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Effect of methanolic extract of *Allium sativum* (AS) in delaying cataract in STZ-induced diabetic rats

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Abstract Glycemic-induced stress is a major culprit contributing to oxidative insult that has far-reaching effects in diabetic cataract worldwide. In an attempt to prevent/delay cataract, many therapeutic agents have been identified, and among these, natural dietary sources have gained pharmacological significance. Hence, we investigated the efficacy of the methanolic garlic extract against diabetic cataract in Wistar rats. Methanolic garlic extract scavenged the transition metal ion-generated H_2O_2 with an IC_{50} of 768.8 ± 1.76 μg/ml, showing its potential ability as an antioxidant. We have noticed lenticular opacity and oxidative damage in streptozotocin (STZ)-induced hyperglycemic rats. This is evident by the elevation of Ca^{2+}, Cu^{2+}, Na^{+}, Mg^{2+}, thiobarbituric acid reacting substances (TBARS), and carbonyl content and increased activities of polyol enzymes, glutathione peroxidase (GPx), superoxide dismutase (SOD), and up regulation of iNOS transcript and protein aggregation/cross-linking followed by a decrease in reduced glutathione (GSH), K^{+} content, and tryptophan fluorescence in the cataractous lenses of STZ-induced diabetic rats. Garlic administration in a dose-dependent manner attenuated the glycemia-mediated oxidative stress as all the parameters have been found normalized more or less to that of control rats and thus delaying the progression of the lens opacity. We conclude that garlic extract has hypoglycemic and anti oxidant properties that can delay the progression of cataract as revealed in this study.

Keywords Methanolic extract · *Allium sativum* · Garlic · Cataract · STZ-induced diabetic rats

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Introduction

Lens is capsulated with an effective blood–aqueous barrier to focus light onto the retina. In cataractous state, an enormous production of reactive oxygen species (ROS) takes place leading to characteristic membrane permeability changes including leakage of structural proteins which is implicated in the opacity of the lens. Hyperglycemia, altered antioxidant/oxidant ratio, dyshomeostasis of cations [1, 2], advanced glycation end products/Maillard products (transition metal-catalyzed reactions) [3], upregulation of iNOS transcript [4], and increased flux of polyols [5] have been documented as important contributing factors in cataractogenesis.

In the management of cataract, a tight metabolic control is vital and this, perhaps, can be achieved by the use of natural dietary ingredients. Garlic, an indigenous dietary component, belongs to the Liliaceae family and is widely used as a condiment. Besides, it is also used widely in home remedies and pharmacotherapy against debilitated pathologies because of its antioxidant [6], anticardiovascular [7], and antihyperglycemic [8] activities. Zhao and Shichi [9] reported that bioactive molecule in garlic oil, diallyl disulfide (DADS) in a dose of 200 mg/kg body weight, prevented the acetaminophen (APAP)-induced cataract in C57BL/6 mice. Sood et al. [10] stated that the administration of aqueous garlic extract prevented the rate

Table 1 Primers used in the study and predicted amplicon size

Gene		Primer sequence	Size of amplicon (bp)
iNOS	S	5′-CTGCATGTGACTCCATCGAC-3′	796
	AS	5′-ACCACTCGTACTTGGGATGC-3′	
GAPDH	S	5′-GCCAAGGTCATCCATGACAAC-3′	600
	AS	5′-GTCCACCACCCTGTTGCTGTA-3′	

S: sense, *AS*: antisense

of hydration and decreased the concentration of fructose and phosphorus in rat eye lenses which were incubated in galactose and xylose medium indicating its protective action against cataract.

In view of the effective hypoglycemic property of garlic, an attempt has been made in the present study to evaluate the efficacy and protective action of methanolic garlic extract against diabetes-induced cataract and its attendant changes on the antioxidant/oxidant status, cation levels, and gene expression of iNOS.

Materials and methods

Chemicals

Streptozotocin (STZ), Tri reagent, DL-glyceraldehyde, sorbitol dehydrogenase, NADPH, acrylamide, bisacrylamide ammonium persulfate, TEMED, SDS, 1,1,3,3-tetraethoxypropane (TEP), and butylated hydroxytoluene (BHT) were all obtained from Sigma Chemical (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were procured from Merck (Darmstadt, Germany). Prestained protein marker and DNA ladder were from New England Biolabs (Beverly, MA, USA). Primers were procured from Bioserve Biotechnologies (India), and reverse transcription-polymerase chain reaction (RT-PCR) system kit from Fermentas. All other chemicals and solvents were of analytical grade and were procured from local companies.

Extraction of garlic bulbs

Fresh garlic bulbs (*A. sativum* L.) were purchased from the local market (A.P., India) and authenticated by the Head of the Department of Botany, Osmania University. The bulbs were chopped into small pieces and kept for drying till there was no moisture left. The methanolic garlic extract was prepared by adding 1:3 ratios of garlic and methanol, respectively, and subjected to Soxhlet extraction for 72 h according to the prescribed method of Eidi et al. [8]. After extraction, the solvent was filtered, lyophilized, and stored at −80°C till use. The residual extract was dissolved in sterile water and used for further investigation.

Assessment of antioxidant activity of methanolic garlic extract

H_2O_2 scavenging capacity (FOX) assay

The efficacy of garlic extract in scavenging H_2O_2 was determined by the ferrous ion oxidation-xylenol orange method [11].

Experimental design

Male Wistar rats were obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad with an average body weight of 218.9±7.7 g (2–3 months old) were housed in individual cages in a temperature- and humidity-controlled room with a 12-h light–dark cycle. All the animals were fed AIN-93 diet ad libitum. The departmental animal ethical committee approved the safety of the rats and protocols. The control (group I: $n=10$) rats received 0.1 M citrate buffer pH 4.5 as a vehicle and the experimental rats received a single intraperitoneal injection of STZ (34 mg/kg body weight) in citrate buffer. After 72 h, fasting blood glucose levels were monitored and the rats having blood glucose <155 mg/dl were excluded from the study. The hyperglycemic rats were distributed into three groups (group II, STZ-induced diabetic rats and the rats in groups III and

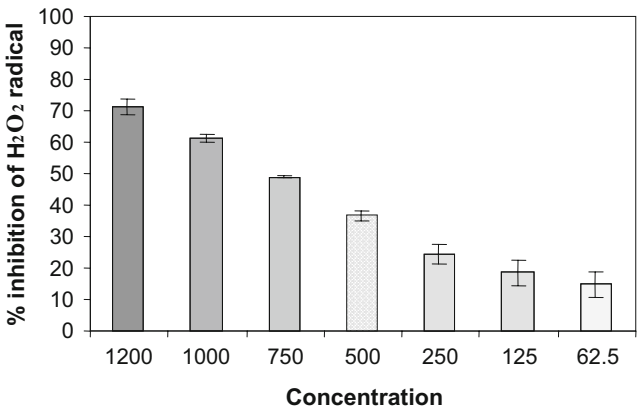

Fig. 1 The effect of methanolic garlic extract on H_2O_2 scavenging capacity by FOX assay. The data are the mean±SD ($n=5$)

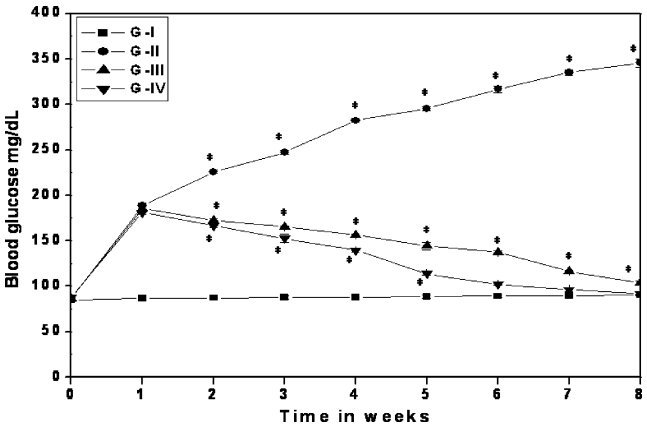

Fig. 2 The effect of methanolic garlic extract on plasma glucose levels in STZ-induced rats. Data are an average of all the animals in a given group. The *asterisk* denotes that data is significantly different from group I at $P<0.05$

IV received garlic in a dose-dependent manner 0.25 and 0.5 g/kg body weight, respectively, on a daily basis ($n=24$) by forcible gut feeding. The rationale behind the selection of the doses is based on experiments done by Eidi et al. on glycemic status [8] and our own pilot experiments where the dosage of garlic above 0.5 g/kg body weight (i.e., 1 g/kg body weight) showed deleterious effect on the hepatic and intestinal tissues and above 0.1 g/kg body weight (i.e., 0.25 g/kg body weight) showed prominent hypoglycemic nature (data not shown). The protocols were approved by the departmental animal ethical committee. Daily food and water intake and weekly body weights were monitored. All the animals had free access to water.

Slit lamp biomicroscope examination and lenticular opacification

To assess the onset and maturation states of cataract, slit lamp biomicroscope examination was carried out at regular intervals and the stages were designated according to Suryanarayana et al. [12]. Briefly, lenses were examined on alternate days and opacities observed were graded into four stages: clear, stage 0; no vacuoles present or clear lens, stage 1; vacuoles of less than one third of the lens radius, stage 2; vacuoles located at the periphery of the lens occupying an area of between one third and two thirds of the radius from the periphery, stage 3; vacuoles extending up to two thirds of the radius from the periphery (nuclear opacity may be seen), stage 4; vacuoles cover the entire lens, which appears white to the naked eye. The incidence of cataract appearance was expressed as the percentage of total lenses in each group.

Blood and lens tissue collection and processing

Blood was collected on weekly basis from the retroorbital plexus for glucose estimation [13]. At the end of 8 weeks, rats were killed by CO_2 asphyxiation; lenses were collected and stored at −80°C till further analysis.

Biochemical estimations

Malondialdehyde (MDA) was quantified as thiobarbituric acid reacting substances (TBARS) by reverse phase HPLC using the Phenomenex column (C18 250× 4.60 mm, 5 μm) [14]. Protein carbonyl content of soluble protein was measured spectrophotometrically using the 2,4-dinitrophenyl-hydrazine [15]; reduced glutathione (GSH) was estimated by the spectrofluorometric method using *O*-pthalaldehyde (OPT) to yield a fluorescent complex [16]; activities of aldose reductase (AR) [17] and sorbitol dehydrogenase (SDH) [18] were assayed spectrophotometrically; specific activity of superoxide dismutase (SOD) was assayed spectrophotometrically by monitoring the rate of inhibition of pyrogallol reduction [19]; glutathione peroxidase (GPx) was assayed spectrofluorometrically using cumene hydroperoxide and GSH as substrate [20]; and tryptophan fluorescence was measured spectrofluorometrically in the soluble protein fraction (0.15 mg/ml in 50 mM sodium phosphate buffer, pH 7.4) at excitation 295 nm and emission between 310 and 400 nm [21] according to prescribed methods. Protein was assayed by the Lowry method [22] using BSA as a standard.

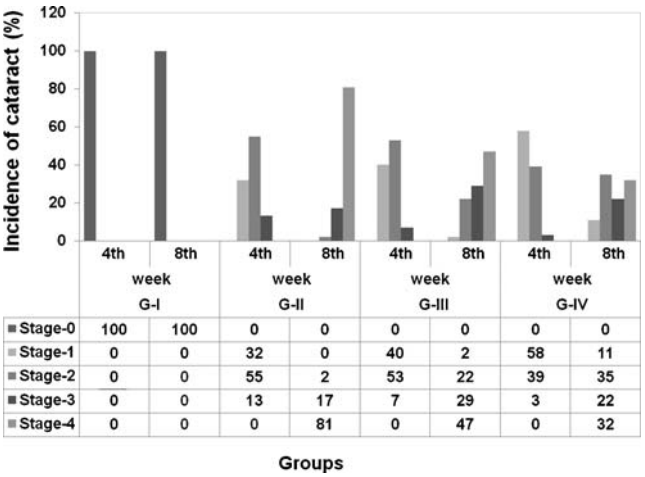

Fig. 3 The effect of methanolic garlic extract on STZ-induced cataract progression during the onset (4th week) and maturation (8th week) states. The total lenses in a group were considered as 100% and the incidence of cataract was calculated

Table 2 The effect of garlic on the oxidative markers on rat lenses

	Group I	Group II	Group III	Group IV
MDA (nmol/g lens)	2.14±0.07	9.36±0.77*	4.60±0.35	3.44±0.30
Protein carbonyls (nmol/mg protein)	1.89±0.11	4.23±0.87*	3.23±0.17*	2.66±0.35*

The data are the mean±SD (n=5); the asterisk denotes that data is significantly different from group I at P<0.05.

Quantification of cation content

Lenticular tissues (approximately 100 mg) was digested with concentrated nitric acid (70% *v/v*), left overnight, and subsequently rota mixed. A fraction of the digested tissues (0.2 ml) was diluted fivefold with deionized water, rota mixed, and subjected to atomic absorption spectrophotometer (AAS) for quantification of Na^+, K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+} using appropriate standards [23].

Analysis of eye lens proteins

The cross-linking and aggregation of soluble proteins were analyzed on 12% polyacrylamide gels in the presence of SDS.

RNA preparation

From all the rats, the total cellular RNA was extracted from lenses (100 mg) using Tri reagent according to manufacturer's instructions. The purity and concentrations of RNA were determined spectrophotometrically at 260, 280, and 320 nm for nucleic acids, proteins, and background, respectively. Samples with a ratio of OD_{260}/OD_{280} values greater than 1.8 were used for quantification.

Semiquantitative reverse transcription-polymerase chain reaction

The RT reaction was performed using the two-step RT kit according to the manufacturer's protocol. Total RNA (approximately 1.2 μg) was reverse transcribed using AMV reverse transcriptase and the cDNA was amplified in the presence of gene-specific primers (Table 1) for iNOS (20 pmol), amplifying the nucleotide region from 128 to 499; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 20 pmol was used as an internal control. The amplification for iNOS consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation for 5 min at 95°C, annealing for 1 min 30 s at 62°C, and extension for 2 min at 72°C and for GAPDH 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and extension for 45 s at 72°C. The final extension was for 10 min at 72°C. The amplicons were electrophoresed on 2.0% agarose gel in Tris–acetate EDTA buffer (pH 8.2). Bands were visualized using ethidium bromide, and then photographed with a UV-trans illuminator (Syngene, USA) and the band intensity was analyzed densitometrically using Gene tools (Syngene, USA) software.

Statistical analysis

The differences between the control and treated groups were analyzed using one-way ANOVA followed by post hoc test (multiple comparisons). The differences were considered significant if P was <0.05 and <0.01.

Results

H_2O_2 scavenging capacity (FOX) of methanolic garlic extract

Methanolic garlic extract scavenged the metal ion catalyzed H_2O_2 with an IC_{50} value of 768.8±1.76 μg/ml (Fig. 1).

Effect of methanolic garlic extract on plasma glucose levels, body weight, and food and water intake

By the end of 8 weeks, the plasma glucose levels were significantly (P<0.05) raised up to 345.6±4.5 in diabetic rats (G-II) in comparison to the controls (G-I) (Fig. 2), representing a persistent hyperglycemia as characterized by

Table 3 The effect of garlic on the redox system and antioxidant enzymes on STZ-induced diabetic cataract

	Group I	Group II	Group III	Group IV
GSH (μmol/g lens)	2.84±0.26	1.36±0.20* 48%	1.78±0.37* 62.7%	2.04±0.14* 71.8%
SOD (IU)	4.61±0.33	5.11±0.12*	4.70±0.23	4.82±0.19
GPx	0.75±0.02	2.13±0.15*	1.57±0.05*	1.09±0.90*

The data are the mean±SD (n=5); the asterisk denotes that data is significantly different from group I at P<0.01. GPx activity is expressed as μmol GSSG formed/min/mg protein. SOD activity is expressed as units/min/mg protein.

Table 4 The effect of polyol pathway enzymes, AR, and SDH in experimental groups

	Group I	Group II	Group III	Group IV
AR	40.55±1.42	51.41±1.31*	44.07±1.53*	42.28±1.11*
SDH	2.20±0.08	5.31±0.10*	4.29±0.12*	3.26±0.07

The data are the mean±SD (n=5); the asterisk denotes that data is significantly different from group I at $P<0.05$. AR activity is expressed as μmol NADPH oxidized/h/100 mg protein. SDH activity as expressed as μmol NADH oxidized/h/100 mg protein.

decrease in body weights (data not shown) and increase in food and water intake, respectively. It is interesting to note that the administration of garlic in a dose-dependent manner to the diabetic rats (G-III and G-IV) significantly ($P<0.05$) decreased the plasma glucose levels by 103.8± 2.1 and 91.6±1.14 (Fig. 2), respectively, proving its effective hypoglycemic nature. However, there was not much improvement in the body weights because of garlic administration despite increased food and water intake.

Slit lamp examination and degree of opacification

The slit lamp examination study revealed the onset state of cataract after 4 weeks. Throughout the course of the study, group I rats did not show any opacification. In STZ-induced (group II) rats, during the onset state, 32% of the lenses were in stage 1, 55% in stage 2, and 13% in stage 3 (Fig. 3) and in the maturation state (8 weeks), 81% of the lenses were in stage 4 and 17% in stage 3, indicating a pronounced opacification (Fig. 3). In groups III and IV rats, during the onset, 40% and 58% of the lenses were in stage 1 and 53% and 39% in stage 2 and during the maturation state, only 47% and 32% were in stage 4 (Fig. 3), suggesting the protective nature of garlic extract in a dose-dependent manner as evidenced by the delay in the onset and maturation of cataract progression with the effect being more prominent with 0.5 g/kg body weight dose.

Oxidative burst biomarkers

A significant ($P<0.01$) elevation of TBARS and protein carbonyl content was noticed in group II rats (Table 2) in comparison to the controls, indicating a clear cut radical generation because of STZ induction followed by lenticular opacification. It is interesting to note that the administration of methanolic garlic in a dose-dependent manner to the diabetic rats (G-III and G-IV) restored the above parameters significantly ($P<0.05$) close to those of the control rats (Table 2).

Redox cycle, antioxidant system

In diabetic rats, lenticular GSH content was significantly ($P<0.05$) reduced by 48%, and the activities of GPX and SOD were significantly ($P<0.05$) increased with respect to the controls (Table 3). The increased activity of these enzymes may be attributed to the overproduction of superoxide and H_2O_2, and this might be an adaptive response for increased oxidative stress. It is interesting to note that a profound improvement was noticed in GSH content (62% and 71%) and the antioxidant levels were restored significantly ($P<0.05$), which are on par with those of control rats (Table 3), suggesting an efficient ROS scavenging property of the extract.

Polyol enzymes

In group II rats, the specific activity of AR and SDH were significantly ($P<0.01$) elevated by approximately 1.3-fold with respect to the controls (Table 4). There is significant ($P<0.05$) restoration of these enzymes in groups III and IV rats because of garlic administration (Table 4).

Cation content

In diabetes-induced cataractous lenses (G-II), significant ($P<0.01$) elevation of Ca^{2+}, Cu^{2+}, Na^{+}, and Mg^{2+} by approximately twofold to fivefold and significant ($P<0.01$) decrease in K^{+} were noticed in comparison to the controls.

Table 5 The effect of methanolic garlic on the lenticular cation content in the experimental rats

	Ca^{2+} (nmol/g lens)	Mg^{2+} (μmol/g lens)	Cu^{2+} (μmol/g lens)	Zn^{2+} (nmol/g lens)	Na^{+} (μmol/g lens)	K^{+} (μmol/g lens)
G-I	0.84±0.02	2.50±0.31	2.00±0.12	1.93±0.12	4.91±0.08	1.92±0.07
G-II	5.14±0.06*	6.28±0.16*	5.99±0.46*	2.11±0.06	8.74±0.26*	1.41±0.05*
G-III	2.95±0.10*	4.84±0.14*	3.72±0.32*	2.09±0.11	6.90±0.22*	1.60±0.06*
G-IV	1.67±0.12*	3.48±0.23*	2.61±0.18*	1.92±0.12	5.38±0.19*	1.86±0.08

The data are the mean±SD (n=5); the asterisk denotes that data is significantly different from group I at $P<0.01$.

Table 6 The effect of garlic on the protein content on rat lenses

	Group I	Group II	Group III	Group IV
Total protein (mg/g lens)	478.6±20.81	320.4±11.76*	404.4±14.27*	435.2±18.55*
Soluble protein (mg/g lens)	331.02±10.01	144.04±11.01*	285.59±15.30*	317.13±10.80
% Soluble protein	69.3	45.6	61.6	67

The data are the mean±SD (n=5); the asterisk denotes that data is significantly different from group I at P<0.05.

This clearly supports the protein aggregate band, opacification, and its associated derangement in membrane permeability characteristic in these rats (Table 5). It is interesting to note that in the rats that have been administrated garlic in a dose-dependent manner, the cation contents were significantly (P<0.01) normalized with respect to controls (Table 5). There is no significant (P< 0.05) variation in the level of Zn^{2+} in all the experimental rats (Table 5).

Effect of garlic on lens protein profile

In the cataractous lenses, aggregation and insolubilization of proteins are related to membrane leakage. This being the main emphasis, we have quantified the total, soluble, and insoluble protein fractions in all the rats. The group II rats showed a significant (P<0.05) decrease in total and soluble protein fractions in comparison to the controls (Table 6). There was a remarkable increase of total and soluble fraction contents in garlic-administered rats in a dose-dependent manner (groups III and IV), and the protective nature is being more prominent with the dose of 0.5 g/kg body weight. The SDS-electrophoretic pattern of the soluble protein fraction showed an aggregated band at 66.2 kDa in relation to the controls and with a clear disappearance of this band in group III and IV rats (Fig. 4). This was further supported by the tryptophan fluorescence studies where a prominent decrease in the spectra was noticed in group II rats with respect to the controls. However, garlic administration had restricted the decrease in spectra with respect to group II (Fig. 5).

RT-PCR analysis

There is no transcript of the candidate gene (iNOS) in group I. However, upregulation of transcript in group II rats by a relative percentage of approximately 95% (Fig. 6) was recorded, which is attributed to the hyperglycemic oxidative burst. It is interesting to note that a downregulation of the transcript was observed because of garlic administration in a dose-dependent manner (Fig. 6).

Discussion

Cataract, a leading cause of blindness, is a major socioeconomic burden for world population. To date, there is no potent therapeutic agent that can prevent/inhibit the lens from opacification. As a part of better strategic management of cataract, metabolic intervention through natural dietary ingredients is gaining importance in recent times. Garlic is the oldest of all cultivated plants and is widely used because of its high pharmacological significance. Thus, the present study aimed to have an insight on the ameliorative action of garlic against diabetes-induced cataract.

The ability of methanolic garlic extract in scavenging the transition metal ion-generated H_2O_2 reflected its antioxidant activity and indicated that it can prevent protein modifications mediated through metal-catalyzed reactions in cataractous lenses, and the findings are in agreement with those of Chaverri et al. [11].

Rats on STZ induction recorded higher plasma glucose levels in a time-dependent manner giving way to a persistent hyperglycemic state. This increased hyperglyce-

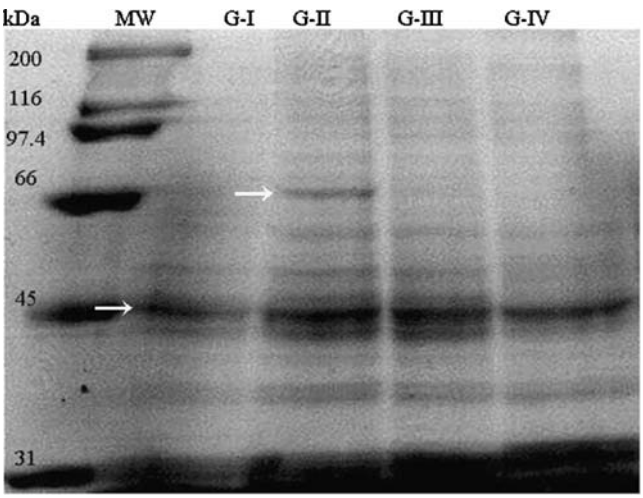

Fig. 4 The effect of methanolic garlic extract on protein cross-linking and aggregation of the soluble fraction of the lens. The *arrow* indicates the cross-linked proteins

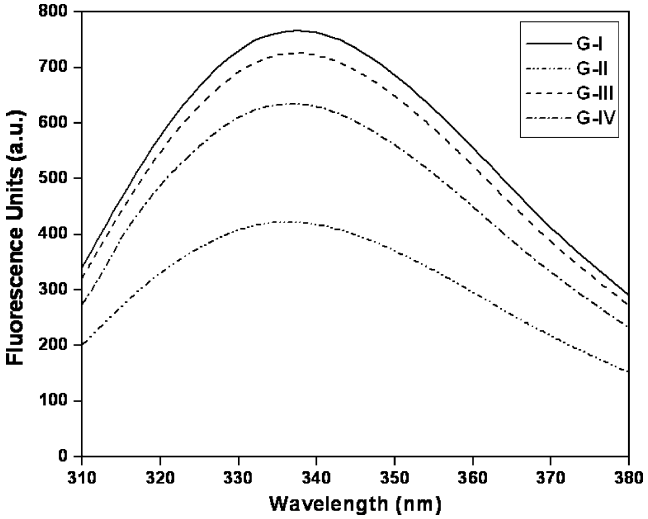

Fig. 5 Tryptophan fluorescence of soluble protein in lenticular tissue of experimental groups. Data are an average of five values

mic state has been responsible for a variety of detrimental changes like oxyradical generation through glucose autooxidation, accumulation of sugar alcohols consequential to an increase of polyol enzymes, and characteristic membrane permeability changes. These altered membrane permeability changes have led to an increased intracellular Ca^{2+}, Cu^{2+}, Na^{+}, and Mg^{2+} and decreased K^{+} levels, which could in turn lead to the overactivation of proteases, protein insolubilization and aggregation/cross-linking culminating finally to increased light scattering, a feature associated with lens opacity.

The hyperglycemic state, altered membrane permeability contributing to imbalance in the electrolytes in diabetes-induced cataractous lenses has led to the upregulation of mRNA iNOS transcript promoting oxidative stress via nitric oxide generation. The increased levels of TBARS, carbonyl content, and altered antioxidant reserves and decrease in GSH content clearly point to the oxidative damage as evidenced in the lenses of diabetic rats, and these observations are in agreement with the previous findings [4, 12, 24–27].

Garlic administration was found to normalize the glucose levels in a dose-dependent manner, suggesting its hypoglycemic potential. The glycemic-mediated oxidative damage was countered by the garlic extract by delaying the progression of cataract. The observed results testify to the

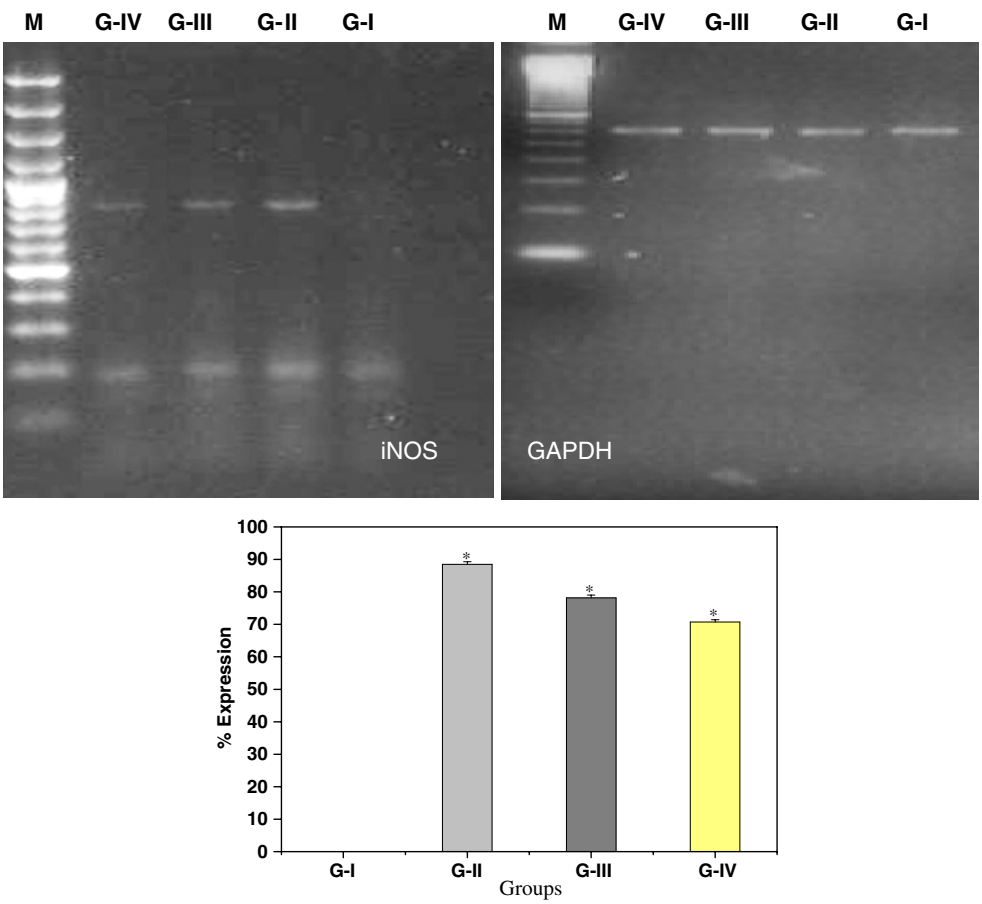

Fig. 6 The effect of methanolic garlic on the expression and quantification of candidate gene (iNOS) in the experimental rats. The % expression values are average of three independent experiments. The *asterisk* denotes that data is significantly different from group I at $P<0.05$

role of the extract in the suppression of the polyol enzymes which are implicated in the maintenance of osmotic/oxidative equilibrium and thus membrane integrity. The extract has also been found to exert its influence on the restoration of electrolytes, GSH content, antioxidant reserves, and very importantly, prevention of protein aggregation, downregulation of mRNA iNOS transcript, TBARS, and carbonyl content. The observed findings of the study could well be because of the ameliorative action of the bioactive compound allicin or ajoene and suggest garlic's efficient antioxidant potential against the changes imposed by hyperglycemia, and our results are in agreement with the works of previous findings [8, 9, 28–31].

The lenticular opacification associated with diabetic stress is a serious affliction mediated through oxidative insult impairing vision. Intervention of the progression of problems related to hyperglycemia through natural supplements may well contribute to the transparency of the lens. The observations/findings of this particular study involving garlic extract in a dose-dependent manner do prove it to be an effective antioxidant in counteracting oxidative insult. Nonetheless, the understanding of the exact mechanism(s) of the ameliorative action of its active compound in stressful situations in general and hyperglycemic stress in particular could open doors a great deal for further investigations in the management of diabetes-related cataract and thereby in the sustenance of quality vision.

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