

Regulatory effect of chrysin on expression of lenticular calcium transporters, calpains, and apoptotic-cascade components in selenite-induced cataract

Mahalingam Sundararajan,¹ Philip A. Thomas,² P. Archana Teresa,² Muniyandi Anbukkarasi,¹ Pitchairaj Geraldine¹

¹Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India;

²Department of Ocular Microbiology, Institute of Ophthalmology, Joseph Eye Hospital, Tiruchirappalli, Tamil Nadu, India

Purpose: Selenite-induced cataract is associated with oxidative stress, loss of calcium homeostasis, activation of calpain enzymes, and apoptotic cell death in the lens. An evaluation of naturally occurring antioxidants that also restrict calcium influx into the lens and calpain activation and thus prevent lenticular cell death may lead to the development of safe and effective anticataractogenic drugs. This study focuses on a naturally occurring flavone, chrysin, and its efficacy in preventing cataractogenic changes in in vitro cultured Wistar rat lenses.

Methods: Lenses from Wistar rats incubated for 24 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) were categorized into four main groups: Group I (control, incubated in DMEM alone); Group II (selenite-challenged and untreated, incubated in DMEM that contained 100 µM/ml of sodium selenite only); Group III (selenite-challenged and chrysin-treated, incubated in DMEM that contained sodium selenite [100 µM/ml of DMEM] and chrysin [200 µM/ml of DMEM]); and Group IV (chrysin-treated, incubated in DMEM that contained chrysin [200 µM/ml of DMEM] only). The Group III (selenite-challenged and chrysin-treated) lenses were further categorized into five sub-groups: Group IIIa (incubated for 24 h in DMEM that contained sodium selenite and chrysin added simultaneously), Group IIIb (first incubated for 2 h in DMEM that contained chrysin only and then for up to 24 h in fresh DMEM that contained sodium selenite only), Group IIIc (first incubated for 30 min in DMEM that contained sodium selenite only and subsequently for up to 24 h in DMEM that contained chrysin only), and Groups IIId and IIIe (first incubated for 1 h and 2 h, respectively, in DMEM that contained sodium selenite only and subsequently for up to 24 h in DMEM that contained chrysin only).

Results: Gross morphological assessment revealed dense opacification (Grade +++) in the selenite-challenged, untreated lenses (Group II); however, seven of the eight selenite-challenged and simultaneously chrysin-treated (Group IIIa) lenses showed no opacification (Grade 0) after 24 h incubation, while the remaining single lens exhibited only a slight degree of opacification (Grade +). In the Group IIIa lenses, the reduced glutathione, protein sulfhydryl, and malondialdehyde concentrations appeared to have been maintained at near-normal levels. The mean lenticular concentration of calcium was significantly lower in the Group IIIa lenses than that in the Group II lenses and approximated the values observed in the normal control (Group I) lenses. The Group IIIa lenses also exhibited significantly ($p < 0.05$) higher mean lenticular activity of calpain, significantly higher mean mRNA transcript levels of genes that encode m-calpain and lenticular preferred calpain (Lp82), and significantly higher mean levels of the m-calpain and Lp82 proteins than the corresponding values in the Group II lenses. Casein zymography results suggested that chrysin prevented calpain activation and autolysis. Significantly ($p < 0.05$) lower mean levels of mRNA transcripts of the genes that encode calcium transporter proteins (plasma membrane Ca^{2+} -ATPase-1 and sarco/endoplasmic reticulum Ca^{2+} -ATPase-2) and lenticular apoptotic-cascade proteins (early growth response protein-1, caspase-3, caspase-8, and caspase-9) and significantly ($p < 0.05$) lower mean concentrations of the proteins themselves were seen in the Group IIIa rat lenses in comparison to the values noted in the Group II rat lenses.

Conclusions: Chrysin appears to prevent selenite-induced cataractogenesis in vitro by maintaining the redox system components at near-normal levels and by preventing the abnormal expression of several lenticular calcium transporters and apoptotic-cascade proteins, thus preventing accumulation of calcium and subsequent calpain activation and lenticular cell death in cultured Wistar rat lenses.

Age-related cataract remains a major cause of blindness, particular in developing countries [1]. At present, there is

no universally accepted pharmacological agent that either prevents or reduces the opacification of the human lens; thus, removal of the opaque lens by surgery remains the principal remedy for human cataract. This pressing need for an inexpensive, non-surgical approach to the treatment of cataract has fueled extensive research on cataract prevention in animal models, particularly the selenite model that

Correspondence to: P. Geraldine, Department of Animal Science, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India; Phone: +91-431-2407040. FAX: +91-431-2414969; email: gerryarchup@yahoo.co.in

mimics some features of human senile cataract [2]. Sodium selenite-induced opacification of the lens is used to study the effects of various stresses on the lens, to model various mechanisms of cataract formation, and to screen potential anticataract agents [3-6].

Intracellular overload with Ca^{2+} in lenticular epithelial cells has been reported to trigger the activation of Ca^{2+} -dependent enzymes, with the irreversible breakdown of important structural proteins and cell death [7], to modify the relative expression of cytochrome c oxidase-1 (*COX-1*; Gene ID: 24,693, OMIM 176805), the gene that encodes the terminal enzyme in the mitochondrial respiratory chain, and to act upon early growth response protein-1 (*EGR-1*; Gene ID: 24,330, OMIM 128990), an immediate-early gene. An important consequence of elevated calcium levels in the lenses of experimental animals is the activation of calpains (EC 3.4.22.17), which are a family of non-lysosomal cysteine proteases found in a variety of organisms.

At least four proteolytically active calpains are expressed in rodent lenses [8]. These calpains include ubiquitous calpain 1 (μ -calpain) and 2 (m-calpain) and lenticular preferred Lp82 and Lp85 [9]. Although m-calpain is the predominant calpain isoform in the human lens, m-calpain is also present in rodent lenses [10,11]. Caspases have been found to transduce the apoptotic signal cascade and to engage cellular targets leading to programmed cell death [12,13]. One of the key effector caspases is caspase-3, which is initiated by caspase-9, and is involved in the mitochondrial-mediated pathway. Induction of apoptosis by exposure to hydrogen peroxide (H_2O_2) is accompanied by increased activation of caspase-9 and caspase-3 at the protein level [14]. Therefore, determining changes in the expression of these caspases is relevant in the context of assessing lenticular apoptosis.

As oxidative stress is reported to be a major factor in inducing cataractogenesis, antioxidants have been administered to prevent cataract formation in animal models. Synthetic antioxidants that exhibit anticataractogenic effects against selenite cataract include acetyl-L-carnitine [15], ellagic acid [16], melatonin [17], resveratrol [18], L-cysteine [19], N-acetylcysteine [20], and rutin [21]. In addition, several medicinal plants and their bioactive flavonoids reportedly protect against selenite-induced cataract by antioxidant mechanisms, including extract of *Emilia sonchifolia* [22], *Vitex negundo* [23], *Ocimum sanctum* [24], *Cineraria maritima* [25], lycopene [26], Drevogenin D [27], and isorhamnetin [28].

Chrysin (5,7-dihydroxy-2-phenylchromen-4-one; Chemical Abstracts Service [CAS] number 480-40-0; PubChem CID:5281607; molecular formula $\text{C}_{15}\text{H}_{10}\text{O}_4$; molecular weight:

254.24; Figure 1) is a major representative of the flavone subclass. Chrysin occurs naturally in the leaves of the Indian trumpet tree, *Oroxylum indicum* [29], passion flower (*Passiflora incarnata* [30]), and silver linden (*Tilia tomentosa*), and is a major constituent of certain edible mushrooms [31], honey, and bee propolis [32], which have long been essential constituents of the normal human diet. Therefore, chrysin was chosen for this study, instead of a synthetic compound. Chrysin exhibits antioxidant [33,34], antiatherogenic [35], anti-inflammatory [36], anticancerous [37], anxiolytic [38], and antidiabetic [39] properties. However, a search of the PubMed databases using the keywords “anticataractogenic effect, chrysin, selenite cataract” did not yield published reports on its anticataractogenic potential. Thus, in the present investigation, an attempt has been made to determine the efficacy of chrysin in modulating or preventing selenite-induced cataractogenesis by virtue of chrysin’s antioxidant properties and its potency in regulating lenticular Ca^{2+} levels and in inhibiting calpain activity and the cascade of apoptotic events.

METHODS

Chemicals: Chrysin (97% pure) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), sodium selenite (Na_2SeO_3) was obtained from LOBA Chemie (Mumbai, India), and Dulbecco’s modified Eagle’s medium (DMEM) and the antibiotics streptomycin and penicillin were obtained from HiMedia (Mumbai, India). Antibodies for m-calpain, Lp82, EGR-1, COX-1, caspase-3, caspase-8, caspase-9, and β -actin were purchased from Sigma-Aldrich Chemical Co. and Cell Signaling Technologies (CST; Danvers, MA).

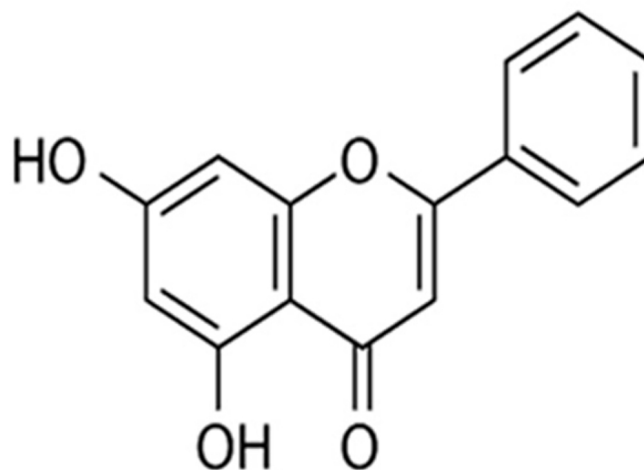


Figure 1. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco’s modified Eagle’s medium: Molecular structure of chrysin.

Anti-rabbit immunoglobulin (IgG) secondary antibody was obtained from Genei (Bengaluru, India). All other chemicals and reagents were of analytical grade. Milli-Q water (Millipore, Bengaluru, India) was used throughout the experiments.

In vitro phase of the study: The animals in the present study were treated in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Research, and the protocols were approved by the Institutional Animal Ethical Committee (IAEC; Approval No. BDU/IAEC/2015/OE/08/Dt.17.03.2015). Wistar rats (75–90 g in weight) were anesthetized with diethyl ether and then killed by cervical dislocation. Their lenses were removed at once and immersed in DMEM, which contained sodium bicarbonate (0.2% w/v) but did not contain calcium; the medium also contained streptomycin (60 µg/ml) and penicillin (60 µg/ml) to prevent microbial contamination. After 2 h, the lenses that developed artifactual opacities were discarded, and only transparent undamaged lenses were collected; they were divided into the following eight groups: (1) Group I (n=8) comprising normal lenses incubated in DMEM alone (control); (2) Group II (n=8) comprising lenses incubated in DMEM that contained sodium selenite (100 µM selenite/ml of DMEM; selenite-challenged, untreated); (3) Group IIIa (n=8) comprising lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 µM selenite/ml of DMEM) and chrysin (200 µM chrysin/ml of DMEM; [simultaneous] selenite-challenge and chrysin treatment); (4) Group IIIb (n=3) comprising lenses first incubated for 2 h in DMEM that contained only chrysin (200 µM chrysin/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only sodium selenite (100 µM selenite/ml of DMEM; 2 h chrysin pretreatment, subsequent selenite-challenge); (5) Group IIIc (n=3) comprising lenses first incubated for 30 min in DMEM that contained only sodium selenite (100 µM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 µM chrysin/ml of DMEM; initial selenite-challenge [30 min], subsequent chrysin treatment); (6) Group IIId (n=3) comprising lenses first incubated for 1 h in DMEM that contained only sodium selenite (100 µM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 µM chrysin/ml of DMEM; initial selenite-challenge [1 h], subsequent chrysin treatment); (7) Group IIIe (n=3) comprising lenses first incubated for 2 h in DMEM that contained only sodium selenite (100 µM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 µM chrysin/ml of DMEM; initial selenite-challenge [2 h], subsequent chrysin treatment); (8) Group IV (n=3)

comprising lenses incubated in DMEM that contained only chrysin (200 µM chrysin/ml of DMEM). The lenses were incubated for 24 h at 37 °C in cell culture test plates maintained in an incubator with an atmosphere of 95% air and 5% CO₂. All lenses were subjected to gross morphological examination at 24 h after the start of the experiment. Following this, all lenses were processed for the determination of reduced glutathione (GSH) and malondialdehyde (MDA).

To exclude the possibility that the simultaneous addition of chrysin and selenite in DMEM affected the concentration of selenite, a time-course stability study of sodium selenite in DMEM that contained chrysin (200 µM/ml; without lenses) was performed. This evaluation was performed in two ways: a) Changes in optical density at 351 nm were measured at different time intervals for 24 h using double-beam ultraviolet-visible spectrophotometer (UV-3600 Plus system, Shimadzu, Kyoto, Japan). b) Sodium selenite concentrations were also measured at these time points using a double-beam ultraviolet-visible spectrophotometer (UV-3600 Plus system), with standard sodium selenite as a reference. To demonstrate that chrysin entered the lenses during the incubation period in DMEM, lenses from Group I (normal control) and Group IV (DMEM that contained only chrysin) were subjected to ultraviolet-visible spectroscopic measurement (UV-3600 Plus system), using standard chrysin as a reference.

Morphological examination of lenses: This was performed with a gross examination, as well as under the magnification of a dissecting microscope against a background of black gridlines. The degree of opacification was graded as follows: (a) 0, the absence of opacification (gridlines clearly visible); (b) +, a slight degree of opacification (minimal clouding of gridlines [gridlines still visible]); (c) ++, the presence of diffuse opacification involving almost the entire lens (moderate clouding of gridlines [gridlines faintly visible]); and (d) +++, the presence of extensive thick opacification involving the entire lens (total clouding of gridlines [gridlines not seen at all]).

Biochemical investigations:

Measurement of reduced glutathione concentration—The reduced GSH content was measured with Moron et al.'s [40] method in which protein in the sample was first precipitated out and then followed by the addition of 4 ml of 0.3 M disodium hydrogen phosphate (pH 8.0) and 0.5 ml of 0.04% (w/v) 5, 5-dithiobis-2-nitrobenzoic acid to the protein-free supernatant to yield a yellow color that was read spectrophotometrically at 412 nm. The GSH level was expressed as micrograms per milligram of protein.

Determination of lipid peroxidation by measurement of MDA: The extent of lipid peroxidation was determined with Ohkawa et al.'s [41] method. The principle of this method is that MDA, an end-product of lipid peroxidation, reacts with thiobarbituric acid to form a pink chromogen. For this assay, lenticular material was homogenized in 1.0 ml of 0.15 M potassium chloride. In a reaction tube, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.81% thiobarbituric acid aqueous solution were added in succession. To this reaction mixture, 0.2 ml of the lenticular homogenate was added and then heated in boiling water for 60 min. After cooling to room temperature, 5 ml of butanol:pyridine (15:1 v/v) solution were added and then centrifuged at $2,000 \times g$ for 15 min. The upper organic layer was aspirated, and the intensity of the resulting pink color was read at 532 nm using tetramethoxypropane as an external standard. The level of lipid peroxide was expressed as nanomoles of malondialdehyde formed per gram of tissue.

Determination of protein sulfhydryl content: The sulfhydryl content of the lenticular proteins was determined using Ellman's procedure as modified by Altomare et al. [42]. Aliquots of total lenticular homogenate (approximately 3 mg of protein) were treated with an equal volume of 4% sulfosalicylic acid. The pellets obtained after centrifugation were washed with 1 ml of 2% sulfosalicylic acid 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 μ l of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The protein sulfhydryl concentration was calculated using a calibration curve prepared with GSH.

Measurement of calcium concentration in the lens: The mean calcium concentrations in Group I (normal control), Group II (selenite-challenged, untreated), and Group IIIa (selenite-challenged simultaneously chrysin-treated) lenses cultured in DMEM were measured. The dry weight of the lens was measured after heating at 100 °C for 20 h. The lenses were then digested with 0.2 ml concentrated HCl at room temperature overnight and adjusted to 1.0 ml with deionized water. The mixtures were centrifuged at $10,000 \times g$ for 10 min to remove insoluble material, if any. The calcium concentration in the supernatant fraction was then measured with an atomic absorption spectrophotometer (model Spectra AA-220, Varian, Santa Clara, CA) operated with a slit width of 0.5 nm, with the wavelength set at 422.7 nm. Standard solutions were prepared from calcium carbonate (CaCO_3) and deionized water. The results were expressed as micromole of calcium per gram dry weight of the lens.

Assay of calpain activity: The combined activity of calpain was determined fluorometrically with casein as the substrate [43] with minor modifications. For this assay, lenses from each group were homogenized thoroughly in 10 volumes of 10 mM sodium borate buffer, pH 8.0, which contained 5 mM EDTA, 3 mM sodium azide, 1 mM dithiothreitol, and 0.1% Triton X-100. After the homogenate was centrifuged at $10,000 \times g$ for 10 min, the supernatant was collected to measure calpain activity. To prepare the substrate, an aqueous solution of casein (60 mg/ml) was subjected to boiling at pH 9.5 (maintained by the addition of 0.1 N NaOH [20 ml]), cooled, adjusted to pH 5.9 with 0.1 N HCl, and then centrifuged to collect the supernatant.

Aliquots of lenticular homogenate corresponding to 500 mg of protein were added to a mixture that contained 3.57 mg of alkali-treated casein, 25 mM of sodium borate, 1 mM of 2-mercaptoethanol, and 5 mM of CaCl_2 to a total volume of 0.35 ml. The pH of the final incubation mixture was 7.5. Incubation was for 1 h at room temperature (the incubation mixture without calcium contained 1 mM ethylene glycol tetraacetic acid). Zero-time incubation blanks were subtracted. The enzyme reaction was stopped by the addition of 200 μ l of 36% (w/v) trichloroacetic acid. The tubes were stored at 4 °C overnight. After centrifugation, 200 μ l of trichloroacetic acid supernatant were mixed with 1.5 ml of 0.1 M sodium borate buffer (pH 8.5), 200 μ l fluorescamine solution (3.4 mg/10 ml of acetone) were added to each tube, and the contents were mixed thoroughly. The number of liberated amino groups was measured fluorometrically [44] in a spectrofluorometer and compared with the standards of known amounts of glutamate in 10% trichloroacetic acid. One unit of calpain activity was defined as the amount that caused the release of 1 μ mol of amino groups/h under the conditions described. Results were expressed as micromole of glutamic acid equivalents released per milligram of protein per hour.

Casein zymography: Casein zymography was performed employing Raser et al.'s [45] method. Casein (0.1%) was copolymerized in an 8% acrylamide separating gel, using 4% acrylamide as a stacking gel. After a 15 min prerun, 50 μ g of each incubated protein sample were wet-loaded and electrophoresed at 125 V (constant) for 2.5 h at 4 °C in running buffer (25 mM Tris HCl, 192 mM glycine, 1 mM ethylene glycol tetraacetic acid, and 1 mM dithiothreitol, pH 8.3). The gel was then incubated in calcium buffer (20 mM Tris HCl, 10 mM dithiothreitol, 2 mM calcium, pH 7.4) overnight at room temperature with slow shaking. The gel was then stained with Coomassie brilliant blue (HiMedia, Mumbai, India) and destained; achromatic bands of caseinolysis appeared white against the stained background.

Molecular investigations:

RT-PCR analysis of mRNA expression—On completion of the experimental period, lenses from the three groups were removed from DMEM and immediately homogenized in TRIzol (Sigma-Aldrich) reagent (1 ml/100 mg tissue); RNA was isolated according to the manufacturer's protocol. RNAs were quantified using a spectrophotometer at A_{260} nm and checked for RNA integrity by assessing 18S and 28S band intensities via agarose gel electrophoresis.

Reverse transcription PCR (RT-PCR) was performed using a one-step RT-PCR kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. Briefly, 1 μ g of template RNA and 0.6 μ M of each of the forward and reverse primers specific to the calcium transporter genes *plasma membrane Ca²⁺-ATPase-1 (PMCA-1)*; Gene ID: 29,598, OMIM 108731), *plasmalemmal Na⁺/Ca²⁺ exchanger-1 (NCX-1)*, Gene ID: 29,715, OMIM 182305) and *sarco/endoplasmic reticulum Ca²⁺-ATPase-2 (SERCA-2)*; Gene ID: 29,693, OMIM 108740), to the calpain family

genes *m-calpain* (Gene ID: 29,154, OMIM 114230) and *Lp82* (Gene ID: 29,155, OMIM 114240) to the apoptotic genes *EGR-1*, *COX-1*, *caspase-3* (Gene ID: 25,402, OMIM 600636), *caspase-8* (Gene ID: 64,044, OMIM 601763), and *caspase-9* (Gene ID: 58,918, OMIM 602234), and to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, Gene ID: 24,383, OMIM 138400; internal control gene; Table 1) were taken into a flat-cap PCR tube (Thermo Scientific, Waltham, MA) along with the enzyme mix (reverse transcriptase and Taq DNA polymerase). Sterile water was added according to the reaction volume and mixed thoroughly.

Amplification was performed in a gradient thermal cycler (Eppendorf, Hamburg, Germany). The RT-PCR conditions were as follows: reverse transcription for 30 min at 50 °C; initial PCR denaturation for 15 min at 95 °C; 30 cycles, each of three steps (denaturation for 30 s at 95 °C; annealing for 30 s at the respective annealing temperature; extension for 1 min at 72 °C); and a final extension for 10 min at 72 °C. The concentration of the template and the cycle

TABLE 1. IN VITRO STUDY ON WISTAR RAT LENSES CULTURED FOR 24 H IN DULBECCO'S MODIFIED EAGLE'S MEDIUM; PRIMER SEQUENCES AND EXPECTED PRODUCT SIZES FOR THE GENES AMPLIFIED.

S. No.	Name of the gene	Primer sequence	Size of the amplicon
1	m-calpain	5' – ATCGCCTCCCTCACCTTGAAT– 3' 5' –AGCTTTCAACCTCCTCGGCTCC– 3'	411 bps
2	Lp82	5' –GTGTTTCCGGCGGGCACCCA– 3' 5' –CGCCGCCAATAATAAAGCGCG– 3'	124 bps
3	EGR-1	5' – CCTCCATCACCTATACTGGCC – 3' 5' – GGGGTTTCAGGCCACAAAGTG– 3'	76 bps
4	COX-1	5' – TTCATCCGAGAAGTACTCATG – 3' 5' – CTGGTGGTGAAAGTGTGTGC – 3'	306 bps
5	PMCA-1	5' –TTGAGTGCTAATGGTGAGGCC– 3' 5'–GGCATATGGTTCTCAGGCCTT– 3'	106 bps
6	NCX-1	5'–ATGGTCTACATGTTTCTCGGAG– 3' 5'–CCCAGGGCCATCAAGGTCAGG– 3'	176 bps
7	SERCA-2	5'–GGTGAAGAAACGATTACAGCC– 3' 5'–TTCTATTGCATTTTCAGCGTT– 3'	111 bps
8	Caspase-3	5'–GTTTCATGTGTATGTTTTATAAG– 3' 5'–GTCCATGGAATTTAACCTCT– 3'	432 bps
9	Caspase-8	5' –GGATTGAAATCTTTTAAGTT– 3' 5' –GGCGAGTCCCACATGTCCTGCA– 3'	297 bps
10	Caspase-9	5' –GAGGTATTTGGTAACACG– 3' 5' –GTGGAGCATCCATAAATGCA– 3'	287 bps
11	GAPDH (internal control)	5'- TCAAGAAGGTGGTGAAGCAGGC –3' 5'- TTCAGTTACTCTCAAGAGCCA –3'	207 bps

Abbreviations: Lp82=lenticular preferred calpain 82; EGR-1=early growth response protein-1; COX-1=cytochrome c oxidase-1; PMCA-1=plasma membrane Ca²⁺-ATPase-1; NCX-1=plasmalemmal Na⁺/Ca²⁺ exchanger-1; SERCA-2=sarco/endoplasmic reticulum Ca²⁺-ATPase-2; GAPDH=glyceraldehyde 3-phosphate dehydrogenase.

number were optimized to ensure the linearity of the response and to avoid saturation of the reaction. After the PCR reaction was completed, a 10 μ l portion of the PCR product was electrophoresed in a 1.8% agarose gel. The ethidium bromide-stained gel was photographed with a DS-34 type Polaroid camera, and the band was scanned with an imaging densitometer (Bio-Rad, Hercules, CA). The *GAPDH* gene was used as an internal standard for the RT-PCR reaction. To measure the transcript level, the ratio of the study gene product to the *GAPDH* gene product was calculated.

Immunoblot analysis: Sodium dodecyl sulfate-PAGE (SDS-PAGE) was performed on 10% gels using the Tris-Glycine buffer system. Immunoblotting for PMCA-1, SERCA-2, m-calpain, Lp82, EGR-1, COX-1, caspase-3, caspase-8, caspase-9, and β -actin was performed by electrotransferring proteins from the SDS-PAGE gel to a nitrocellulose membrane at 30 V (constant) for 100 min at an ice-cold temperature using Tris-Glycine buffer (12 mM Tris, 96 mM glycine, 20% methanol). The monoclonal primary antibodies were used at a 1:1,000 dilution, and alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody was at a 1:2,000 dilution. Immunoreactivity was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The intensity of the bands was calculated using densitometric software (GelQuant.NET, Version 1.7.8, University of California, San Francisco, CA).

Statistical analysis: The values are expressed as the mean \pm standard deviation of multiple readings. Differences between groups were assessed with a one-way ANOVA test using SPSS for Windows (version 21, IBM Corporation, New York, NY). Where one-way ANOVA yielded significant results, post-hoc testing was performed for inter-group comparisons using the least significant difference test. The chi-square test with Yates' correction (GraphPad Prism, version 6, GraphPad Software Inc., San Diego, CA) was applied wherever relevant. Values corresponding to $p < 0.05$ were considered statistically significant and are denoted by distinct symbols in the tables and figures.

RESULTS

In vitro testing for the anticataractogenic potential of chrysin in Wistar rat lenses:

Morphological assessment of cultured Wistar rat lenses—None of the eight lenses incubated in DMEM alone (Group I control) exhibited opacification (Grade 0 opacification) even after 24 h incubation. All (100%) lenses in Group II (incubated in DMEM and sodium selenite) exhibited thick opacification (Grade ++++) after 24 h incubation. In contrast, only one out of the eight lenses (12.5%) in Group

IIIa (incubated in DMEM + sodium selenite + chrysin added simultaneously) exhibited slight opacification (Grade +) after 24 h incubation, while the remaining seven lenses (87.5%) did not show any opacification (Grade 0). The difference was statistically significant (Yates' χ^2 [degree of freedom=1]=9.143; $p < 0.05$) when the proportion of opaque lenses in Group II (100%) was compared with that in Group IIIa (12.5%). Diffuse opacification (Grade ++) was noted in all three Group IIIb lenses (chrysin-pretreated, subsequent selenite-challenge; Table 2 and Figure 2). Interestingly, in all three Group IIIc (initial selenite-challenge [30 min], subsequent chrysin treatment) lenses, a slight degree of opacification (Grade +) was noted. Similarly, in all three Group IIId (initial selenite-challenge [1 h], subsequent chrysin treatment) lenses, diffuse opacification (Grade ++) was noted, while in all three Group IIIe (initial selenite-challenge [2 h], subsequent chrysin treatment) lenses, extensive thick opacification (Grade +++) was noted. The results obtained for Group IIIe lenses were essentially similar to those seen in Group II lenses (Table 2 and Figure 2). However, Group IV (incubated in DMEM that contained only chrysin) lenses exhibited no opacification after 24 h incubation; the appearance of the lenses was similar to that observed in control (Group I) lenses.

The mean concentration of chrysin in chrysin only-treated (Group IV) lenses was found to be 4.711 mM/g of wet lenticular tissue after 24 h incubation; however, chrysin was not detected in the Group I (control) lenses even after 24 h incubation.

In the stability study of sodium selenite in DMEM that contained chrysin (200 μ M/ml of DMEM) but without lenses, sodium selenite was found to be stable even after 24 h incubation in the presence of chrysin; this was inferred by the presence of a "peak" at 351 nm, the same peak being observed at 0, 6, 12, and 24 h (Appendix 1). A time-course study showed that the mean concentration of selenite in the presence of chrysin in DMEM was 15.38 mg/l at 0 h, 15.35 mg/l at 6 h, 15.33 mg/l at 12 h, and 15.31 mg/l at 24 h.

Determination of reduced glutathione and malondialdehyde levels in cultured rat lenses:

Reduced GSH—A significantly ($p < 0.05$) lower mean level of GSH was observed in the selenite-challenged, untreated (Group II) lenses (3.57 ± 0.59 μ mol) when compared to that in the control (Group I; 8.28 ± 0.25 μ mol), selenite-challenged simultaneously chrysin-treated (Group IIIa; 6.27 ± 0.63 μ mol), and chrysin only-treated (Group IV; 8.23 ± 0.15 μ mol) lenses. Similarly, the mean GSH level was significantly lower in the Group IIIb (chrysin-pretreated,

subsequent selenite-challenged; 5.73 ± 0.27 μmol), Group IIIc (selenite-challenged [30 min], subsequent chrysin treatment; 4.14 ± 0.73 μmol), Group IIId (selenite-challenged [1 h], subsequent chrysin treatment; 3.78 ± 0.35 μmol), and Group IIIe (selenite-challenged [2 h], subsequent chrysin treatment; 3.69 ± 0.26 μmol) lenses than the mean levels in the Group I, Group IIIa, and Group IV lenses (Table 3). However, the mean GSH level in the selenite-challenged simultaneously chrysin-treated lenses was significantly ($p < 0.05$) lower than the mean levels in the Group I (control) and Group IV lenses.

Malondialdehyde: The mean concentration of MDA in the selenite-challenged, untreated lenses (Group II; 85.81 ± 3.44 nmol) was significantly ($p < 0.05$) higher than the mean concentration in the control (Group I; 60.47 ± 3.15 nmol),

selenite-challenged simultaneously chrysin-treated (Group IIIa; 71.99 ± 2.86 nmol), and chrysin only-treated (Group IV; 60.54 ± 3.86 nmol) lenses. The mean MDA level was also significantly higher in the Group IIIb (chrysin-pretreated, subsequent selenite-challenged; 76.63 ± 3.81 nmol), Group IIIc (selenite-challenged [30 min], subsequent chrysin treatment; 80.25 ± 4.02 nmol), Group IIId (selenite-challenged [1 h], subsequent chrysin treatment; 82.55 ± 4.07 nmol), and Group IIIe (selenite-challenged [2 h], subsequent chrysin treatment; 83.89 ± 5.03 nmol) lenses than the mean MDA levels in Group I, Group IIIa, and Group IV lenses (Table 3). However, the mean MDA concentration in selenite-challenged simultaneously chrysin-treated lenses (Group IIIa) was also

TABLE 2. IN VITRO STUDY ON WISTAR RAT LENSES CULTURED IN DULBECCO'S MODIFIED EAGLE'S MEDIUM: ASSESSMENT OF LENTICULAR OPACIFICATION AFTER 24 H INCUBATION.

Groups of lenses (incubated in DMEM)*	Total number of lenses	Grades of opacification **	Number of lenses with different grades of lenticular opacification
Group I	8	0	all 8
Group II	8	+++	all 8
^a Group IIIa	8	0	7 of 8
		+	1 of 8
Group IIIb	3	++	all 3
Group IIIc	3	+	all 3
Group IIId	3	++	all 3
Group IIIe	3	+++	all 3
Group IV	3	0	all 3

* Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM containing sodium selenite (100 μM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μM selenite/ml of DMEM) and chrysin (200 μM chrysin /ml of DMEM; selenite-challenged simultaneously chrysin-treated); Group IIIb: Lenses first incubated for 2 h in DMEM containing only chrysin (200 μM chrysin/ml of DMEM) and then for up to 24 h in fresh DMEM containing only sodium selenite (100 μM selenite/ml of DMEM; 2 h chrysin pre-treatment, subsequent selenite-challenge); Group IIIc: Lenses first incubated for 30 min in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [30 min], subsequent chrysin-treatment); Group IIIId: Lenses first incubated for 1 h in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [1 h], subsequent chrysin- treatment); Group IIIe: Lenses first incubated for 2 h in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [2 h], subsequent chrysin- treatment); Group IV: Lenses incubated in DMEM containing only chrysin (200 μM chrysin/ml of DMEM). ** Grades of opacification 0: when there was absence of opacification (gridlines clearly visible); +: when there was a slight degree of opacification (minimal clouding of gridlines [gridlines still visible]); ++: when there was presence of diffuse opacification involving almost the entire lens (moderate clouding of gridlines [gridlines faintly visible]); +++: when there was presence of extensive thick opacification involving the entire lens (total clouding of gridlines [gridlines not seen at all]). Statistical analysis: ^aStatistically significant difference ($p < 0.05$) when compared with group II values (Chi-square with Yates' correction). Abbreviation: DMEM - Dulbecco's modified Eagle's medium.

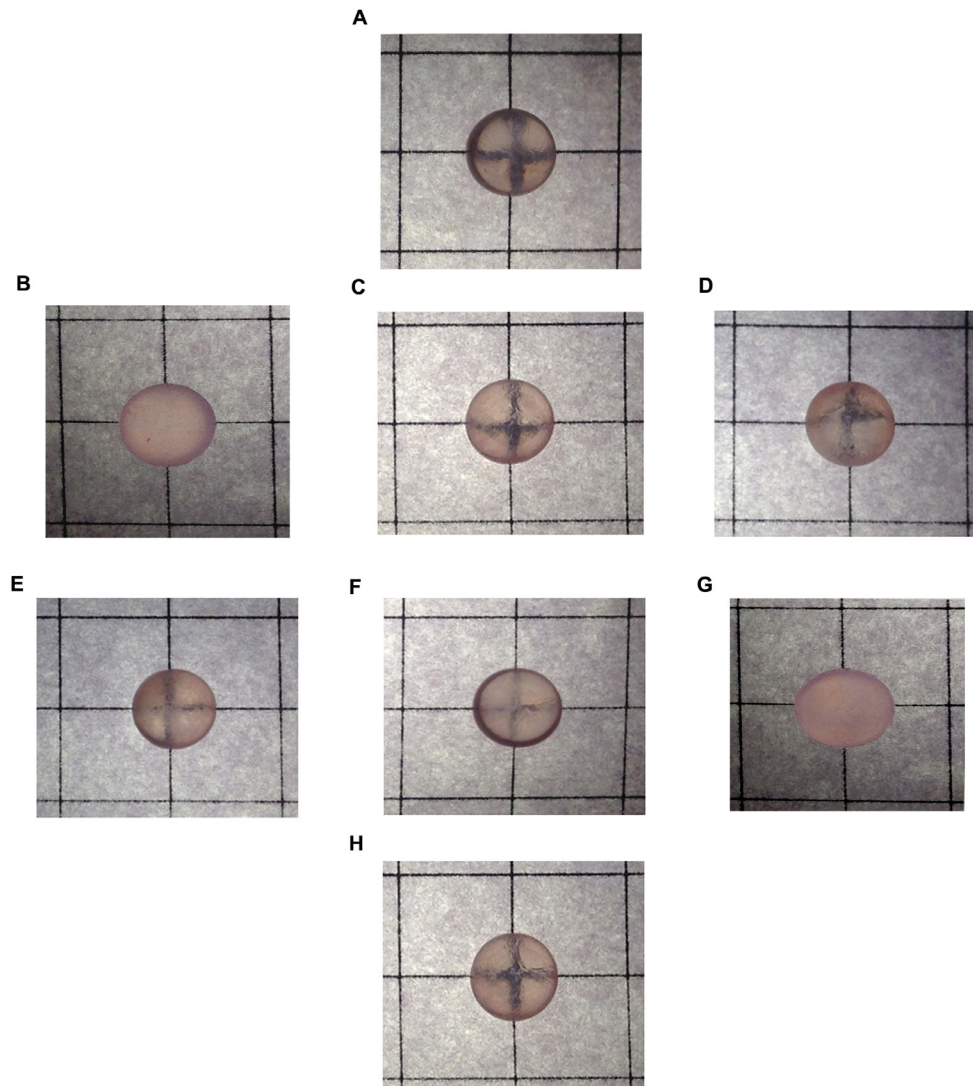


Figure 2. In vitro study on Wistar rat lenses cultured in Dulbecco's modified Eagle's medium: Morphological assessment of lenticular opacification after 24 h incubation **A**: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control; Group I). Grade 0 opacification (the absence of opacification [gridlines clearly seen]) in all eight lenses. **B**: Lenses incubated in DMEM that contained sodium selenite (100 μ M selenite/ml of DMEM; selenite-challenged, untreated; Group II). Grade +++ opacification (extensive thick opacification involving the entire lens [total clouding of gridlines; gridlines not seen at all]) in all eight lenses. **C**: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μ M selenite/ml of DMEM) and chrysin (200 μ M chrysin/ml of DMEM) selenite-challenged simultaneously chrysin-treated; Group IIIa). Grade + opacification (slight degree of opacification [minimal clouding of gridlines; gridlines still visible]) in one of the eight lenses. **D**: Lenses first incubated for 2 h in DMEM that contained only chrysin (200 μ M chrysin/ml of DMEM)

and then for up to 24 h in fresh DMEM that contained only sodium selenite (100 μ M selenite/ml of DMEM; 2 h chrysin pretreatment, subsequent selenite-challenge; Group IIIb). Grade ++ opacification (diffuse opacification involving almost the entire lens (moderate clouding of gridlines [gridlines faintly visible]) in all three lenses. **E**: Lenses first incubated for 30 min in DMEM that contained only sodium selenite (100 μ M selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 μ M chrysin/ml of DMEM; initial selenite-challenge [30 min], subsequent chrysin treatment; Group IIIc). Grade + opacification (slight degree of opacification [minimal clouding of gridlines; gridlines still visible]) in all three lenses. **F**: Lenses first incubated for 1 h in DMEM that contained only sodium selenite (100 μ M selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 μ M chrysin/ml of DMEM; initial selenite-challenge [1 h], subsequent chrysin treatment; Group IIIId). Grade ++ opacification; diffuse opacification involving almost the entire lens (moderate clouding of gridlines [gridlines faintly visible]) in all three lenses. **G**: Lenses first incubated for 2 h in DMEM that contained only sodium selenite (100 μ M selenite/ml of DMEM) and then for up to 24 h in fresh DMEM that contained only chrysin (200 μ M chrysin/ml of DMEM; initial selenite-challenge [2 h], subsequent chrysin treatment; Group IIIe). Grade +++ opacification (extensive thick opacification involving the entire lens [total clouding of gridlines; gridlines not seen at all]) in all three lenses. **H**: Lenses incubated in DMEM that contained only chrysin (200 μ M chrysin/ml of DMEM; Group IV). Grade 0 opacification (absence of opacification [gridlines clearly seen]) in all three lenses.

TABLE 3. IN VITRO STUDY ON WISTAR RAT LENSES CULTURED FOR 24 H IN DULBECCO'S MODIFIED EAGLE'S MEDIUM: ACTIVITY OF REDOX COMPONENT SYSTEM AND MALONDIALDEHYDE CONCENTRATION.

Groups of lenses (incubated in DMEM)*	Reduced glutathione ($\mu\text{mol/g}$ wet weight of lens)	MDA (nmol/g wet weight of lens)
Group I	8.28 \pm 0.25 ^{b,c,d,e,f,g}	60.47 \pm 3.15 ^{b,c,d,e,f,g}
Group II	3.57 \pm 0.59 ^{a,c,d,e,h}	85.81 \pm 3.44 ^{a,c,d,e,h}
Group IIIa	6.27 \pm 0.63 ^{a,b,d,c,f,g,h}	71.99 \pm 2.86 ^{a,b,d,c,f,g,h}
Group IIIb	5.73 \pm 0.27 ^{a,b,c,e,f,g,h}	76.63 \pm 3.81 ^{a,b,c,e,f,g,h}
Group IIIc	4.14 \pm 0.73 ^{a,b,c,d,f,g,h}	80.25 \pm 4.02 ^{a,b,c,d,g,h}
Group IIId	3.78 \pm 0.35 ^{a,c,d,e,h}	82.55 \pm 4.07 ^{a,c,d,h}
Group IIIe	3.69 \pm 0.26 ^{a,c,d,e,h}	83.89 \pm 5.03 ^{a,c,d,e,h}
Group IV	8.23 \pm 0.15 ^{b,c,d,e,f,g}	60.54 \pm 3.86 ^{b,c,d,e,f,g}

*Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM containing sodium selenite (100 μM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μM selenite/ml of DMEM) and chrysin (200 μM chrysin /ml of DMEM; selenite-challenged simultaneously chrysin-treated); Group IIIb: Lenses first incubated for 2 h in DMEM containing only chrysin (200 μM chrysin/ml of DMEM) and then for up to 24 h in fresh DMEM containing only sodium selenite (100 μM selenite/ml of DMEM; 2 h chrysin pre-treatment, subsequent selenite-challenge); Group IIIc: Lenses first incubated for 30 min in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [30 min], subsequent chrysin-treatment); Group IIId: Lenses first incubated for 1 h in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [1 h], subsequent chrysin- treatment); Group IIIe: Lenses first incubated for 2 h in DMEM containing only sodium selenite (100 μM selenite/ml of DMEM) and then for up to 24 h in fresh DMEM containing only chrysin (200 μM chrysin/ml of DMEM; initial selenite-challenge [2 h], subsequent chrysin- treatment); Group IV: Lenses incubated in DMEM containing only chrysin (200 μM chrysin/ml of DMEM). Statistical analysis: ^aStatistically significant difference ($p<0.05$) when compared with groups I values. ^bStatistically significant difference ($p<0.05$) when compared with group II values. ^cStatistically significant difference ($p<0.05$) when compared with group IIIa values. ^dStatistically significant difference ($p<0.05$) when compared with group IIIb values. ^eStatistically significant difference ($p<0.05$) when compared with group IIIc values. ^fStatistically significant difference ($p<0.05$) when compared with group IIId values. ^gStatistically significant difference ($p<0.05$) when compared with group IIIe values. ^hStatistically significant difference ($p<0.05$) when compared with group IV values. Abbreviation: DMEM - Dulbecco's modified Eagle's medium.

significantly ($p<0.05$) higher than the mean concentration in the Group I (normal control) and Group IV lenses.

The results of the morphological assessment and the mean GSH, and mean MDA levels in the groups of lenses appeared to suggest that selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses showed better in vitro morphological appearance and mean GSH and mean MDA levels than that observed in the other subgroups. Therefore, the Group I, Group II, and Group IIIa lenses were evaluated for additional parameters.

Protein sulfhydryl content: In the selenite-challenged, untreated (Group II) lenses, the protein sulfhydryl content was significantly ($p<0.05$) lower (10.17 \pm 0.165 μg) compared to that in the control (Group I; 16.36 \pm 0.128 μg) lenses and

that in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (13.57 \pm 0.35 μg). However, in the Group IIIa lenses the mean lenticular protein sulfhydryl concentration appeared to have been maintained at a level similar to that noted in the Group I (control) lenses (Figure 3).

Calcium parameters in cultured rat lenses:

Mean calcium concentration in rat lenses—With reference to the mean calcium ion concentrations in the lenticular homogenates from the three groups of lenses (Figure 4), the mean calcium concentration was significantly ($p<0.05$) higher in the Group II (selenite-challenged, untreated) lenses (1.81 \pm 0.067 μmol) than the mean concentrations in the Group IIIa (selenite-challenged simultaneously chrysin-treated)

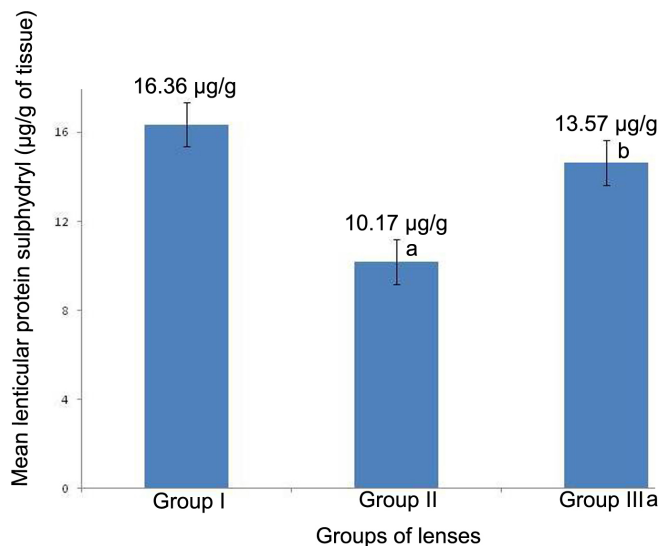


Figure 3. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Mean lenticular levels of protein sulphhydryl. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 µM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 µM selenite/ml of DMEM) and chrysin (200 µM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis was one-way ANOVA with post-hoc test (least significant difference). All values are expressed as mean \pm standard deviation (SD) of six determinations. ^aStatistically significant difference ($p < 0.05$) when compared with the Group I and III values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values.

lenses (0.85 ± 0.03 µmol) and in the Group I (control) lenses (0.55 ± 0.05 µmol; Figure 4).

Effect of chrysin on expression profiles of calcium transporter genes and proteins: The mean mRNA transcript levels of the calcium transporter genes, *PMCA-1* and *SERCA-2*, were found to be significantly ($p < 0.05$) higher in the selenite-challenged, untreated (Group II) lenses (87% and 79%, respectively) than those in the control (Group I) lenses (59% and 68.4%, respectively; Figure 5). However, such an elevation in the mean mRNA transcript levels was not observed in the selenite-challenged lenses that had been treated with chrysin (Group IIIa; 71% and 67.1%, respectively; Figure 5). On the contrary, the mean mRNA transcript level of the lenticular *NCX-1* gene was found to be lower in the cultured selenite-challenged, untreated (Group II) lenses (54%) than that in the cultured normal (Group I; control) lenses (89%). However, such a decline in the mRNA transcript level appeared to have been prevented in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (Figure

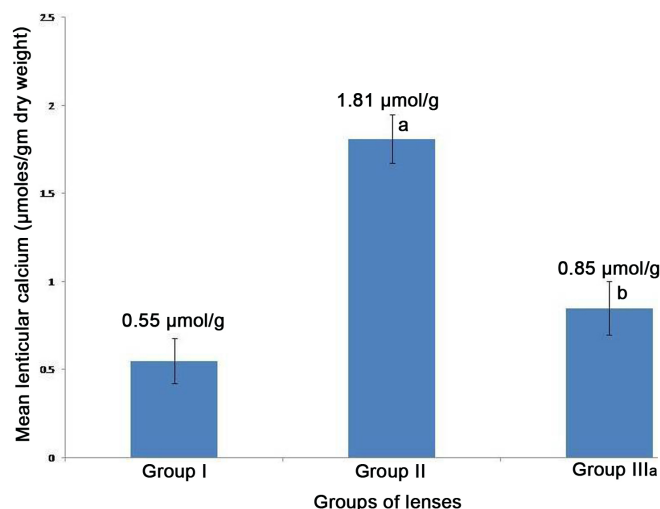


Figure 4. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Mean lenticular levels of calcium. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 µM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 µM selenite/ml of DMEM) and chrysin (200 µM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis was one-way ANOVA with post-hoc test (least significant difference). All values are expressed as the mean \pm standard deviation (SD) of six determinations. ^aStatistically significant difference ($p < 0.05$) when compared with the Group I and III values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values.

5), in which the mean level (86%) approached the mean level noted in the control (Group I) lenses.

To further validate the data obtained from studying the mRNA transcript levels of genes that encode calcium transporters in the cultured lenses, the expression of the calcium transporter proteins, *PMCA-1* and *SERCA-2*, was evaluated with western blot analysis. Densitometric scans of the protein bands showed that the *PMCA-1* and *SERCA-2* protein concentrations were significantly ($p < 0.05$) higher in the selenite-challenged, untreated (Group II) lenses (89.56% and 106.45%, respectively) than those in the control (Group I) lenses (71.4% and 81.78%, respectively; Figure 6). However, such an increase in protein band intensity was not observed in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (76.1% and 87.23%, respectively; Figure 6).

Effect of chrysin on calpain activity:

Total calpain activity assay—With reference to the mean calpain activity in the three different groups of lenses

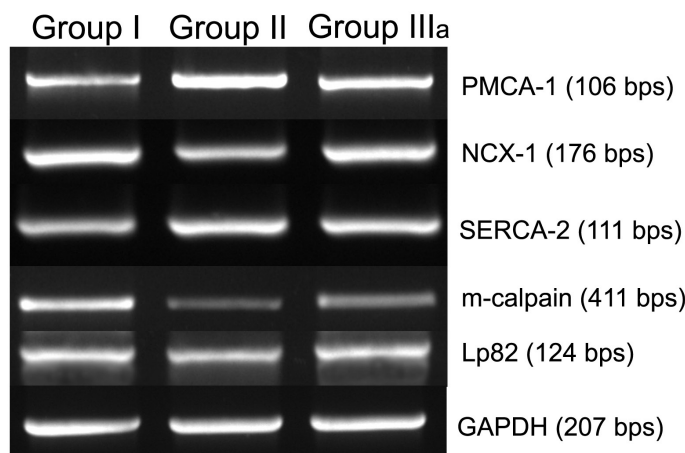
(Figure 7A), in the Group II (selenite-challenged, untreated) lenses, the mean lenticular calpain activity ($0.34 \pm 0.05 \mu\text{mol}$) was significantly ($p < 0.05$) lower than that in the Group IIIa (selenite-challenged simultaneously chrysin-treated) lenses ($0.61 \pm 0.03 \mu\text{mol}$) and that in the Group I (control) lenses ($0.65 \pm 0.06 \mu\text{mol}$; Figure 7A).

Casein zymography: Differential alterations in calpain activity in the lens in selenite-induced cataractogenesis were measured with zymography by looking for calcium-activated calpain-mediated lysis of casein on native PAGE gels (Figure 7B). Based on molecular weight, the calpain isoforms were separated; the uppermost band was Lp82, followed by

m-calpain. The activity of these two calpain isoforms was found to be reduced in the selenite-challenged, untreated (Group II; 71.15% and 65.25%, respectively) lenses. The selenite-challenged simultaneously chrysin-treated (Group IIIa; 75.63% and 76.29%, respectively) lenses showed a markedly intense clearing that was marginally less than that observed in the normal control (Group I; 86.28% and 81.32%, respectively) cultured lenses (Figure 7B). Decreased calpain activity in the Group II lenses was possibly partly due to autolysis.

Analysis of expression of calpain genes and proteins: Lenticular m-calpain and Lp82 gene transcript levels in the three groups were compared to determine the differential

A



B

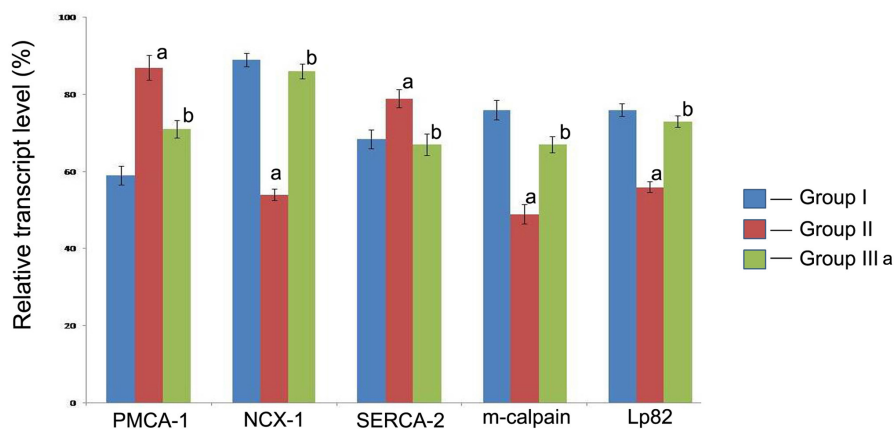


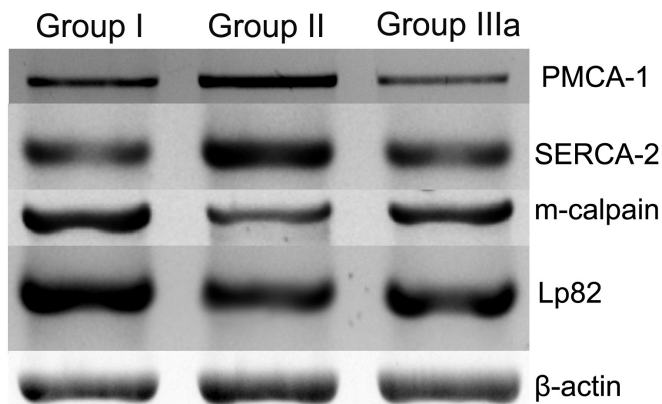
Figure 5. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: RT-PCR investigations. **A:** Ethidium bromide-stained agarose gel showing differential staining intensity of mRNA transcripts of genes that encode calcium transporters and calpain isoforms in the three groups of lenses. **B:** Bar graphs of mean normalized densitometry readings of the mRNA transcripts of genes that encode calcium transporters and calpain isoforms in the three groups of lenses. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 μM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μM selenite/ml of DMEM) and chrysin (200 μM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis of values derived with densitometric scanning of the agarose gel (values

shown as a bar diagram to below the agarose gel). Values represent the mean \pm standard deviation (SD) of the band intensity (densitometric reading) of each gene (experiments run in triplicate). Statistical analysis was one-way ANOVA with post hoc testing (least significant difference). ^aStatistically significant difference ($p < 0.05$) when compared with the Group I values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values. Abbreviations: PMCA-1=plasma membrane Ca^{2+} -ATPase-1; NCX-1=plasmalemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger-1; SERCA-2=sarco/endoplasmic reticulum Ca^{2+} -ATPase-2; Lp82=lens preferred calpain 82; GAPDH=glyceraldehyde 3-phosphate dehydrogenase.

expression of genes that encode calpain activity. The mean mRNA transcript levels of the *m-calpain* and *Lp82* genes (49% and 56%, respectively) were found to be lower in the selenite-challenged, untreated (Group II) lenses than those in the normal control (Group I; 76% and 76%, respectively) lenses (Figure 5). However, in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses, the mean mRNA transcript levels of the *m-calpain* and *Lp82* genes (67% and 73%, respectively) were found to be higher than those in the selenite-challenged, untreated (Group II) lenses (Figure 5). To further validate these data, immunoblot

analysis was performed. Expression of m-calpain and Lp82 proteins was inferred to be significantly ($p < 0.05$) lower in the selenite-challenged, untreated (Group II) lenses (62.12% and 81.5%, respectively) than that in the control (Group I) lenses (88.98% and 107.2%, respectively; Figure 6). However, reduced expression of the m-calpain and Lp82 proteins was inferred to have been prevented in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (75.62% and 94.1%, respectively; Figure 6).

A



B

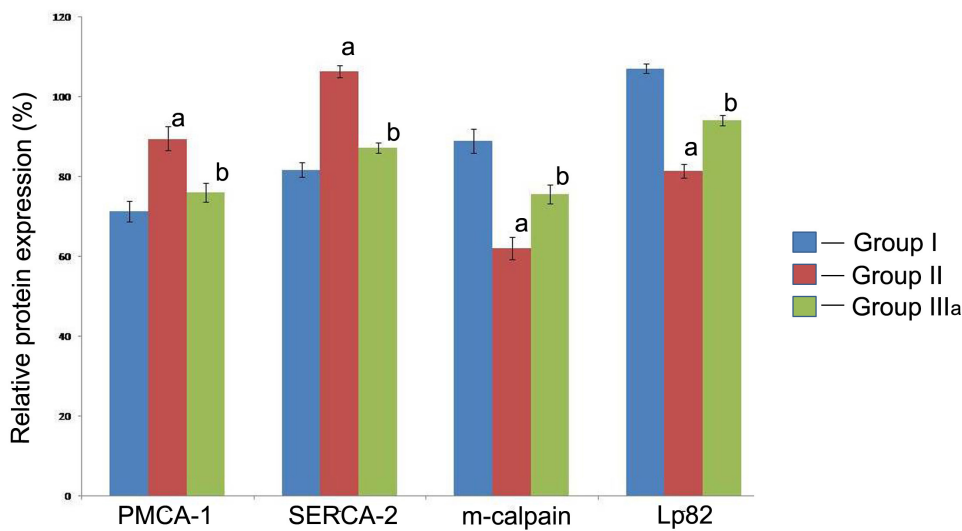


Figure 6. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Immunoblot studies. **A:** Immunoblots showing differential staining intensity of the calcium transporter and calpain isoform proteins in the three groups of lenses. **B:** Bar graphs of the mean normalized densitometric readings of the calcium transporter and calpain isoform proteins in the three groups of lenses. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 μ M selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μ M selenite/ml of DMEM) and chrysin (200 μ M chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis of values derived with densitometric scanning of the immunoblots (values shown as a bar diagram to below the immunoblots). Values represent the mean \pm standard deviation (SD) of the band intensity (densitometric reading) of each protein (experiments run in triplicate). Statistical analysis was one-way ANOVA with

post hoc testing (least significant difference). ^aStatistically significant difference ($p < 0.05$) when compared with the Group I values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values. Abbreviations: PMCA-1=plasma membrane Ca^{2+} -ATPase-1; SERCA-2=sarco/endoplasmic reticulum Ca^{2+} -ATPase-2; Lp82=lens preferred calpain 82.

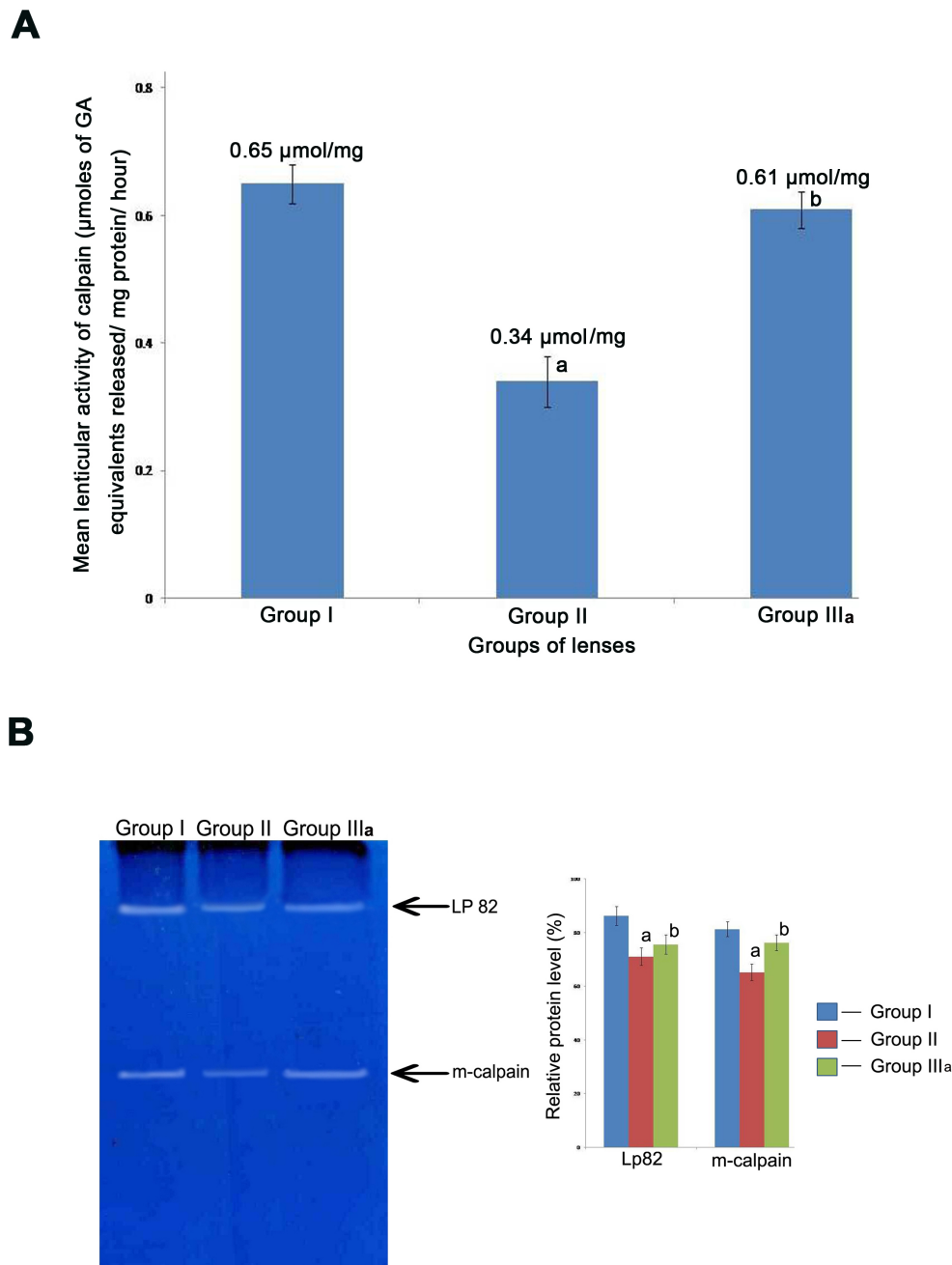


Figure 7. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Effect of chrysin on calpain activity. **A:** Mean lenticular activity of calpain. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 µM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 µM selenite/ml of DMEM) and chrysin (200 µM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis was one-way ANOVA with post-hoc test (least significant difference). Values are expressed as the mean ± standard deviation (SD) of six determinations. ^aStatistically significant difference (p<0.05) when compared with the Group I and III values. ^bStatistically significant difference (p<0.05) when compared with the Group II values. **B:** Analysis of casein zymography. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 µM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite

(100 µM selenite/ml of DMEM) and chrysin (200 µM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis of values derived with densitometric scanning of the casein zymography (values shown as a bar diagram to the right of the casein zymogram) and one-way ANOVA with the post-hoc test (least significant difference). Values are expressed as mean ± standard deviation (SD) of six determinations. ^aStatistically significant difference (p<0.05) when compared with the Group I and III values. ^bStatistically significant difference (p<0.05) when compared with the Group II values. Abbreviations: GA=glutamic acid; Lp82=lenticular preferred calpain 82.

Effect of chrysin on mRNA transcript levels of genes encoding proteins involved in apoptosis and on apoptosis-related protein levels: The mean levels of the mRNA transcripts of the *EGR-1*, *COX-1*, *caspase-3*, *caspase-8*, and *caspase-9* genes were analyzed with semiquantitative RT-PCR with *GAPDH* as an internal control. The levels of *EGR-1*, *caspase-3*, *caspase-8*, and *caspase-9* (Figure 8) mRNA transcripts were found to be significantly ($p < 0.05$) higher in the selenite-challenged, untreated (Group II) lenses (84%, 98.7%, 69.8%, and 85.5%, respectively) than those in the normal control (Group I) lenses (62%, 71.8%, 52.5%, and 66.1%, respectively). However, such elevations in these mRNA transcript levels appeared to have been prevented in the selenite-challenged simultaneously chrysin-treated

(Group IIIa) lenses, where the mean mRNA transcripts levels (68.6%, 83.4%, 55.1%, and 69.7%, respectively) approximated normal levels (Figure 8). Conversely, the mean mRNA transcript level of the *COX-1* gene was found to be significantly ($p < 0.05$) lower in the selenite-challenged, untreated lenses (Group II; 63.2%) than that in the normal control lenses (Group I; 96.5%; Figure 8) but approached near-normal levels in selenite-challenged simultaneously chrysin-treated lenses (Group IIIa; 79.1%; Figure 8).

Immunoblot analysis suggested a relatively higher mean intensity of the EGR-1 protein in the soluble fraction of the cultured selenite-challenged, untreated (Group II) lenses (92.7%) than that in the normal control (Group I) lenses

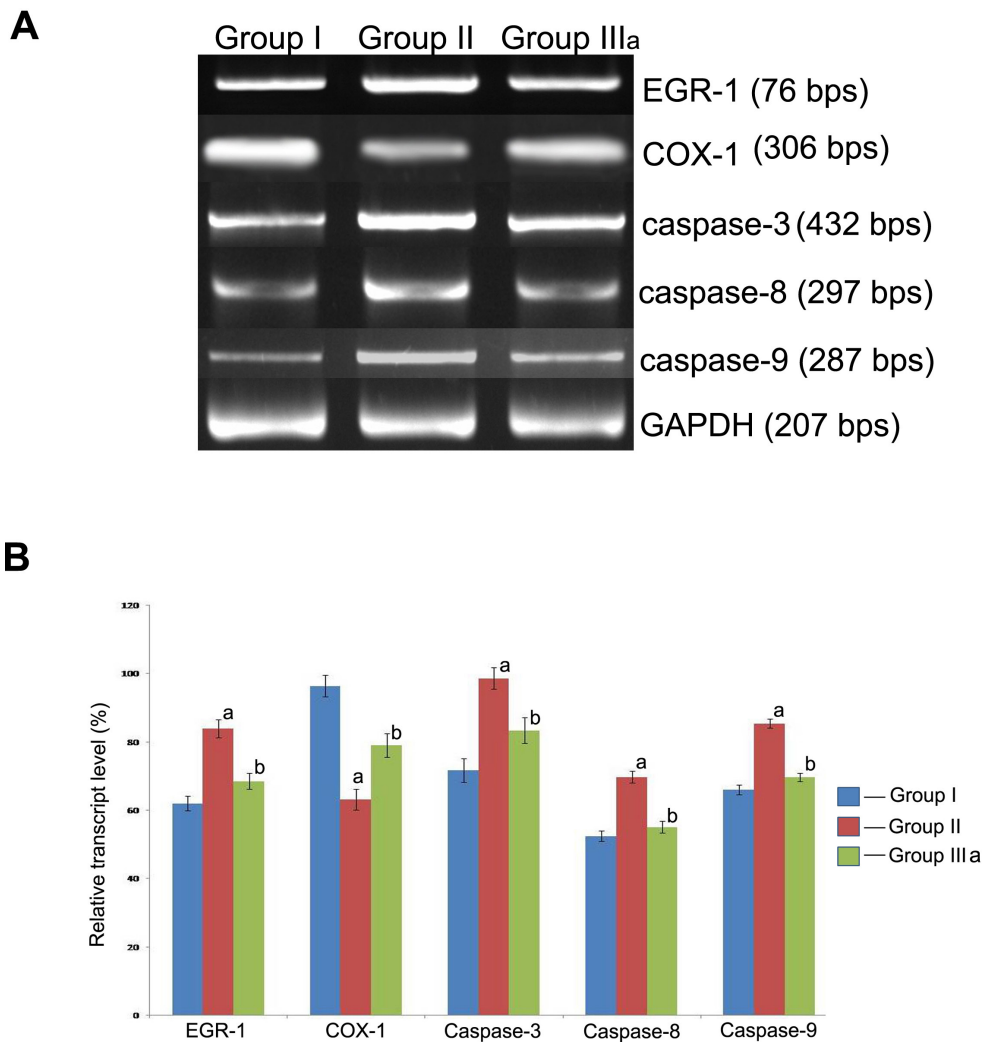


Figure 8. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: RT-PCR investigations. A: Ethidium bromide-stained agarose gel showing differential staining intensity of mRNA transcripts of genes encoding apoptotic cascade proteins in the three groups of lenses. B: Bar graphs of mean normalized densitometry readings of mRNA transcripts of genes that encode apoptotic cascade proteins in the three groups of lenses. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 μ M selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μ M selenite/ml of DMEM) and chrysin (200 μ M chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis of values derived with densitometric scanning of the agarose gel (values shown as a bar diagram to below the agarose gel). Values represent the mean \pm

standard deviation (SD) of the band intensity (densitometric reading) of each gene (experiments run in triplicate). Statistical analysis was one-way ANOVA with post hoc testing (least significant difference). ^aStatistically significant difference ($p < 0.05$) when compared with the Group I values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values. Abbreviations: EGR-1=early growth response protein-1; COX-1=cytochrome c oxidase-1; GAPDH=glyceraldehyde 3-phosphate dehydrogenase.

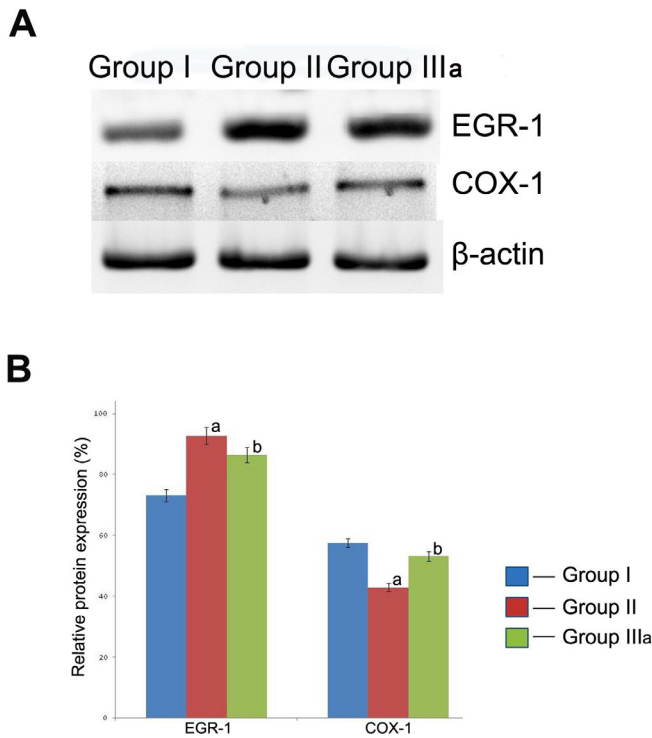


Figure 9. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Immunoblot studies. **A:** Immunoblots showing differential staining intensity of some apoptotic cascade proteins in the three groups of lenses. **B:** Bar graphs of mean normalized densitometric readings of some apoptotic cascade proteins in the three groups of lenses. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 μM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μM selenite/ml of DMEM) and chrysin (200 μM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated). Statistical analysis of values derived by densitometric scanning of the immunoblots (values shown as a bar diagram to below the immunoblots). Values represent the mean ± standard deviation (SD) of the band intensity (densitometric reading) of each protein (experiments run in triplicate). Statistical analysis was one-way ANOVA with post hoc testing (least significant difference). ^aStatistically significant difference ($p < 0.05$) when compared with the Group I values. ^bStatistically significant difference ($p < 0.05$) when compared with the Group II values. Abbreviations: EGR-1=early growth response protein-1; COX-1=cytochrome c oxidase-1.

(73.1%; Figure 9). However, no such elevation in the mean band intensity was noted in the cultured selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (86.4%; Figure 9). Immunoblot analysis suggested a lower mean level of COX-I protein in the soluble fraction of the cultured selenite-challenged, untreated lenses (Group II; 42.9%) than that in the cultured normal (control; Group I) lenses (57.5%). However, such a reduction in the mean COX-I protein level

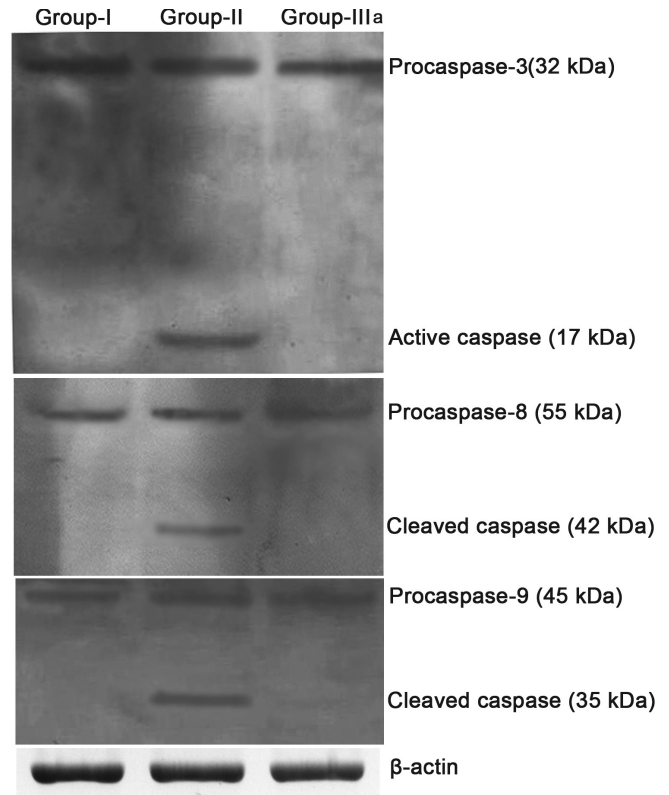


Figure 10. In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Immunoblots showing differential staining intensity of some apoptotic cascade proteins in the three groups of lenses. Groups of lenses; Group I: Normal lenses incubated in Dulbecco's modified Eagle's medium (DMEM) alone (control); Group II: Lenses incubated in DMEM that contained sodium selenite (100 μM selenite/ml of DMEM; selenite-challenged, untreated); Group IIIa: Lenses incubated in DMEM and simultaneously exposed to sodium selenite (100 μM selenite/ml of DMEM) and chrysin (200 μM chrysin/ml of DMEM; selenite-challenged simultaneously chrysin-treated).

appeared to have been prevented in the cultured selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses (51.2%; Figure 9).

To further examine the involvement of the mitochondrial apoptotic pathway, the expression of caspase-3, caspase-8, and caspase-9 was determined with western blotting analysis, while the proteolytic processing of procaspase-3 was detected with a monoclonal antibody to caspase-3. Western blotting revealed a band at 32 kDa, which represented uncleaved caspase-3; this was most intense in the normal control (Group I) lenses. In the selenite-challenged, untreated (Group II) lenses, an additional band was present at 17 kDa, possibly representing a fragment of cleaved caspase-3. However, in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses, such cleavage of caspase-3 did not appear to have occurred as there was no additional 17 kDa band (Figure 10).

Immunoblot analysis was also performed to detect the activation of lenticular caspase-8 protein in the lenses. Western blot analysis appeared to confirm the activation of caspase-8 in the selenite-challenged, untreated (Group II) lenses, as there was a reduction in procaspase-8 (55 kDa) with the simultaneous appearance of its cleaved product, a 42 kDa fraction. However, in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses, such activation appeared to have been prevented, which was inferred from the absence of the cleaved product, the 42 kDa fraction (Figure 10). The level of caspase-9 was also determined as it has been reported that, upon activation, procaspase-9 (45 kDa) cleaves into a fragment of approximately 35 kDa [46]. In the present investigation, this was clearly evident in the selenite-challenged untreated (Group II) lenses. However, in the selenite-challenged simultaneously chrysin-treated (Group IIIa) lenses, there did not appear to be activation of either caspase-8 or caspase-9 (Figure 10).

DISCUSSION

From the data generated by numerous human epidemiological and experimental animal studies, it can be inferred that oxidative stress is a key factor in cataract formation [47-49]. Production of reactive oxygen species and reduction in endogenous antioxidant levels are believed to contribute to cataract formation [50]. Exposure to sodium selenite has been reported to increase lenticular lipid peroxidation and formation of H_2O_2 within the aqueous humor of the eye in experimental animals [51]. In the present study, gross morphological assessment of the selenite-challenged, untreated cultured lenses revealed thick opacification (Grade +++) in all eight lenses (100%). However, seven out of the eight (87.5%) selenite-challenged simultaneously chrysin-treated lenses did not show any opacification (Grade 0) after 24 h incubation; the remaining lens (12.5%) showed a slight degree of opacification (Grade +; Table 2 and Figure 2). This suggests that chrysin partially prevented the occurrence of lenticular opacification caused by exposure to selenite in the Group IIIa lenses. The Group IIIb (chrysin-pretreated, subsequent selenite-challenged) lenses exhibited diffuse opacification. The Group IIIc lenses (initial selenite-challenge [30 min], subsequent chrysin treatment) showed a greater degree of opacification (Table 2, Figure 2) than that of the Group IIIa lenses but less intense opacification than that of the Group II lenses. However, in the Group IIId (initial selenite-challenge [1 h], subsequent chrysin treatment) and Group IIIe (initial selenite-challenge [2 h], subsequent chrysin treatment) lenses, no anticataractogenic effect was noted. All three Group IV (chrysin only-treated) lenses did not show any opacification

(Grade 0), an appearance similar to that noted in the control (Group I) lenses.

The activity of chrysin was best seen when sodium selenite and chrysin were added simultaneously to DMEM (Table 2). Interestingly, the lenses initially exposed to sodium selenite for 30 min and subsequently treated up to 24 h with chrysin exhibited less intense opacification (Grade +) than that seen in the Group II lenses (Grade +++). These results suggest that chrysin did not merely act by blocking the uptake of selenite from the test medium.

Results of the stability study of sodium selenite in DMEM that contained chrysin (but no lenses) suggest that sodium selenite was stable even after 24 h incubation, as inferred by the presence of a “peak” at 351 nm, the same peak observed at 0, 6, 12, and 24 h. Moreover, a time-course study showed that the sodium selenite concentrations in the medium were 15.38 mg/l (0 h), 15.35 mg/l (6 h), 15.33 mg/l (12 h), and 15.31 mg/l (24 h). Thus, these results strongly suggest that sodium selenite is stable in the presence of chrysin in DMEM; that is, there is no obvious physical or chemical interaction with chrysin in DMEM.

The mean concentration of chrysin in the Group IV lenses was 4.711 mM/g of wet lenticular tissue after 24 h incubation. However, chrysin was not detected in the Group I lenses after 24 h incubation. The results suggest that chrysin is able to enter the lens and thus emphasize the potential of chrysin for intercellular lens protection.

To assess the putative role of chrysin as an antioxidant in slowing the progression of selenite-induced cataractogenesis, the status of the redox system and the intensity of lipid peroxidation were determined in the current study. A lower mean level of GSH and a higher mean level of MDA were observed in the selenite-challenged, untreated (Group II), chrysin-pretreated, subsequent selenite-challenged (Group IIIb), and initial selenite-challenged, subsequent chrysin-treated (Group IIIc, IIId, and IIIe) lenses. However, such alterations appeared to have been prevented to a greater extent in the selenite-challenged simultaneously chrysin-treated lenses (Group IIIa) than that in the lenses of Groups IIIb, IIIc, IIId, and IIIe (Table 3). Other groups of investigators have also reported that treatment with antioxidants could prevent selenite-induced cataractogenesis in a Wistar rat model [15,16,52].

Protein sulfhydryl content is an important indicator of tissue protein oxidation. In the process of cataractogenesis, lenticular proteins lose sulfhydryl groups and become thiolated or cross-linked by disulfide bonds [50]. In the current study, the mean protein sulfhydryl level was significantly lower in the selenite-challenged, untreated lenses than that

in the normal (control) lenses; however, such a reduction in mean lenticular protein sulfhydryl content appeared to have been prevented in the selenite-challenged simultaneously chrysin-treated lenses (Figure 3). These results are consistent with studies on selenite-induced cataract in Wistar rats that had been treated with the flavonoid fraction of leaves of *Vitex negundo* [23] and methanolic extract of saffron [52] that have antioxidant potential.

The function of a protein is usually governed by its shape and charge. The influx of Ca^{2+} ions results in activation of calcium-binding proteins, thus triggering changes in the shape and charge of proteins [53]. Therefore, calcium homeostasis must be maintained at an appropriate level to avert pathological conditions. It has been suggested that unregulated Ca^{2+} -mediated proteolysis of essential lenticular proteins by calpains is a major contributor to some forms of cataract in animals and humans [54]. Moreover, elevated levels of Ca^{2+} associated with certain forms of injury to the lens appear to induce proteolysis of crystallins by calpains [55]. In the present study, an attempt was made to determine the mean calcium concentrations in in vitro cultured Wistar rat lenses. The mean lenticular calcium concentration was significantly higher in the Group II (selenite-challenged, untreated) lenses than that in the Group IIIa (selenite-challenged simultaneously chrysin-treated) and the Group I (normal control) lenses (Figure 4). The observed higher level of lenticular calcium was possibly due to the damaged lenticular epithelium as a result of oxidation of sulfhydryl groups or changes in membrane polarity arising out of lipid peroxidation by selenite [2,56]. However, treatment with chrysin appeared to prevent such calcium influx, possibly by preventing selenite-induced oxidative insults. Several other antioxidants, such as Drevogenin D in vitro [27] and ellagic acid [16], acetyl-L-carnitine [57], and the flavonoid fraction of *Brassica oleracea* var. *italica* in vivo [58], have been reported to regulate calcium influx in selenite-induced cataractogenesis.

Cellular calcium homeostasis is achieved by a balance between an inward leak and an outward push of calcium by PMCA and NCX [59,60]. Thus, in the present study, an attempt was made to measure the mRNA transcript levels of the genes that encode PMCA-1, SERCA-2, and NCX-1 and were further traced at the translational level of calcium transporters, such as PMCA-1 and SERCA-2, in the three groups of cultured lenses (Figure 5 and Figure 6). The mean mRNA transcript levels of *PMCA-1* and *SERCA-2* were found to be significantly higher in the selenite-challenged, untreated (Group II) lenses, when compared to those of the normal control (Group I) lenses; the expression of the corresponding

proteins was also found to be enhanced (Figure 6). This upregulated expression of the *PMCA-1* and *SERCA-2* genes in the cultured cataractous lenses was possibly a compensatory mechanism by the cell to increase lenticular calcium, as has been suggested by Marian et al. [61]. A notable study on Upjohn Pharmaceuticals Limited (UPL) rats (a model for hereditary cataract formation) revealed that the expression of the calcium transporter genes, *PMCA-1* and *SERCA-2* (at the mRNA transcript level) was increased during cataractogenesis [62]. Thus, in the present investigation, elevation of mRNA transcript and protein levels was also observed in cultured selenite-challenged, untreated lenses. Interestingly, this enhanced expression of *PMCA-1* and *SERCA-2* genes, as inferred by measuring mRNA transcript levels, as well as elevated levels of PMCA-1 and SERCA-2 proteins, appeared to have been prevented in the selenite-challenged simultaneously chrysin-treated cultured Wistar rat lenses (Figure 5 and Figure 6).

The plasma membrane of most animal cells contains another Ca^{2+} transport system, namely, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) that functions in parallel with the Ca^{2+} -selective channels and the ATP-driven Ca^{2+} pump. NCX can promote either influx or efflux of Ca^{2+} in cells, depending on the net electrochemical driving force on the exchanger [63]. Wang et al. [64] reported that preweaning rats challenged with sodium selenite exhibited reduced NCX activity in their lenses. Similarly, in the present study, the mean level of the mRNA transcripts of *NCX-1* was significantly lower in the selenite-challenged, untreated lenses than that in the normal control lenses and that in the selenite-challenged simultaneously chrysin-treated lenses (Figure 5). This decreased expression of the *NCX-1* gene is apparently consistent with the increase in the lenticular calcium concentration in cultured Group II (selenite-challenged, untreated) lenses. However, in the Group IIIa lenses, the mean level of the mRNA transcripts of *NCX-1* was significantly higher than that in the Group II lenses (Figure 5). Overall, the regulatory mechanism of chrysin on calcium transporters appears to have prevented the elevation of calcium levels that occurred due to selenite-challenge. This finding corroborates those noted in studies on the prevention of calcium increase in lenticular cells by known antioxidants such as Drevogenin D [27], a naturally occurring polyphenol, ellagic acid [16], acetyl-L-carnitine [57], and the flavonoid fraction of the leaves of *Vitex negundo* [23].

An elevated calcium level in the lens leads to the activation of lenticular calpains, thus resulting in subsequent proteolysis of lenticular crystallins [65]. In the current investigation, an attempt was made to assess the in vitro potential

of chrysin to inhibit activation of calpain by determining the calpain activity in three groups of cultured Wistar rat lenses. Decreased mean calpain activity was observed in the cultured selenite-challenged, untreated lenses. However, such a decrease appeared to have been prevented in the selenite-challenged simultaneously chrysin-treated lenses, with the mean calpain activity approximating that observed in the normal control lenses (Figure 7A). Decreased lenticular calpain activity appeared to occur concomitantly with increased lenticular calcium levels in the present study (Figure 4), presumably due to the well known autolysis of calpain that results from the exposure of the enzyme to an elevated calcium concentration. Yoshida et al. [66] also postulated that loss of calpain activity after the formation of cataract in the Nakano mouse model was due to autolysis. In the present study, the selenite-challenged simultaneously chrysin-treated lenses appeared to maintain intracellular calcium levels at near-normal levels (Figure 4), thus preventing the activation of calpain, as inferred from the inhibition of autolysis. A similar finding has been reported for other antioxidants such as ellagic acid [16], the flavonoid fraction of leaves of *Vitex negundo* [23], and acetyl-L-carnitine [57] in preventing calpain activation.

The activation of Lp82, presumably by transient, localized spikes in the cellular calcium concentration resulting in the breakdown of lenticular crystallins, has been found to lead to its autolysis [2]. The addition of calcium to partially purified Lp82 has been found to cause partial loss of calpain, possibly due to autolysis [67]. Similar activation of purified m-calpain causes autodegradation [68]. In the present study, calpain zymography confirmed autolysis of activated Lp82 and m-calpain in the selenite-challenged, untreated lenses (Figure 7B), which was possibly due to the observed elevation in the calcium levels in this group of lenses. However, casein zymography did not reveal autolysis in the selenite-challenged simultaneously chrysin-treated lenses (Figure 7B); thus, chrysin possibly prevented calpain activation and autolysis. These zymographic results appeared to mirror the maintenance of gene (mRNA transcript) and protein expression of m-calpain and Lp82 at near-normal levels, since significant reduction in the mRNA transcript levels and protein levels of these two calpains appeared to have been prevented by treatment with chrysin (Figure 5 and Figure 6). Overall, the evaluation of the lenticular calcium level and total calpain activity and the expression of calpain isoforms strongly suggests that chrysin exhibits anticalpain activity. Anticalpain compounds have also been shown to exhibit anticataractogenic effects in various studies [54,57,69-71].

We also sought to examine the possibility that the effect of calcium extends up to the activation of the apoptotic pathway and that chrysin possibly plays a role in modulating or blocking the caspase cascade, thus slowing selenite-induced cataractogenesis. This hypothesis was tested by evaluating the expression of five important genes involved in the apoptotic pathway, namely, genes that encode EGR-1, COX-1, caspase-3, caspase-8, and caspase-9. Freyssenet et al. [72] demonstrated a direct correlation between increased intracellular calcium and upregulation of the gene that encodes EGR-1. A similar association appears to have occurred in the present investigation in that the selenite-challenged, untreated lenses exhibited elevated mean calcium levels as well as increased expression of the gene *EGR-1* at the transcriptional and translational levels (Figure 8 and Figure 9). Nakajima et al. [73] were of the opinion that the loss of epithelial barrier function contributes to an increase in the intracellular calcium pool, thus leading to increased expression of *EGR-1*. However, treatment of selenite-challenged lenses with chrysin appeared to maintain *EGR-1* at near-normal levels, at the mRNA transcript level and the translational level (Figure 8 and Figure 9). Decreased expression levels of *EGR-1* have been reported in human gastric cancer AGS cells following treatment with chrysin [74]. A similar pattern of *EGR-1* expression has also been reported following treatment with other antioxidants, such as curcumin in endothelial cells and fibroblasts [75] and acetyl-L-carnitine in selenite-induced cataractous Wistar rat lenses [76].

Dysfunction of the COX-1 enzyme leads to compromised mitochondrial membrane potential and a decreased ATP level [77]. In the present investigation, selenite-challenged, untreated lenses demonstrated significantly lower mean levels of *COX-1* mRNA transcripts (Figure 8) and COX-I protein than those in the normal control lenses (Figure 9). Decreased expression of *COX-1* has also been noted in selenite-cataractous lenses [76,78] and in UPL rats [62]. The observed downregulation of *COX-1* might have resulted in decreased synthesis of ATP, as suggested by Yang et al. [79]. However, in the present study, such a decline in *COX-1* expression, at the transcriptional and translational levels, appears to have been prevented in the selenite-challenged simultaneously chrysin-treated lenses, suggesting a protective role for this compound. Similarly, acetyl-L-carnitine was found to maintain mRNA transcript levels of *COX-1* at near-normal levels in lenses challenged with selenite [76].

Following activation, caspase-9 and caspase-8 activate procaspase-3 [80], thus forming active caspase-3, an “executioner caspase,” which is reported to play a central role in regulating and executing apoptosis in mammalian cells. The

activation of caspase-3 has been shown to be an essential step in multiple apoptotic signaling pathways triggered by different apoptotic signals [81]. In the present study, the mean mRNA transcript levels of genes that encode caspase-3, caspase-8, and caspase-9 were analyzed with semiquantitative RT-PCR (Figure 8). In the selenite-challenged, untreated lenses, the mean mRNA transcript levels of genes that encode caspase-3, caspase-8, and caspase-9 were found to be significantly higher than those in the cultured normal lenses. However, in the cultured selenite-challenged simultaneously chrysin-treated lenses, the mean mRNA transcript levels of the genes that encode caspase-3, caspase-8, and caspase-9 were found to be less than those in the selenite-challenged, untreated lenses (Figure 8). To confirm the finding at the translational level with detection of the protein, a specific antibody was used. In the samples of the selenite-challenged, untreated lenses, the band intensity of caspase-3 was lower than that seen in the samples of the cultured normal lenses; in addition, a proteolytic fragment of active caspase-3 (17 kDa) was also noted. However, the anti-caspase-3 antibody could not detect such a proteolytic fragment (active form) in the samples of the cultured selenite-challenged simultaneously chrysin-treated lenses (Figure 10).

Caspase-8 was found to be activated in the samples of the selenite-challenged, untreated lenses as observed by a cleaved fragment of 42 kDa (Figure 10). However, in the selenite-challenged simultaneously chrysin-treated lenses, the activation of caspase-8 appeared to have been prevented, as there was absence of these 42 kDa fragments (Figure 10). Procaspase-9 (approximately 45 kDa), upon activation, is reported to cleave into fragments of approximately 35 kDa [46,82]. In the present set of experiments, similar activation of procaspase-9 was also noted in the samples from the selenite-challenged, untreated lenses that showed cleaved products of 35 kDa fragments (Figure 10). However, the chrysin treatment appeared to prevent such activation, and thus, it appeared as intact procaspase-9 (Figure 10). Thus, the results of the molecular investigation of apoptotic-related genes in the current study suggest that chrysin confers protection against apoptotic cell death in lenticular cells of selenite-challenged lenses by reducing or blocking the activation of the apoptotic cascade and thus preventing caspase-mediated cell death. Numerous antioxidants have been reported to possess antiapoptotic properties in various animal models; these include quercetin [83], beta-carotene [84], acetyl-L-carnitine [76], melatonin [85], and extract of leaves of *Nerium oleander* [86].

A possible limitation of the current study using an in vitro model of selenite-induced cataractogenesis may be the perception that selenite cataract differs in many features

from actual human cataract. However, there is no doubt that selenite cataract is an extremely rapidly induced and convenient model of cataractogenesis. Moreover, experienced investigators have stated that selenite cataract shows several similarities to the human senile nuclear cataract, such as the formation of vesicles, increased levels of calcium, the presence of insoluble protein and decreased water-soluble protein, the occurrence of proteolysis, and diminished amounts of reduced glutathione [2]. The rodent model of selenite cataract is a popular choice for rapid screening of possible anticataract agents. Thus, this model was chosen for the current study to test the anticataractogenic potential of chrysin in an in vitro model of cataractogenesis. Another limitation of the present study is that Lp82 calpain is found only in rodent lenses and is not relevant to human cataractogenesis. However, Lp82 represents a major splice variant for calpain-3 in the rat, and other splice variants for calpain-3 have been found in human and monkey lenses [87-89]. Therefore, we believe that the results related to Lp82 in rodent lenses may have some relevance for the splice variants for calpain-3 that occur in human and monkey lenses, as these variants may perform some hitherto-undefined role in cataractogenesis in human lenses. Moreover, these rodent calpains provide a framework for understanding the role of other low-abundance proteolytic enzymes in the human lens. We also believe that our observations, particularly those related to m-calpain (calpain-2), which occurs in human and rodent lenses, are relevant to the context of cataractogenesis in human lenses.

In conclusion, the results of the current study strongly support the hypothesis that the flavonoid chrysin possesses a novel paradigm of maintaining lenticular calcium homeostasis and the calpain cascade in Wistar rat lenses cultured in DMEM that contained sodium selenite, thus preventing cataractogenesis. Chrysin's effect on calcium homeostasis, inhibition of calpain, and antiapoptotic potential have been demonstrated by chrysin's modulatory effects on the expression of genes involved in calcium transport, calpain activation, and apoptosis. Chrysin may therefore be considered a potential candidate for prevention of selenite-induced cataract. Further investigation is needed to validate the possible contribution of chrysin in the prevention of human cataract.

APPENDIX 1.

In vitro study on Wistar rat lenses cultured for 24 h in Dulbecco's modified Eagle's medium: Absorption spectra of sodium selenite at different time intervals in DMEM containing chrysin. To access the data, click or select the words "Appendix 1."

ACKNOWLEDGMENTS

The instrumentation facility provided by the University Grant Commission-Special Assistance Programme-Departmental Research Support-II (UGC-SAP-DRS-II; F.3-9/2013[SAP-II]Dt.31.03.2013) of the Department of Animal Science, Bharathidasan University, is acknowledged. Financial assistance provided by the UGC-SAP-DRS-II as Technical Assistant to the first author is gratefully acknowledged. The corresponding author is gratefully acknowledged to the financial assistance provided by the University Grants Commission-Basic Scientific Research (UGC-BSR) New Delhi.

REFERENCES

- Nirmalan PK, Krishnadas R, Ramakrishnan R, Thulasiraj RD, Katz J, Tielsch JM, Robin AL. Lens Opacities in a Rural Population of Southern India: The Aravind Comprehensive Eye Study. *Invest Ophthalmol Vis Sci* 2003; 44:4639-43. [PMID: 14578379].
- Shearer TR, Ma H, Fukiage C, Azuma M. Selenite nuclear cataract: review of the model. *Mol Vis* 1997; 3:8-[PMID: 9238097].
- Ghosh MP, Zigler JS Jr. Lack of fiber cell induction stops normal growth of rat lenses in organ culture. *Mol Vis* 2005; 11:901-8. [PMID: 16280975].
- Son HY, Kim H, Y HK. Taurine prevents oxidative damage of high glucose-induced cataractogenesis in isolated rat lenses. *J Nutr Sci Vitaminol (Tokyo)* 2007; 53:324-30. [PMID: 17934237].
- Spector A, Kuszak JR, Ma W, Wang RR, Ho Y, Yang Y. The effect of photochemical stress upon the lenses of normal and glutathione peroxidase-1 knockout mice. *Exp Eye Res* 1998; 67:457-71. [PMID: 9820794].
- Zigler JS Jr, Qin C, Kamiya T, Krishna MC, Cheng Q, Tumminia S, Russell P. Tempol-H inhibits opacification of lenses in organ culture. *Free Radic Biol Med* 2003; 35:1194-202. [PMID: 14607518].
- Gupta PD, Johar K, Vasavada A. Causative and preventive action of calcium in cataractogenesis. *Acta Pharmacol Sin* 2004; 25:1250-6. [PMID: 15456524].
- Ma H, Hata I, Shih M, Fukiage C, Nakamura Y, Azuma M, Shearer TR. Lp82 is the Dominant Form of Calpain in Young Mouse Lens. *Exp Eye Res* 1999; 68:447-56. [PMID: 10192802].
- Shih M, Ma H, Nakajima E, David LL, Azuma M, Shearer TR. Biochemical properties of lens-specific calpain Lp85. *Exp Eye Res* 2006; 82:146-52. [PMID: 16054132].
- Nakamura Y, Fukiage C, Shih M, Ma H, David LL, Azuma M, Shearer TR. Contribution of calpain Lp82-induced proteolysis to experimental cataractogenesis in mice. *Invest Ophthalmol Vis Sci* 2000; 41:1460-6. [PMID: 10798663].
- Ueda Y, Fukiage C, Shih M, Shearer TR, David LL. Mass measurements of C-terminally truncated alpha-crystallins from two-dimensional gels identify Lp82 as a major endopeptidase in rat lens. *Mol Cell Proteomics* 2002; 1:357-65. [PMID: 12118077].
- Bantsev V, Youn HY. Mitochondrial "movement" and lens optics following oxidative stress from UV-B irradiation: cultured bovine lenses and human retinal pigment epithelial cells (ARPE-19) as examples. *Ann N Y Acad Sci* 2006; 1091:17-33. [PMID: 17341599].
- Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993; 74:597-608. [PMID: 8358789].
- Yao K, Ye P, Zhang L, Tan J, Tang X, Zhang Y. Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells. *Mol Vis* 2008; 14:217-23. [PMID: 18334937].
- Elanchezian R, Ramesh E, Sakthivel M, Isai M, Geraldine P, Rajamohan M, Jesudasan CN, Thomas PA. Acetyl-L-carnitine prevents selenite-induced cataractogenesis in an experimental animal model. *Curr Eye Res* 2007; 32:961-71. [PMID: 18027172].
- Sakthivel M, Elanchezian R, Ramesh E, Isai M, Jesudasan CN, Thomas PA, Geraldine P. Prevention of selenite-induced cataractogenesis in Wistar rats by the polyphenol, ellagic acid. *Exp Eye Res* 2008; 86:251-9. [PMID: 18068705].
- Yağci R, Aydin B, Erdurmus M, Karadag R, Gurel A, Durmus M, Yigitoglu R. Use of melatonin to prevent selenite-induced cataract formation in rat eyes. *Curr Eye Res* 2006; 31:845-50. [PMID: 17050276].
- Doganay S, Borazan M, Iraz M, Cigremis Y. The Effect of Resveratrol in Experimental Cataract Model Formed by Sodium Selenite. *Curr Eye Res* 2006; 31:147-53. [PMID: 16500765].
- Jahadi Hosseini H, Aminlari M, Khalili M. Prevention of Selenite-induced Cataract by L-Cysteine and Vitamin C in Rats. *Iran Red Crescent Med J* 2008; 10:281-7. .
- Aydin B, Yagci R, Yılmaz FM, Erdurmus M, Karadağ R, Keskin U, Durmus M, Yigitoglu R. Prevention of Selenite-Induced Cataractogenesis by N-Acetylcysteine in Rats. *Curr Eye Res* 2009; 34:196-201. [PMID: 19274526].
- Isai M, Sakthivel M, Ramesh E, Thomas PA, Geraldine P. Prevention of selenite-induced cataractogenesis by rutin in Wistar rats. *Mol Vis* 2009; 15:2570-7. [PMID: 20011628].
- Lija Y, Biju PG, Reeni A, Cibir TR, Sahasranamam V, Abraham A. Modulation of selenite cataract by the flavonoid fraction of Emilia sonchifolia in experimental animal models. *Phytother Res* 2006; 20:1091-5. [PMID: 17009203].
- Rooban BN, Lija Y, Biju PG, Sasikala V, Sahasranamam V, Abraham A. Vitex negundo attenuates calpain activation and cataractogenesis in selenite models. *Exp Eye Res* 2009; 88:575-82. [PMID: 19094987].

24. Gupta SK, Srivastava S, Trivedi D, Joshi S, Halder N. Ocimum sanctum modulates selenite- induced cataractogenic changes and prevents rat lens opacification. *Curr Eye Res* 2005; 30:583-91. [PMID: 16020293].
25. Anitha TS, Annadurai T, Thomas PA, Geraldine P. Prevention of selenite-induced cataractogenesis by an ethanolic extract of *Cineraria maritima*: an experimental evaluation of the traditional eye medication. *Biol Trace Elem Res* 2011; 143:425-36. [PMID: 20949376].
26. Gupta SK, Trivedi D, Srivastava S, Joshi S, Halder N, Verma SD. Lycopene attenuates oxidative stress induced experimental cataract development: an in vitro and in vivo study. *Nutrition* 2003; 19:794-9. [PMID: 12921892].
27. Biju PG, Devi VG, Lija Y, Abraham A. Protection against selenite cataract in rat lens by drevogenin D, a triterpenoid aglycone from *Dregea volubilis*. *J Med Food* 2007; 10:308-15. [PMID: 17651067].
28. Devi VG, Rooban BN, Sasikala V, Sahasranamam V, Abraham A. Isorhamnetin-3-glucoside alleviates oxidative stress and opacification in selenite cataract in vitro. *Toxicol In Vitro* 2010; 24:1662-9. [PMID: 20566334].
29. Chen LJ, Games DE, Jones J. Isolation and identification of four flavonoid constituents from the seeds of *Oroxylum indicum* by high-speed counter-current chromatography. *J Chromatogr A* 2003; 988:95-105. [PMID: 12647824].
30. Beaumont DM, Mark TM Jr, Hills R, Dixon P, Veit B, Garrett N. The effects of chrysin, a *Passiflora incarnata* extract, on natural killer cell activity in male Sprague-Dawley rats undergoing abdominal surgery. *AANA J* 2008; 76:113-7. [PMID: 18478816].
31. Jayakumar T, Thomas PA, Sheu JR, Geraldine P. In-vitro and in-vivo antioxidant effects of the oyster mushroom *Pleurotus ostreatus*. *Food Res Int* 2011; 44:851-61. .
32. Williams CA, Harborne JB, Newman M, Greenham J, Eagles J. Chrysin and other leaf exudate flavonoids in the genus *Pelargonium*. *Phytochemistry* 1997; 46:1349-53. [PMID: 9419900].
33. Malarvili T, Veerappan R. Effects of chrysin on free radicals and enzymatic antioxidants in N ω -nitro-l-arginine methyl ester: Induced hypertensive rats. *International Journal of Nutrition, Pharmacology, Neurological Diseases* 2014; 4:112-7. .
34. Pushpavalli G, Kalaiarasi P, Veeramani C, Pugalendi KV. Effect of chrysin on hepatoprotective and antioxidant status in D-galactosamine-induced hepatitis in rats. *Eur J Pharmacol* 2010; 631:36-41. [PMID: 20056116].
35. Anandhi R, Thomas P, Geraldine P. Evaluation of the anti-atherogenic potential of chrysin in Wistar rats. *Mol Cell Biochem* 2014; 385:103-13. [PMID: 24065391].
36. Brown E, Hurd NS, McCall S, Ceremuga TE. Evaluation of the anxiolytic effects of chrysin, a *Passiflora incarnata* extract, in the laboratory rat. *AANA J* 2007; 75:333-7. [PMID: 17966676].
37. Sathiavelu J, Senapathy GJ, Devaraj R, Namasivayam N. Hepatoprotective effect of chrysin on prooxidant-antioxidant status during ethanol-induced toxicity in female albino rats. *J Pharm Pharmacol* 2009; 61:809-17. [PMID: 19505373].
38. Zhang T, Chen X, Qu L, Wu J, Cui R, Zhao Y. Chrysin and its phosphate ester inhibit cell proliferation and induce apoptosis in Hela cells. *Bioorg Med Chem* 2004; 12:6097-105. [PMID: 15519155].
39. Woo KJ, Jeong YJ, Inoue H, Park JW, Kwon TK. Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA- binding activity. *FEBS Lett* 2005; 579:705-11. [PMID: 15670832].
40. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta* 1979; 582:67-78. [PMID: 760819].
41. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95:351-8. [PMID: 36810].
42. Altomare E, Grattagliano I, Vendemaile G, Micelli-Ferrari T, Signorile A, Cardia L. Oxidative protein damage in human diabetic eye: evidence of a retinal participation. *Eur J Clin Invest* 1997; 27:141-7. [PMID: 9061308].
43. Banay-Schwartz M, DeGuzman T, Faludi G, Lajtha A, Palkovits M. Alteration of protease levels in different brain areas of suicide victims. *Neurochem Res* 1998; 23:953-9. [PMID: 9690737].
44. Nakai N, Lai CY, Horecker BL. Use of fluorescamine in the chromatographic analysis of peptides from proteins. *Anal Biochem* 1974; 58:563-70. [PMID: 4827397].
45. Raser KJ, Posner A, Wang KK. Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch Biochem Biophys* 1995; 319:211-6. [PMID: 7771786].
46. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; 91:479-89. [PMID: 9390557].
47. Shui YB, Fu JJ, Garcia C, Dattilo LK, Rajagopal R, McMillan S, Mak G, Holekamp NM, Lewis A, Beebe DC. Oxygen distribution in the rabbit eye and oxygen consumption by the lens. *Invest Ophthalmol Vis Sci* 2006; 47:1571-80. [PMID: 16565394].
48. Truscott RJ. Age-related nuclear cataract-oxidation is the key. *Exp Eye Res* 2005; 80:709-25. [PMID: 15862178].
49. Wu SY, Leske MC. Antioxidants and cataract formation: a summary review. *Int Ophthalmol Clin* 2000; 40:71-81. [PMID: 11064858].
50. Ho M-C, Peng Y-J, Chen S-J, Chiou S-H. Senile cataracts and oxidative stress. *Journal of Clinical Gerontology and Geriatrics* 2010; 1:17-21. .
51. David LL, Shearer TR. State of sulfhydryl in selenite cataract. *Toxicol Appl Pharmacol* 1984; 74:109-15. [PMID: 6729815].

52. Makri OE, Ferlemi AV, Lamari FN, Georgakopoulos CD. Saffron administration prevents selenite- induced cataractogenesis. *Mol Vis* 2013; 19:1188-97. [PMID: 23734088].
53. Strynadka NC, James MN. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu Rev Biochem* 1989; 58:951-98. [PMID: 2673026].
54. Biswas S, Harris F, Dennison S, Singh J, Phoenix DA. Calpains: targets of cataract prevention? *Trends Mol Med* 2004; 10:78-84. [PMID: 15102361].
55. Biswas S, Harris F, Phoenix DA. Treatment of cataracts - vision for the future. *Biologist (London)* 2001; 48:273-7. [PMID: 11740079].
56. Borchman D, Paterson CA, Delamere NA. Oxidative inhibition of Ca²⁺-ATPase in the rabbit lens. *Invest Ophthalmol Vis Sci* 1989; 30:1633-7. [PMID: 2545647].
57. Elanchezian R, Sakthivel M, Geraldine P, Thomas PA. The effect of acetyl-L-carnitine on lenticular calpain activity in prevention of selenite-induced cataractogenesis. *Exp Eye Res* 2009; 88:938-44. [PMID: 19150348].
58. Vibin M, Siva Priya SG, B NR, Sasikala V, Sahasranamam V, Abraham A. Broccoli regulates protein alterations and cataractogenesis in selenite models. *Curr Eye Res* 2010; 35:99-107. [PMID: 20136419].
59. Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000; 1:11-21. [PMID: 11413485].
60. Guerini D, Coletto L, Carafoli E. Exporting calcium from cells. *Cell Calcium* 2005; 38:281-9. [PMID: 16102821].
61. Marian MJ, Mukhopadhyay P, Borchman D, Paterson CA. Plasma membrane Ca-ATPase isoform expression in human cataractous lenses compared to age-matched clear lenses. *Ophthalmic Res* 2008; 40:86-93. [PMID: 18223301].
62. Nabekura T, Tomohiro M, Ito Y, Kitagawa S. Changes in plasma membrane Ca²⁺ -ATPase expression and ATP content in lenses of hereditary cataract UPL rats. *Toxicology* 2004; 197:177-83. [PMID: 15003327].
63. Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. *Physiol Rev* 1999; 79:763-854. [PMID: 10390518].
64. Wang Z, Hess JL, Bunce GE. Calcium efflux in rat lens: Na/Ca-exchange related to cataract induced by selenite. *Curr Eye Res* 1992; 11:625-32. [PMID: 1325893].
65. David LL, Azuma M, Shearer TR. Cataract and the acceleration of calpain-induced beta-crystallin insolubilization occurring during normal maturation of rat lens. *Invest Ophthalmol Vis Sci* 1994; 35:785-93. [PMID: 8125740].
66. Yoshida H, Murachi T, Tsukahara I. Age-related changes of calpain II and alpha-crystallin in the lens of hereditary cataract (Nakano) mouse. *Curr Eye Res* 1985; 4:983-8. [PMID: 2998702].
67. Ma H, Fukiage C, Azuma M, Shearer TR. Cloning and expression of mRNA for calpain Lp82 from rat lens: splice variant of p94. *Invest Ophthalmol Vis Sci* 1998; 39:454-61. [PMID: 9478008].
68. Varnum MD, David LL, Shearer TR. Age-related changes in calpain II and calpastatin in rat lens. *Exp Eye Res* 1989; 49:1053-65. [PMID: 2558903].
69. Muralidharan AR, Selvaraj C, Singh SK, Sheu JR, Thomas PA, Geraldine P. Structure-Based Virtual Screening and Biological Evaluation of a Calpain Inhibitor for Prevention of Selenite-Induced Cataractogenesis in an in Vitro System. *J Chem Inf Model* 2015; 55:1686-97. [PMID: 26270943].
70. Payne RJ, Brown KM, Coxon JM, Morton JD, Lee HY, Abell AD. Peptidic Aldehydes Based on α - and β -Amino Acids: Synthesis, Inhibition of m-Calpain, and Anti-Cataract Properties. *Aust J Chem* 2004; 57:877-84. .
71. Qian W, Shichi H. Cataract formation by a semiquinone metabolite of acetaminophen in mice: possible involvement of Ca(2+)and calpain activation. *Exp Eye Res* 2000; 71:567-74. [PMID: 11095908].
72. Freyssenet D, Irrcher I, Connor MK, Di Carlo M, Hood DA. Calcium-regulated changes in mitochondrial phenotype in skeletal muscle cells. *Am J Physiol Cell Physiol* 2004; 286:C1053-61. [PMID: 15075204].
73. Nakajima T, Belusko PB, Walkup RD, Azuma M, Shearer TR. Involvement of Egr-1 in lens epithelial cell death induced by selenite. *Exp Eye Res* 2006; 82:874-8. [PMID: 16368091].
74. Xia Y, Lian S, Khoi PN, Yoon HJ, Han JY, Chay KO, Kim KK, Jung YD. Chrysin inhibits cell invasion by inhibition of Recepteur d'origine Nantais via suppressing early growth response-1 and NF- kappaB transcription factor activities in gastric cancer cells. *Int J Oncol* 2015; 46:1835-43. [PMID: 25625479].
75. Pendurthi UR, Rao LV. Suppression of transcription factor Egr-1 by curcumin. *Thromb Res* 2000; 97:179-89. [PMID: 10674404].
76. Elanchezian R, Sakthivel M, Geraldine P, Thomas PA. Regulatory effect of acetyl-l-carnitine on expression of lenticular antioxidant and apoptotic genes in selenite-induced cataract. *Chem Biol Interact* 2010; 184:346-51. [PMID: 20067779].
77. Li Y, Park JS, Deng JH, Bai Y. Cytochrome c oxidase subunit IV is essential for assembly and respiratory function of the enzyme complex. *J Bioenerg Biomembr* 2006; 38:283-91. [PMID: 17091399].
78. Nakajima T, Nakajima E, Fukiage C, Azuma M, Shearer TR. Differential gene expression in the lens epithelial cells from selenite injected rats. *Exp Eye Res* 2002; 74:231-6. [PMID: 11950233].
79. Yang J, Zhu J, Xu W-H. Differential expression, phosphorylation of COX subunit 1 and COX activity during diapause phase in the cotton bollworm, *Helicoverpa armigera*. *J Insect Physiol* 2010; 56:1992-8. [PMID: 20816850].
80. Cain K, Bratton SB, Cohen GM. The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* 2002; 84:203-14. [PMID: 12022951].

81. Fernandes-Alnemri T, Takahashi A, Armstrong R, Krebs J, Fritz L, Tomaselli KJ, Wang L, Yu Z, Croce CM, Salvesson G, Eamshaw WC, Litwack G, Alnemri ES. Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res* 1995; 55:6045-52. [PMID: 8521391].
82. Zhivotovsky B, Samali A, Gahm A, Orrenius S. Caspases: their intracellular localization and translocation during apoptosis. *Cell Death Differ* 1999; 6:644-51. [PMID: 10453075].
83. Marchionatti AM, Tolosa de Talamoni N. Antioxidant and antiapoptotic properties of quercetin prevent oxidative stress caused by menadione in chick intestine. *Bone* 2009; 45:S157-.
84. Peng HC, Chen JR, Chen YL, Yang SC, Yang SS. beta-Carotene exhibits antioxidant and anti-apoptotic properties to prevent ethanol-induced cytotoxicity in isolated rat hepatocytes. *Phytother Res* 2010; 24:Suppl 2S183-9. [PMID: 19957244].
85. Carpentieri A, Marchionatti A, Areco V, Perez A, Centeno V, Tolosa de Talamoni N. Antioxidant and antiapoptotic properties of melatonin restore intestinal calcium absorption altered by menadione. *Mol Cell Biochem* 2014; 387:197-205. [PMID: 24234419].
86. Benson KF, Newman RA, Jensen GS. Antioxidant, anti-inflammatory, anti-apoptotic, and skin regenerative properties of an Aloe vera-based extract of Nerium oleander leaves (nae-8((R))). *Clin Cosmet Investig Dermatol* 2015; 8:239-48. [PMID: 26005354].
87. Fougereousse F, Bullen P, Herasse M, Lindsay S, Richard I, Wilson D, Suel L, Durand M, Robson S, Abitbol M, Beckmann JS, Strachan T. Human-mouse differences in the embryonic expression patterns of developmental control genes and disease genes. *Hum Mol Genet* 2000; 9:165-73. [PMID: 10607827].
88. Kawabata Y, Hata S, Ono Y, Ito Y, Suzuki K, Abe K, Sorimachi H. Newly identified exons encoding novel variants of p94/calpain 3 are expressed ubiquitously and overlap the alpha-glucosidase C gene. *FEBS Lett* 2003; 555:623-30. [PMID: 14675785].
89. Nakajima T, Fukiage C, Azuma M, Ma H, Shearer TR. Different expression patterns for ubiquitous calpains and Capn3 splice variants in monkey ocular tissues. *Biochim Biophys Acta* 2001; 1519:55-64. [PMID: 11406271].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 30 April 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.