and is further purified and screened by column and TLC.

High-performance column chromatography

HPLC analysis was performed on Ultra-Fast Liquid Chromatography instrument (Shimadzu) equipped with a quaternary pump, Photodiode array detector, autosampler and a column compartment. The samples were separated on a C₁₈ column (250 mm × 4.6 mm × 5µm) for a total run time of 30 min. The mobile phase consisted of acetonitrile and water containing 0.1% (w/v) formic acid with a gradient from 20 to 90% acetonitrile over the first 25 min and held at 20% acetonitrile for 5 min. The flow rate was 1ml/min. Detection was by monitoring of absorption at 254 nm for anthraquinones and 360 nm for flavonoids. The chromatographic peaks of the analytes were confirmed by comparing their retention time with corresponding standards.

Electrospray ionization mass spectrometric (ESI-MS) analysis

ESI-MS analysis of column fractions was performed using an Agilent 1100LC/MSD ion trap MS (Agilent, Palo Alto, CA, USA) equipped with an ESI interface. Nitrogen was used as a nebulizing gas at a pressure of 50 psi at 10L/min, temperature of 35⁰C, capillary voltage of -4 kV and in the positive-ion mode with the scan range m/z 100–600.

Statistical analysis

All statistical calculations were carried out¹⁹ with the statistical package for social sciences (SPSS) software program (version 17.0 for Windows). The values were expressed as the mean \pm SEM. The data were statistically analyzed using one-way analysis of variance and the significant difference of means was determined using Duncan's multiple range tests at the level of $p < 0.05$. Comparison is carried out between groups; Cataract group (group III) is compared with control group (group I) and treatment group (group IV) is compared with cataract group.

Results

Cataract incidence. Selenite administration resulted in nuclear opacities in all the Group III animals with 50% of them at stage 6. But ECT treatment prevented cataract maturation in group IV with 60% lenses was clear and transparent and comparable with Group I and Group II lenses. The remaining treated lenses were at stage 1 and 2 indicating the anticataractogenic potential of *Cassia tora* leaves (Table 2).

Activities of xanthine oxidase and transglutaminase. The activities of pro-oxidant enzymes xanthine oxidase and transglutaminase were significantly ($P < 0.05$) increased in selenite induced cataractous lenses compared with controls. But the administration of ECT significantly ($P < 0.05$) reduced the activities of these enzymes in the group IV (Fig. 1 and 2).

Thiol transferase activity and sulfhydryl content. The activity of thiol transferase and the content of sulfhydryl was reduced in lenses of group III animals and is augmented significantly ($P < 0.05$) in ECT lenses of pre-treated animals (Fig.3 and 4).

Protein carbonyl level. Protein carbonyl level was significantly increased in lenses of cataract induced rats compared to control ones. However, pretreatment of ECT in Group IV resulted in a marked decrease ($P < 0.05$) in the level of carbonyl accumulation (Fig. 5).

Calcium homeostasis. There was a decrease in the activity of membrane pump Ca^{2+} ATPase in the cataract group, but supplementation of ECT in group IV was found to significantly ($P < 0.05$) increase the activity to near normal. Lens calcium level augmented significantly in the cataract group when compared with control and was significantly declined ($P < 0.05$) as a result of pre-treatment with ECT in group IV. Caspase 3 activity also showed a similar pattern of Ca^{2+} ATPase (Table 3).

Gene and protein expression studies. Lens crystallin gene expression (αA , αB and γ Crystallin) and (αB Crystallin) were significantly altered in the selenite induced group and was normalized in ECT supplemented group (Fig. 6, 7).

HPLC and ESI-MS analysis. HPLC profiling of ECT indicates the presence of flavonoid kaemferol and anthraquinones such as chrysophanol and emodin when compared to the retention time of corresponding standards (Fig. 8). The mass spectrum of ECT is shown in Fig.8. The ion at m/z 287.3 represents kaemferol, the ion at m/z 255.2 represents chrysophanol, the ion at m/z 271.24 represents emodin and the ion at m/z 285.3 represents physcion (Fig. 9).

Discussion

The eye lens loses its transparency with time due to aging, environmental factors, habitats etc., which, in turn, form cataract. Understanding of the basis for cataract pathology may provide a valuable tool for a novel approach to prevention and treatment cataract. As cataract is a protein aging disorder so that the modified proteins cannot be reverted back by treatment. So the only remedy is to prevent or delay cataractogenesis. We designed the study to evaluate the preventive potential of ECT against selenite induced cataract models. Selenite induced cataract model is the globally accepted nuclear cataract model with all the characteristics of the aged lens.

Preliminary studies were carried out to assess the toxicity and to fix minimal effective dose of plant material (Results not given). Activities of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in serum confirmed the nontoxic nature of ECT. A dose response study was carried using ECT at different concentrations (1, 2.5, 5 and 10 $\mu\text{g}/\text{g}$ body weight) against selenite induced cataract. The minimal effective dose was fixed as 5 $\mu\text{g}/\text{g}$ body weight by measuring the activities of Superoxide dismutase, Catalase and Calcium ATPase.

Morphological evaluation of lenses in different groups provides an insight to the anticataractogenic effect of ECT that more than 50% of ECT pre-treated lenses were free from any opacity when compared to opaque group III lenses.

Xanthine oxidase is a pro-oxidant enzyme normally present in ocular tissues. Under pathological conditions, its activity is raised and forms an important source of free radicals, mainly superoxides and is a lens oxidative stress marker.²⁰ Oxidants generated by xanthine oxidase system imposes cross-linking and aggregation of crystallins, loss of cellular redox balance, oxidation of DNA bases and lipid peroxidation of polyunsaturated fatty acids and inflict the damage to the lens membrane active transport pumps and implicated in cataractogenesis.²¹ Lens xanthine oxidase activity was increased after administration of sodium selenite²² and decreased to a near normal level by ECT treatment, which might be due to the radical scavenging activity of ECT and similar results were obtained by Padmaja and Raju (2004)²³ when curcumin was used against selenite cataract.

Deamidation is a major protein modification in aging and it occurs non-enzymatically and enzymatically. TGase 2 is an inducible transamidating acyltransferase that catalyzes Ca^{2+} dependent protein modifications by inducing of covalent cross-links between peptide bound glutamine and lysine residues. It is up-regulated often in cells undergoing oxidative stress and apoptosis²⁴ and lens crystallins are excellent substrates for TGase. Deamidation is the major modification in the lens and implicated in cataractogenesis by cross-linking of crystallins.²⁵ It introduces a negative charge at physiological pH by replacing an amide with a carboxyl group, causing protein unfolding and instability, perhaps allowing disulfide bonds to form and disrupt the secondary and tertiary structure

of crystallins.²⁶ Consistent with the above reports, TGase2 activity was increased significantly in Group III lenses but ECT administration prevents the raise of TGase2 in Group IV

The lens depends on a balanced redox state and sulfhydryl/disulfide ratio for maintaining its transparency. Protein S-thiolation is a process in which under stressed conditions, vulnerable sulfhydryl groups of proteins are conjugated to non-protein thiols such as glutathione (GSH) or cysteine resulting in the formation of protein-thiol mixed disulfides, protein-S-S-glutathione (PSSG) and protein-S-S-cysteine (PSSC). This process spontaneously disrupts the redox homeostasis of the cells, which in turn leads to functional disturbances in the respective tissue. In the lens, such modification of proteins precedes a cascade of events starting with protein disulfide cross-links, alteration of protein conformation, protein/enzyme deactivation, protein solubility loss and eventually lens opacification.²⁷ Lens uses thioltransferase repair system to reduce oxidized proteins/enzymes and to maintain redox homeostasis. It dethiolates the protein thiols that have been oxidized into mixed disulfides and restore protein free sulfhydryl groups.²⁸ Thioltransferase was far more efficient in dethiolating GSH and crystallins. Lowered thioltransferase activity upon selenite induction was improved by ECT treatment supporting the effect of ECT in cataract prevention.

Proteins may contain several actual or potential sulfhydryl groups and are the potential sites for reversible oxidation-reduction reactions. Such groups appear to be of importance not only for purely redox mechanisms but for the enhanced functioning of certain enzymes too. Sulfhydryl groups in lens proteins are very sensitive to oxidative stress and can easily conjugate with non-protein thiols to form protein-thiol mixed disulfides. These damaged proteins accumulate in the cell and result in degenerative disorders. Sulfhydryl oxidation is thought to be one of the main pathological events leading through disulfide cross-linking and molecular aggregation, to protein precipitation and lens opacification.²⁹ In the lenses of Group III rats (cataract induced) the level of sulfhydryl groups was found to be significantly lower than those in lenses of Groups I and II and ECT treatment restores their mean levels significantly in Group IV.

Protein carbonyl content is a stable marker of oxidative damage. *In vivo*, Protein carbonylation is a metal (copper or iron) accelerated modification occurring to protein side chain of many amino acids such as lysine, arginine, proline or histidine to produce carbonyls. It makes the protein functionally inactive and resistant to hydrolysis.³⁰ The prevailing oxidative stress induced by selenium in Group III reflects the increased protein carbonyl content which is reduced significantly in ECT treated animals by trapping free radicals and preventing oxidation of biomolecules and is supported by the improved level of sulfhydryl level upon ECT treatment.

Calcium homeostasis is recognized as having fundamental importance in lens pathophysiology and more than a century ago, calcium has been recognized as a key mediator of cataractogenesis.³¹ Normally the calcium levels are maintained at micromolar concentrations inside the cell. In the lens, the ionized calcium is less than 1% of the total concentration and below 0.1% of the level in surrounding aqueous humor.³² This is maintained by $\text{Na}^+ \text{K}^+$ exchanger; $\text{Na}^+ \text{Ca}^{2+}$ exchanger and calcium ATPase pump. The plasma membrane calcium ATPase is a high-affinity calcium pump which uses the energy from ATP hydrolysis to drive calcium out of the cell against its electrochemical gradient.³³ The decrease of calcium ATPase activity with cataract is due to lipid structural changes³⁴, increase in oxidation of sulfhydryl groups³⁵ and down regulated ATP utilization.³⁶

Apoptosis is a fundamental biological process that enables normal cellular homeostasis and any abnormalities in cell death regulation can be a significant component of neurodegenerative diseases. Induction of apoptosis is reliant on the activation of intracellular cysteine proteases called caspases and caspase 3 is the most important effector molecule to induce apoptosis. In the lens, a single layer of epithelial cells is essential to maintain the entire metabolism and lens epithelial cell apoptosis is correlated with cataract formation.³⁷ The loss of epithelial cells through apoptosis and diminished activity of calcium ATPase causes an influx of calcium into the underlying fiber cells. Elevated calcium content in the lens has been shown to activate caspases and calpain, a calcium dependent protease. Proteolysis of crystallins by calpain would lead to abnormal interactions of the crystallins, insolubilization of proteins, and lens opacity.³⁸

Selenite administration resulted in the diminished activity of calcium ATPase and increased activity and level of caspase 3 and calcium respectively. ECT treatment effectively maintained calcium homeostasis by restoring calcium ATPase activity and prevents caspase activation through regulating the calcium level in Group IV lenses. Our results are in agreement with the results of Rooban et al., 2009³⁹ and Sreelakshmi et al., 2015⁴⁰ when using flavonoid rich fraction of *Vitex negundo* and Luteolin respectively in selenite induced models.

As evident from the forgoing discussion, the proper packing and functioning of the lens is the key aspect in visual acuity. Crystallins are the major structural proteins responsible for the lens transparency through short range interactions. Crystallin aggregation and accumulation of high molecular weight complexes have been reported in cataract⁴¹ and an up-regulation of crystallins plays a major role in the management of cataract complications.⁴² Calcium activated proteolysis and crystallin modifications⁴³ and transglutaminase mediated cross-linking of crystallins are implicated in cataractogenesis.⁴⁴ Our results showed that the mean levels and gene transcripts of α A Crystallin, α B Crystallin and γ Crystallin and protein expression of α B Crystallin were significantly up-regulated by the administration of ECT as evidenced by the normalized calcium level and decreased activity of transglutaminase and other pro-oxidant enzymes.

HPLC analysis of column fractions of ECT showed the presence of kaemferol, chrysophanol and emodin and presence of these components was confirmed by ESI-MS analysis. In addition to the above compounds presence of one more compound physcion was revealed by ESI-MS analysis. Previous studies have shown that these flavonoids and anthraquinones possess strong free radical quenching and alleviate oxidative damage⁴⁵⁻⁴⁷ and the result justifying the time-honored uses of the plant in the healing of eye disorders.

Conclusion

In short, we concluded that the supplementation of ECT at the dose of 5 μ g/g body weight is effective in delaying the onset and pathology of cataract by maintaining lenticular homeostasis by modulating antioxidant defense, keeping ionic balance, preventing lens protein deterioration and normalizing crystallin gene expression in selenite induced

neonatal rats. This may be mediated by the cumulative action of anthraquinones and flavonoids in the leaves and as it is an edible plant, incorporation of these leaves in daily food is a good attempt to prevent or delay cataract.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

The authors are thankful to University Grants Commission (UGC), New Delhi for the financial support and University of Kerala for providing infrastructure facility for carrying out this work.

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Figure captions

Figure 1: Activity of xanthine oxidase in the lenses of experimental groups and expressed in units per milligram lens protein. One unit of activity was defined as 1 μ mol of uric acid formed per minute at 37⁰C, pH 7.5. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Figure 2: Activity of transglutaminase (absorbance/mg protein) in the lenses of experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Figure 3: Activity of thiol transferase (absorbance/mg protein) in the lenses of experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Figure 4: The level of sulfhydryl groups (micromoles/g tissue) in the lenses of experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

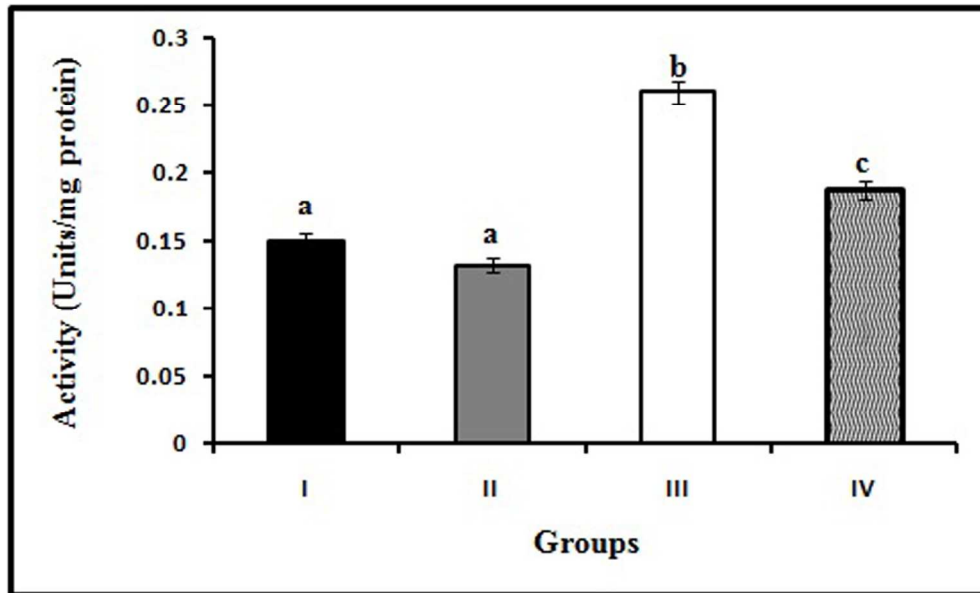
Figure 5: The level of protein carbonyl (micromoles/mg protein) in the lenses of experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Figure 6: Photographic and graphical representation of mRNA expression of α A Crystallin, α B Crystallin and γ Crystallin in the lenses of different experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

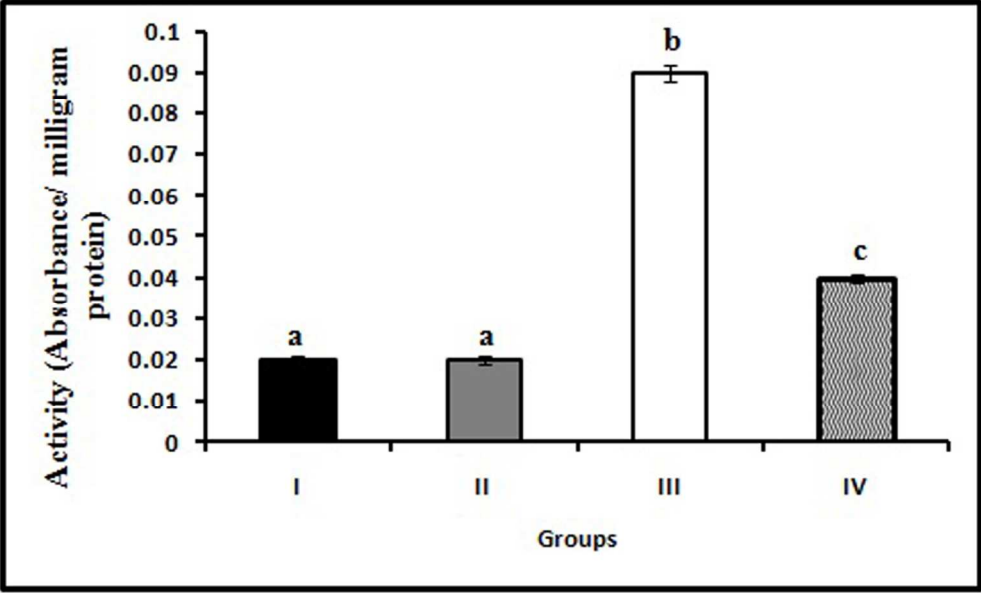
Figure 7: Protein expression of α B Crystallin in the lenses of different experimental groups. Each value represents mean \pm SEM of six values. Different alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Figure 8: HPLC profile. (A): Column fraction A; (B); Kaemferol standard; (C): Column fraction B; (D); Chrysophanol standard; (E) Emodin standard (F) Retention time of different fractions and standards.

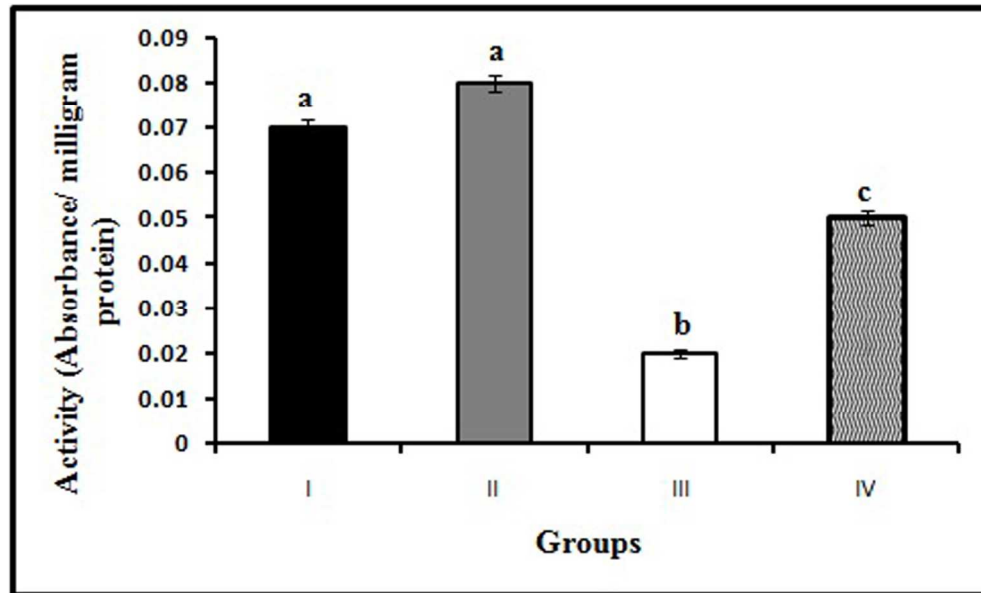
Figure 9: ESI MS analysis. (A): Column fraction A; (B): Column fraction B.



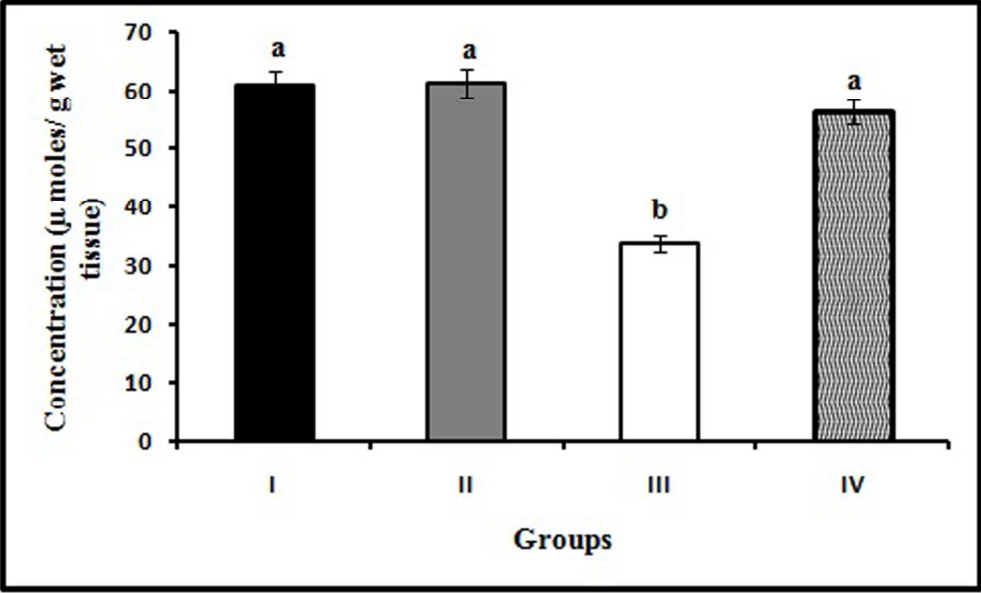
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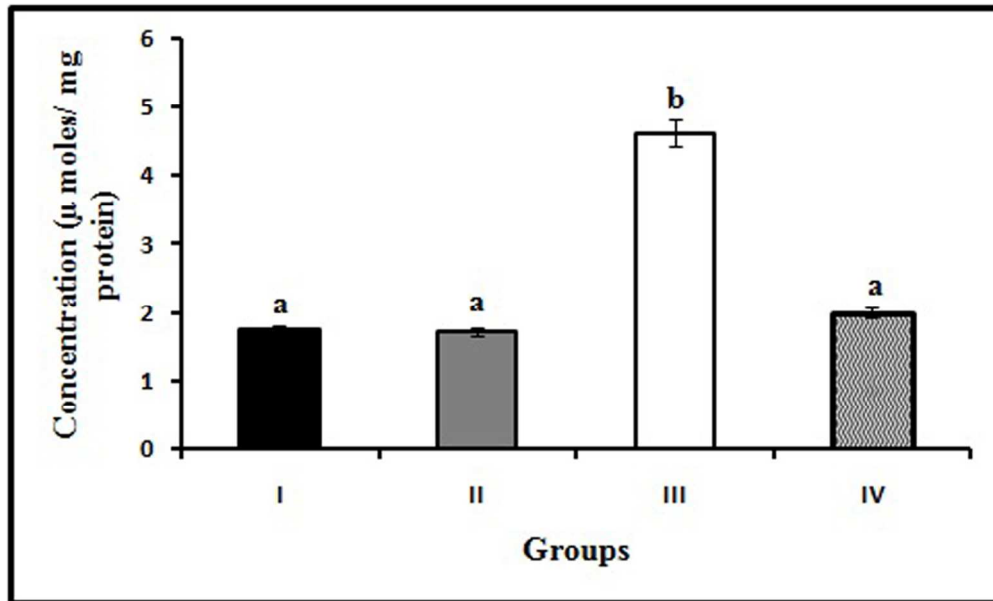
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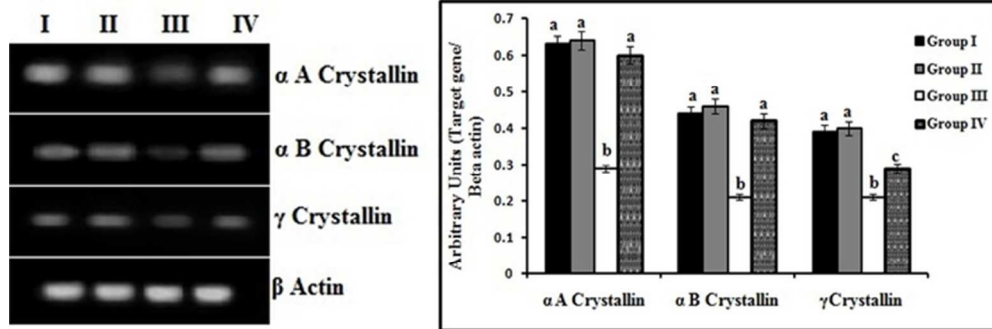
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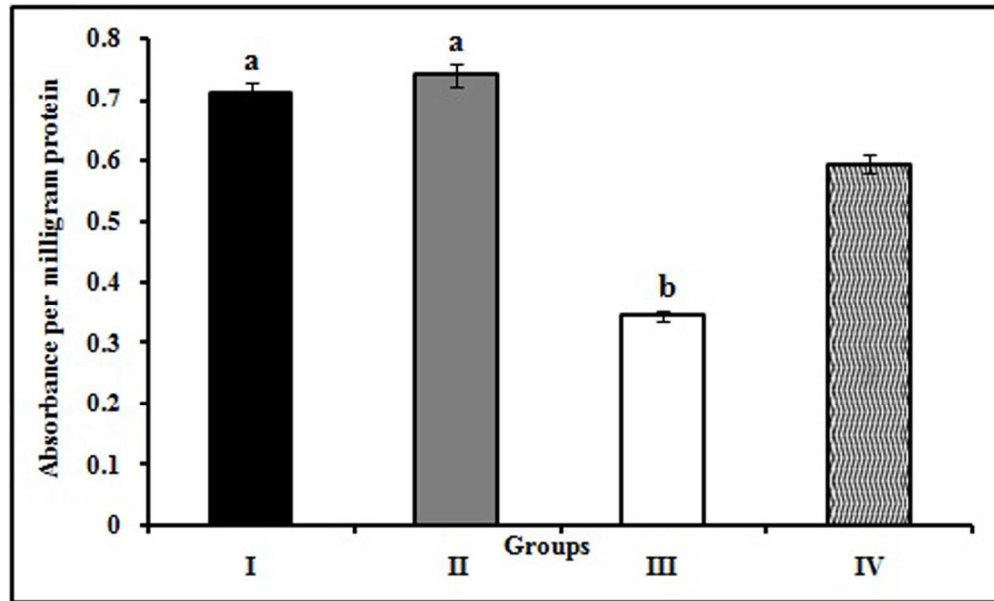
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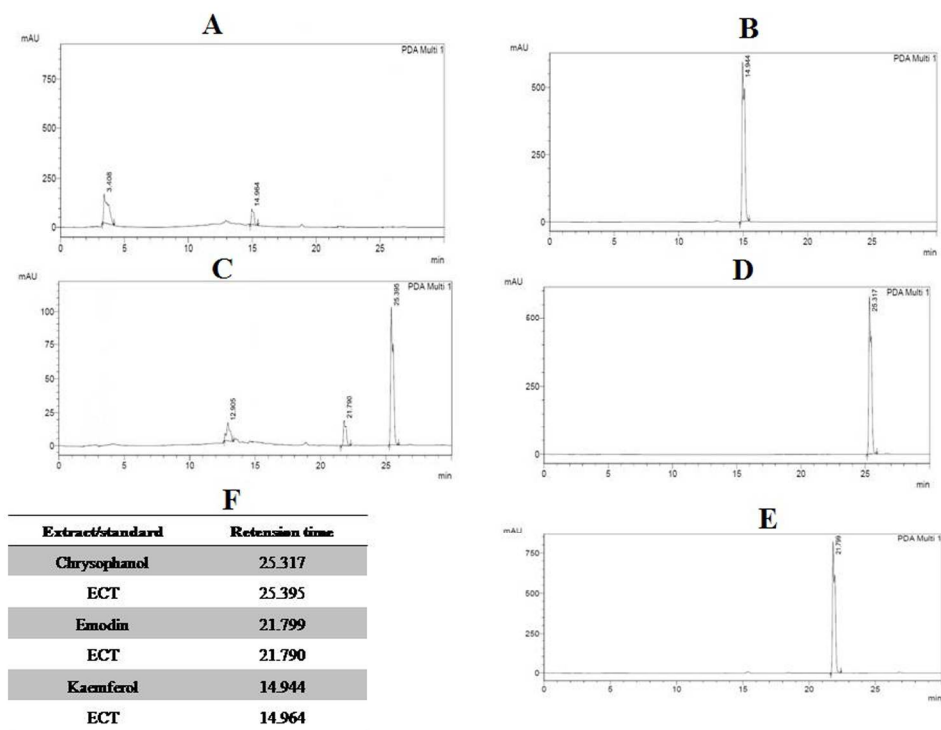
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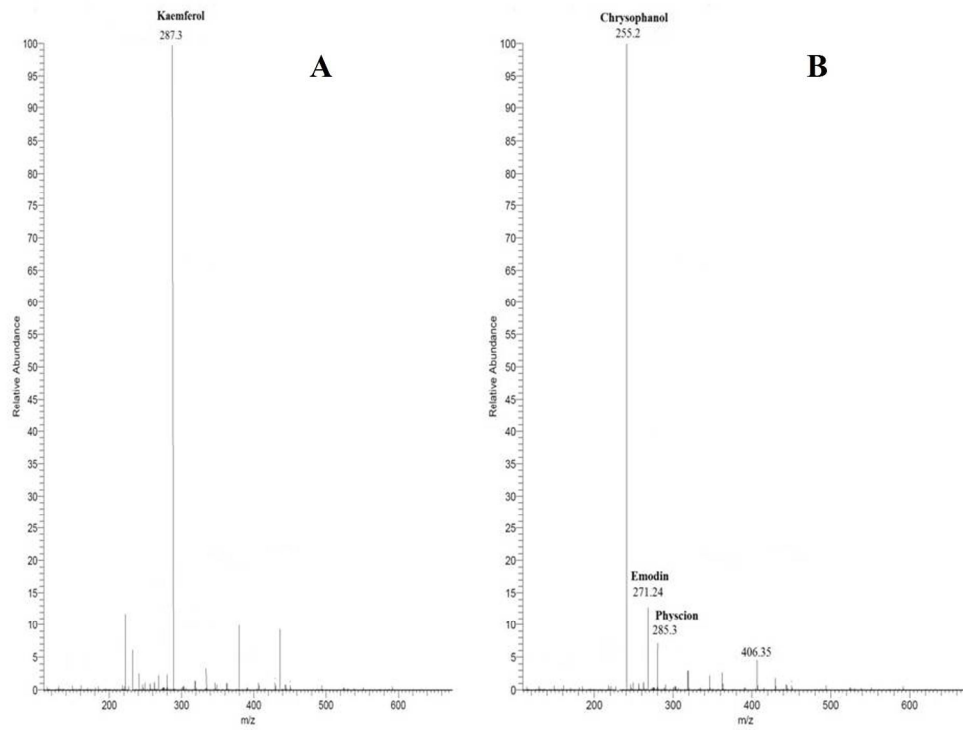
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128x77mm (300 x 300 DPI)



254x190mm (300 x 300 DPI)



254x190mm (300 x 300 DPI)

Tables

Genes	Forward primer	Reverse primer
α A Crystallin	5'CACACTTACCGCAGGCGT3'	5'TGATCATCATAGCGAGGACCCC3'
α -B Crystallin	5'GCCGCCATGGACATCGCCA3'	5'CTACTTCTTGGGGGCTGCAG3'
γ Crystallin	5'CTAGAGGAGAAAAGTAGAGTCTCAAATGCC3'	5'CGAAAGAGATGACTTCAGAGGACAAATGTC3'
β actin	5'TCCTGTGGCATCCATGAAACTAC3'	5'AGCACTGTGTTGGCATAGAGGTC3'

Table 1: Rat specific PCR primer sequences

Groups (n=10)	Stage 0	Stage 1	Stage 3	Stage 4	Stage 5	Stage 6
Group I ^a	10	0	0	0	0	0
Group II ^a	10	0	0	0	0	0
Group III ^a	0	0	0	2	3	5
Group IV ^c	6	3	1	0	0	0

Table 2: Grading of cataract in different experimental groups. Each value represents mean \pm SEM of six values. Different superscript alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

Parameters	Group I	Group II	Group III	Group IV
Ca ²⁺ ATPase(U/mg protein)	0.931 \pm 0.032 ^a	1.01 \pm 0.037 ^a	0.35 \pm 0.013 ^b	0.794 \pm 0.026 ^c
Calcium (μ g/g weight)	20.3 \pm 0.828 ^a	20.1 \pm 0.821 ^a	38.6 \pm 1.575 ^b	25.8 \pm 1.053 ^c
Caspase 3 (U/mg protein)	0.22 \pm 0.009 ^a	0.22 \pm 0.009 ^a	0.795 \pm 0.037 ^b	0.253 \pm 0.010 ^a

Table 3: Activity of Calcium ATPase (μ moles of phosphate liberated/ hour / mg protein), activity of caspase 3 (μ mol of p- nitroaniline released/min/mg protein) and level of calcium (μ g/g weight) in the lenses of different experimental groups. Each value represents mean \pm SEM of six values. Different superscript alphabets indicate significant difference between different groups at $p < 0.05$. Comparison is carried out between the groups; ^a indicates normal group, ^b indicates significantly different from normal group and ^c indicates significantly different from cataract group.

