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Aqueous Garlic Extract Alleviates Oxidative Stress and Inflammation in Retinal Tissue of Rats with Diabetes Type 2

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ABSTRACT

Background & Objective: Retinopathy is a common difficulty in diabetic subjects. Hyperglycemia damages the tissues through stimulation of oxidative stress and inflammation. Since the antioxidant function of garlic has been proven, in the current report the activity of aqueous garlic extract (AGE) upon the oxidative stress and inflammation in the retinal substances of rats with diabetes was investigated.

Materials & Methods: 24 male Wistar rats were distributed in 4 groups: the healthy rats, the rats with diabetes (DM2),the garlic- treated rats with diabetes (DM2+AGE), and the garlic- treated healthy rats (AGE). After the treatment was finished, oxidative stress, total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), thiol group (SH), and lipid peroxidation (LPO) were assayed. For the evaluation of inflammation, mRNA and protein levels of transforming growth factor beta-2 (TGF-β2) and interleukin-1 beta (IL-1β) were measured in retinal homogenates using real-time PCR and ELISA, correspondingly.

Results: In the DM2 rats, TAC and thiol group diminished (p<0.05 and p<0.001, respectively), whereas TOS and LPO increased (p<0.01 and p<0.001, correspondingly) compared to the control rats. In the DM2+AGE rats TAC and thiol group increased (p<0.01 and p<0.05, correspondingly), whereas TOS and LPO diminished (p<0.05 and p<0.001, correspondingly) compared with DM2 rats. The gene expression and protein concentrations of TGF-β2 and IL-1β increased in the DM2 rats compared to the healthy group, whereas these parameters decreased in the DM2+AGE rats compared to the untreated rats with diabetes (p<0.001).

Conclusion: The findings revealed the antioxidant and anti-inflammatory results of garlic extract. Thus, garlic extract could be beneficial for lessening diabetes-induced retinopathy.

Keywords: Diabetes mellitus, Garlic extract, Inflammation, Oxidative stress, Retina

Introduction

Diabetes mellitus as a very familiar endocrine chaos is identified by hyperglycemia (1). The most common problems in two types of diabetes are nephropathy, neuropathy and retinopathy (2).

Diabetic retinopathy is a major concern for blindness all over the world. It is predicted that 191 million people will be identified with diabetic retinopathy until the year 2030 (3). The main cause of retinopathy is chronic hyperglycemia that causes neurodegeneration, endothelial dysfunction and retinal damage. In this pathway mitochondrial dysfunction causes the increased reactive oxygen species (ROS), oxidative stress, and inflammation. Oxidative stress through nuclear factor kappa B (NF-κB) enhances the expression of inflammatory cytokines involving vascular endothelial growth factor (VEGF), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemo attractant protein-1 (MCP-1), transforming growth factor beta-2 (TGF-β2) and interleukin-1 beta (IL-1β). These elements increase in the retina of cases with diabetes mellitus and animal models of diabetes. TGF-β probably has an important function in this pathway (4), because it is strictly promoted in fibrosis, remodeling of the extracellular matrix, and angiogenesis (5). Since oxidative stress and inflammation have a key function in expansion of diabetes mellitus, any factor that can reduce oxidative stress and inflammation can be a cure for the diabetic complications (6).

Herbal medicine is widely used due to the reasonable price, accessibility and minimal complications **(7)**. Medical, *in vitro* and *in vivo* studies have suggested that the medicinal plants and their extracts have meaningful properties in the management of diabetic retinopathy **(8)**. Garlic (*Allium sativum*) is an herb that is used in tradetional treatment. Garlic has various properties including antioxidant, hypoglycemic, anti-bacterial, lipid lowering and anti-plaque formation effects **(9)**, but its effect on the retinal tissues in diabetes disorder has not been investigated. So in the present research, the results of water -soluble garlic extract treatment on oxidative stress and inflammation in retina of rats with diabetes were examined.

Materials and Methods

Production of aqueous garlic extracts (AGE)

Fresh garlic was prepared from public bazaar in Hamadan, Iran. The garlic bulbs were skinned, rinsed and sliced into small pieces. Roughly 50 g was mingled in 250 mL of distilled water, and beaten with a mixing system. The supernatant was passed through Whatman no. 1 filter paper. The obtained aqueous fresh material was consumed or rapidly stored in freezer until use. The rats were given 200 mg of the material/100 g body weight /day by gavage.

In vivo study proposal

The research was conducted with 24 adult male Wistar rats (8- week-old) from Hamadan University of Medical Sciences, Iran. The rats were sheltered in usual coops (equal time of dark/light runs, and fixed heat of 25 ± 2°C were allowed to standard rodent chow and water). The rats were adapted in a minimum of five days to this status before the beginning of the research.

The animals were assigned randomly into four classes with six animals each. Streptozotocin (STZ) (Sigma, USA) and nicotinamide (Sigma, USA) were administered for diabetes mellitus type 2 (DM2) (10).

The control group included healthy rats that were treated with only one dosage of citrate buffer (0.1 M, pH 4.5).

The DM2group included rats that were treated with only one dosage of STZ (65 mg/kg body weight, *i.p*) dissolved in fresh cold citrate buffer (0.1 M, pH 4.5) 15 min following the injection of nicotinamide (110 mg/kg, *i.p.*) for stimulation of DM2. The whole blood glucose was assessed 7 days following the injection of STZ with a strip- functioned blood glucose device (Accuchek; Roche, Germany). A fasting blood sugar level of 250 mg/dL indicated that the animal was diabetic.

The DM2+AGEgroup included rats with diabetes (similar to group 2) that received AGE (200 mg/100 g BW/day, gavages, 5 weeks).

The AGE group lncluded normal rats that were treated with only AGE (200 mg/100 g BW/day, gavages, 5 weeks).

Once the treatment period was over, the rats' eyes were removed, and put rapidly in nitrogen and saved at -80°C for separation of retinal tissues.

For preparation of retinal tissue homogenates lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1% triton x100, 10 mM HEPES, 0.5 mM DTT, protease inhibitor cocktail, pH 7.9) was used. For evaluation of oxidative stress status, total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI), thiol group (SH), and lipid peroxidation (LPO) were assayed. For evaluation of inflammation, mRNA and protein concentrations of TGF-β2 and IL-1β were measured using real-time PCR and ELISA in retinal homogenates. For normalization, total protein level was measured in retinal homogenate samples by Bradford method.

Oxidative stress status assay

The level of TAC in retinal sample homogenates was measured by ferric reducing antioxidant potential (FRAP) analysis in line with Benzie and Strain procedures. This assay was established for the reduction of the ferric tripyridyltriazine (TPTZ) to the ferrous shape with blue dye at lower pH. This reaction was checked through measurement of the absorption variations at 593 nm. A solution of Fe (II) concentration (FeSO4.7H2O) was used as the standard **(11)**.

The evaluation of TOS was established for the oxidation of Fe^{+2} form to Fe^{+3} form in the attendance of oxidant mediators at a low pH media (FOX1 assay). Discernment was established for the development of a dyed compound between the produced Fe^{+3} and xylenol orange that had highest absorption at 560 nm. Hydrogen peroxide was used aimed at the standardization of the analysis **(12)**.

OSI was estimated consistent with the subsequent formula: OSI (Arbitrary unit) = TOS/TAC.

Total serum thiol concentration assay according to Ellman is based on 2, 2-dithiobisnitrobenzoic acid (DTNB) which is reduced with SH groups to produce an acidic anion, 2-nitro-5-mercaptobenzoic. This complex yellow color has maximal absorption at 412 nm **(13)**.

The evaluation of LPO is based on the formation of aldehydes during lipid peroxidation in retinal tissue homogenate through reactive thiobarbituric acid (TBA). The fluorescence power of the result is measured at 515 nm excitation and 553 nm emission wavelength. The used standard was tetraethoxy propane **(14)**.

Real time PCR protocol for determination of TGF-β2 and IL-1β gene expression

The whole RNA of the retinal tissues was obtained through RNX-Plus chemical (CinnaGen, Iran), consistent with the producer's protocol and was measured by spectrophotometry. The integrity of the extracted total RNA was investigated by agarose gel electrophoresis. The entire RNA was reverse-transcribed into one strand cDNA by RevertAid™ First Strand cDNA synthesis kit (Thermo Scientific, Lithuania). Quantitative Real-time

PCR was completed for cDNA tests by the SYBR Premix ExTaq real-time PCR kit (Takara Bio Inc, Japan), as mentioned by the producer's procedures on a Light-Cycler® 96 System (Roche Life Science, Deutschland GmbH Sandhofer, Germany). β-actin was considered as the standard. The sequences of the β-actin, TGF-β2, and IL-1β primers used in the qRT-PCR method are listed in table 1. The $2^{-\Delta\Delta ct}$ formula is used to calculate the comparative gene expression in the studied animals **(15)**.

Table 1. The orders of the primers in the qRT-PCR results

Gene	Forward	Reverse	Product size (bp)
β-actin	ATCAGCAAGCAGGAGTACGAT	AAAGGGTGTAAAACGCAGCTC	94
IL-1β	GTGACTCGTGGGATGATGAC	TCGTTGCTTGTCTCTCCTTG	199
TGF-β2	AAGCGACGAGGAGTACTACG	ATGGCATCAAGGTACCCACA	159

ELISA method for determination of TGF-β2 and IL-1β proteins level

The level of TGF-β2 and IL-1β in retinal tissue homogenates was calculated with rat ELISA kits (R&D, IBL International, respectively) as stated by the manufacturer's instruction.

Statistical Analysis

Data evaluation was made by SPSS software version 16 (SPSS, Chicago, IL, USA). Differences in the variation between the four groups were investigated by Analysis of Variance (ANOVA) and then Tukey`s test. All data were expressed as mean ± SD. Possibility (p) value < 0.05 was considered meaningful in statistical studies.

Results

Findings of retinal tissues oxidative stress parameters

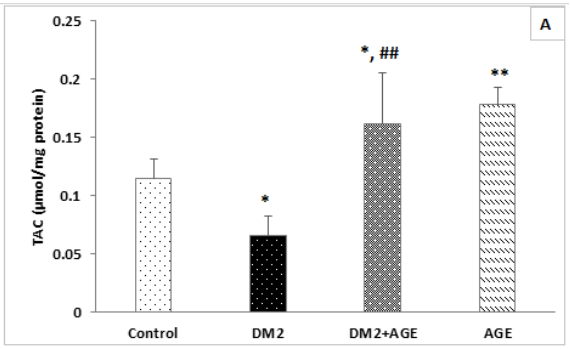
In rats with diabetes, TAC level was reduced drastically compared to the control (p<0.05) and improved significantly in rats with diabetes that were given garlic compared to untreated rats with diabetes and control groups (p<0.01 and p<0.05 correspondingly). In healthy animals fed on garlic extract orally, retinal TAC increased meaningfully compared to control animals (p<0.01) (Fig.1A).

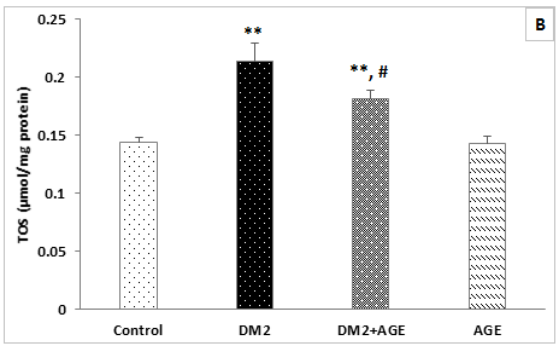
In rats with diabetes, TOS level increased compared to control animals (p<0.01). The level of this parameter decreased meaningfully in the DM2+AGE group compared to untreated rats with diabetes (p<0.05), and it was considerably higher than the control animals (p<0.01). There were not any meaningful differences between the AGE and control animals (p>0.05) (Fig.1B).

The data of oxidative stress index is shown in Fig.1C. In rats with diabetes, OSI ratio increased drastically compared to the control (p<0.001) and reduced meaningfully in rats with diabetes that were fed on garlic extract orally compared to control animals (p<0.001), so this rate reached the normal level and was not different from control animals (p> 0.05) (Fig.1C).

In rats with diabetes, thiol group level declined compared to control subjects (p<0.001). The concentration of this matter increased outstandingly in the DM2+AGE group compared to untreated diabetic rats (p<0.05). The amount of this substance reached the normal level and there was no difference compared to control animals (p> 0.05). In healthy rats that received garlic extract, thiol group level was equal to control animals (p> 0.05) (Fig.1D).

The data of lipid peroxidation is shown in Fig.1E. In rats with diabetes, LPO level increased meaningfully compared to the control (p<0.001) and reduced significantly in rats with diabetes that were given garlic extract orally compared to animals with diabetes (p<0.001), so that it reached the normal level and was equal to the control group (p> 0.05). In healthy rats that received garlic extract, retinal LPO declined drastically compared to control class (p<0.05) (Fig.1E).





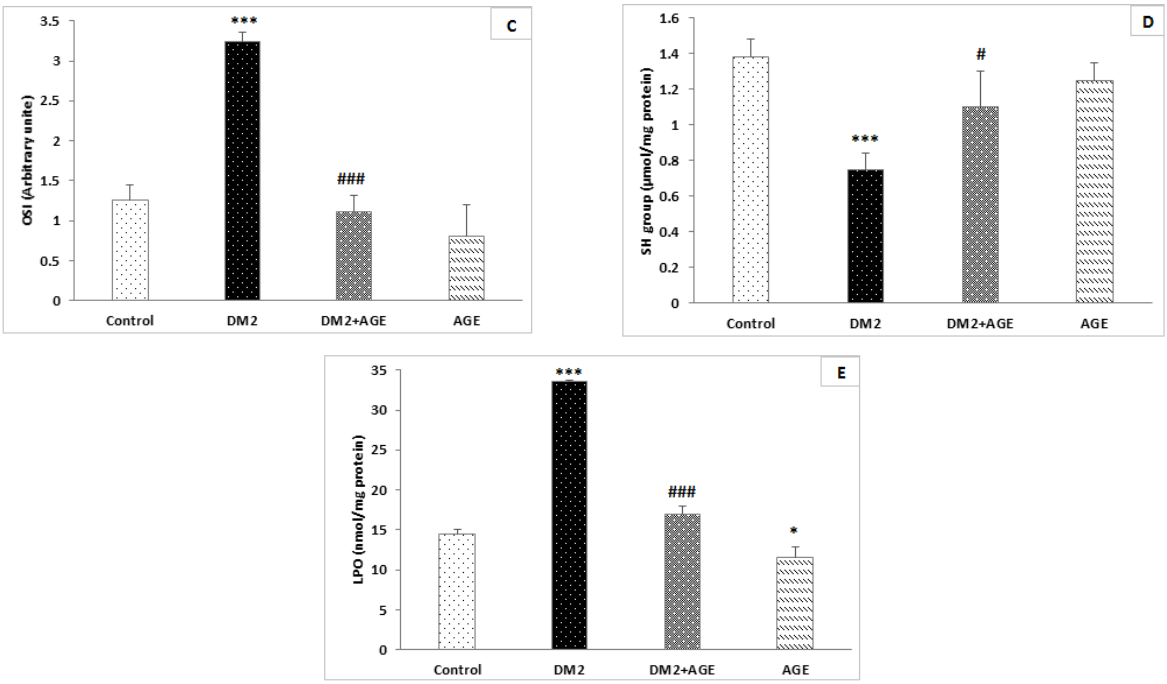


Figure 1. The findings of oxidative stress parameters affected by garlic extract

A: total antioxidant capacity (TAC), B: total oxidative status (TOS), C: oxidative stress index (OSI), D: total thiol (SH) group and E: lipid peroxidation (LPO).

Control: normal rats, DM2: diabetic rats, DM2+AGE: garlic- treated diabetic rats, AGE: normal rats that received garlic extract.

Results are mean ± SD (n=6). $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to Control. $^{\#}p<0.05$, $^{\#\#}p<0.01$and $^{\#\#\#}p<0.001$ compared to DM2.

Findings of retinal tissues mRNA expression and protein level of TGF-β2

The mRNA expression of TGF-β2 in the retinal tissues increased in rats with diabetes compared to control animals ($p<0.001$). This parameter diminished in rats with diabetes fed on garlic extract orally compared to untreated diabetic animals ($p<0.05$). In healthy rats fed on garlic extract, mRNA expression of TGF-β2was similar to control animals ($p> 0.05$) (Fig.2A).

The protein level of TGF-β2 in retinal tissues increased in rats with diabetes compared to control animals ($p<0.001$), while it declined in DM2+AGE animals compared to untreated diabetic rats ($p<0.001$), so that the amount of this substance reached the normal level and was not different from control subjects ($p>0.05$). In healthy rats that received garlic extract, protein expression of TGF-β2 was similar to control rats ($p> 0.05$) (Fig.2B).

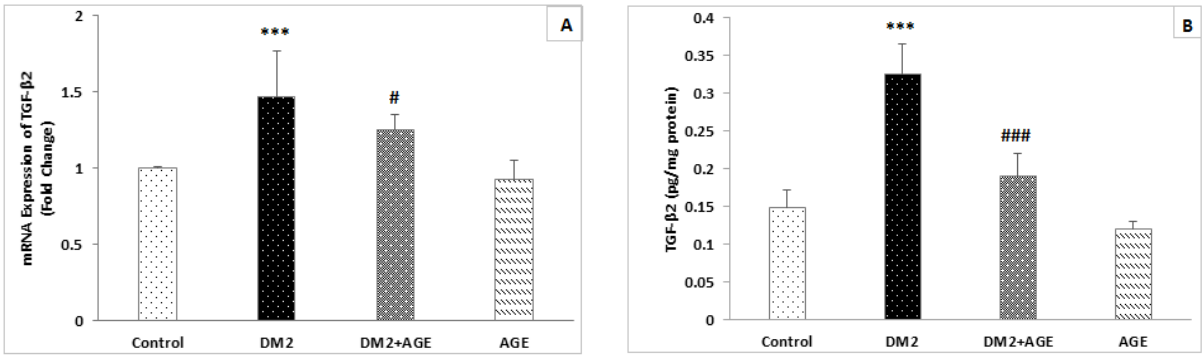


Figure 2. The findings of gene expression (A) and protein level (B) of transforming growth factor beta-2 (TGF-β2) in retinal tissues of rats affected by garlic extract.

Control: normal rats, DM2: diabetic rats, DM2+AGE: garlic treated diabetic rats, AGE: normal rats that received garlic extract.

Results are mean ± SD (n=6). $^{***}p<0.001$ compared to Control. $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$compared to DM2.

Findings of retinal mRNA expression and protein level of IL-1β

The mRNA expression of IL-1β decreased in rats with diabetes compared to control animals (p<0.001). This parameter was reduced in rats with diabetes that received garlic extract orally compared to untreated diabetic animals (p<0.001), while it was still higher than control animals (p<0.01). In healthy rats that were fed on garlic extract, mRNA expression of TGF-β2 was similar to control rats (p>0.05) (Fig.3A).

The level of retinal tissues IL-1β protein in animals with diabetes was high compared to control animals (p<0.001), although it was reduced in the DM2+AGE group compared to untreated diabetic rats (p<0.001), so that it reached the normal level and was not different from control animals (p>0.05). In healthy rats that were given garlic extract orally, mRNA expression of IL-1β was similar to control rats (p>0.05) (Fig.3B).

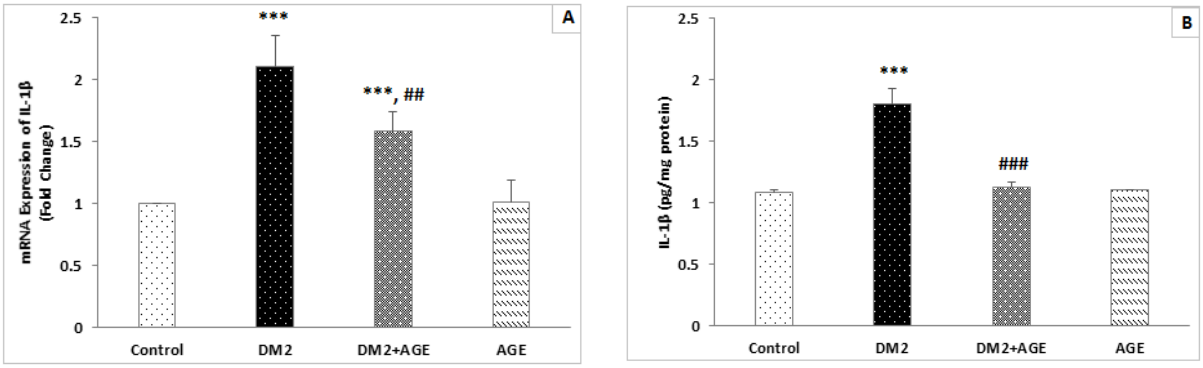


Figure 3. The findings of gene expression (A) and protein level (B) of interleukin-1 beta (IL-1β) in retinal tissues of rats affected by garlic extract.

Control: normal rats, DM2: diabetic rats, DM2+AGE: garlic- treated diabetic rats, AGE: normal rats t treated with garlic extract.

Results are mean ± SD (n=6). $^{***}p$<0.001 compared to Control. $^{\#\#}p$<0.01and $^{\#\#\#}p$<0.001compared to DM2.

Discussion

Diabetes mellitus is a common prolonged endocrine condition. Some current reports have revealed that diabetes is linked to microvascular complications. Although retinopathy resulted from diabetes is a common complication of diabetes, it might occur without symptoms or any sight disorders in the initial phases. However, as the illness develops, the eyesight will be affected. The main reason for diabetic retinopathy is chronic hyperglycemia. The increase in the blood sugar through the development of oxidative stress can cause diabetes complications, such as retinopathy (6). Malondialdehyde (MDA) as a lipid peroxidation indicator, was elevated in the plasma and vitreous of diabetic retinopathy groups (16). In retinopathy-induced diabetes, except for the blood level, there are lower concentrations of TAC at the vitreous part and aqueous humor, (17), and meaningfully lower and higher serum total thiol levels and TOS levels respectively, compared to control groups (18).

In this study, we used animal model of diabetes. Streptozotocin and nicotinamide induced mild form of the disease, similar to type 2 diabetes in rats (10). Oxidative stress factors were examined in the retinal tissues of diabetic subjects. The findings showed that there was an increase in the concentration of total oxidative status and lipid peroxidation and a decrease in total antioxidant capacity, in the retinal thiol group rats with diabetes in comparison to control rats. Overall, with increase in oxidative stress index, induction of oxidative stress in retinal tissues was confirmed.

The pathogenesis of hyperglycemia in retinopathy-induced diabetes is associated with principal biochemical modifications: increased (i) polyol pathway flux, (ii) advanced glycation end-product formation, (iii) activation of protein kinase C (PKC) isoforms, and (iv) hexosamine pathway flux (19). Since one of the important causes of diabetic retinopathy is oxidative stress, any compound that has antioxidant effect may decrease oxidative stress and subsequently reduce retinopathy. Thus, in this report, aqueous extract of garlic was selected to treat rats with diabetes. The results of present research indicated the reduced level of total oxidative status and lipid peroxidation and an increase in whole antioxidant capacity and thiol group in retinal tissues of rats with diabetes that orally received garlic extract in comparison to untreated rats with diabetes. Overall, with decrease in oxidative stress index, reduction of oxidative stress in retinal tissues was confirmed. Garlic consumption alone can reduce oxidative stress via increasing TAC and decreasing lipid peroxidation in garlic- treated normal rats. Antioxidant capacity of garlic has been shown in previous studies. Anwar et al., reported that in rats with diabetes that were given garlic extract orally plasma concentrations of total thiol increased. In lysate of erythrocytes, lipid peroxides and total thiol are meaningfully reduced and increased, respectively.

Their findings suggest that garlic oil could efficiently stabilize the reduced antioxidants status in STZ–stimulated diabetes **(20)**. Al-brakati et al., showed protecting properties of garlic on diabetic retinopathy in STZ-stimulated diabetes in rats. Their results showed morphological changes in inner and outer nuclear layers in retinal histopathological observation and improvement of these changes after administration of garlic **(21)**. It seems that garlic bioactives like S-Allylcysteine, allicin, saponins, and ajoene, can attenuate oxidative stress via ROS trapping and free radical scavenging **(22)**.It has been shown that in diabetic animals or patients, the activity of multiple cell pathways, usually associated with hyperglycemia, may be a major cause of the pathogenesis of retinopathy.

Increase in the blood sugar, through the increase of oxidative stress and inflammation, along with a rise in inflammatory cytokines, may cause diabetes complications, such as retinopathy, which is one of the significant sources of blindness in the universe. Oxidative Stress by activating the NF-κB factor, increases the expression of inflammatory cytokines such as connective tissue growth factor (CTGF), tumor necrosis factor alpha (TNFα), VEGF, TGF-β2 and IL-1β **(6, 23)**.

Fibrotic scarring may compromise vision and ultimately lead to blindness in diabetic retinopathy. In diabetes, each part of the eye is susceptible to the fibrosis that is characterized by the appearance of myofibroblasts. The process of generation of myofibroblasts from either fibroblast of epithelial cell types is mediated by the growth factors including TGF-β2, one of the most potent factors involved in tissue fibrosis **(24)**. Kowluru et al., showed that antioxidant ability and the quantities of intracellular antioxidant, reduced glutathione (GSH) concentrations and oxidetively- modified DNA (8-OHdG), and nitrotyrosine remained elevated in the diabetic rats' retina. The quantities of IL-1β and VEGF were elevated, and NF-κB was activated **(25)**.

The findings of current research presented higher mRNA expression and protein level of TGF-β2 and IL-1β in the retinal tissues of animals with diabetes than control rats. These results indicated inflammation in the retinal tissue of STZ+Nicotinamide-stimulated diabetes in rats.

The anti-cytokine treatments seem to be of temporary value in retinopathy. This makes the evaluation of the further molecular ways a possible goal in diabetic retinopathy, and initial findings suggest 'retinal inflammation' as a vital function in the etiology of retinopathy due to diabetes. So blocking the signal activated by cytokines through chemical or natural inhibitors is one of the powerful tools to prevent or treat the diseases **(19)**.

Curcumin administration can prevent diabetes-induced oxidative stress. Curcumin also repressed diabetes-stimulated rise in the quantities of IL-1β, VEGF and NF-kB **(25)**. Lycopuslucidus Turcz (LT) has therapeutical advantage through ameliorating inflammation and angiogenesis of diabetic retinopathy via decreasing the IL-6, IL-1β, VEGF, and TNF-α **(26)**. In another study the effects of grape seed proanthocyanidin extract (GSPE) in cases with non-proliferative retinopathy (NPDR) from diabetes were evaluated. Oral GSPE therapy for one year corrected retinal thickening by hard exudates (HEs) in patients with NPDR **(27)**. Gupta et al., studied the protecting results of Trigonellafoenum-graecum Linn (Fenugreek) in the retinal tissues of STZ-stimulated diabetes in rats. Critically increased expressions of TNF-α, and IL-1β, VEGF, and PKC-β as indicators were detected in retina of rats with diabetes compared to normal retina **(28)**.

Although there are studies on the impacts of plant extracts on oxidative stress and inflammation in the retina of animals with diabetes, there are not any reports on the effects of garlic on the rats' retina with diabetes, whereas the effects of garlic on other tissues and serum of diabetic patients or animals have been studied. Garlic, as expected, supports an anti-inflammatory situation through cytokine alteration in mans' blood that results in the whole blockage of NF-κB activity in the adjacent tissues **(29)**.

Conclusion

The outcomes of present report revealed that aqueous garlic extract decreased TOS, LPO and OSI and raised TAC and thiol group in the retinal tissues of STZ+nicotinamide- induced diabetes in rats compared to untreated diabetic rats. In other words, this extract can reduce oxidative stress and has anti-oxidant property. Also, we showed that oral usage of the garlic extract decreased mRNA expression and protein level of TGF-β2 and IL-1β in the retinal tissues of animals with diabetes type 2 compared to untreated rats with diabetes. These results showed anti-inflammatory effect of garlic. So TGF-β2 and IL-1β can be as a target for anti-inflammatory drugs that are used for diabetic retinopathy.

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Conflict of Interest

The writers announce that there is no competing interest.

Ethical Statement

All procedures of the research were adapted to the ethical standards of the Institutional Animal Ethics

Committee of Hamadan University of Medical Sciences (Approval No.IR.UMSHA.REC.1395.78).

Limitations of the Study

It would have been better if we had measured the other cytokines, which was not possible. Because the size of retinal tissue was very small and we did not have enough tissue for the assay of other cytokines.

Funding Sources

None.

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