Garlic ameliorates long-term pre-diabetes induced retinal abnormalities in high fructose fed rat model

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Retinopathy is one of the micro vascular complications of diabetes and can also be observed in pre-diabetic state. However, there are only limited studies available on the pathophysiology of retinopathy in pre-diabetic state and its preventive strategies. In this study, we investigated the retinal functional, structural and molecular alterations using high fructose (HF) induced pre-diabetic rat model and also the protective role of garlic. Feeding of HF to Wistar NIN (WNIN) rats had developed insulin resistance (IR) and impaired glucose tolerance (IGT) by three months, while retinal functional abnormalities by ten months as evidenced by decrease of Electroretinogram (ERG) scotopic, photopic b-wave amplitudes, oscillatory potentials (OPs) when compared to controls. Supplementation of garlic (3%) to HF+G group rats had marginally protected these changes. Elevated expression of glial fibrillary acidic protein (GFAP), vascular endothelial growth factor (VEGF), aldose reductase (AR) and decreased rhodopsin (Rho) in HF group rats as evidenced by immunohistochemistry, immunoblot methods, which were further supported by gene expression studies, indicate the initiation of retinal abnormalities. Increased immune-fluorescence signal of carboxymethyl lysine (CML) and 4-hydroxynonenal (4-HNE) in retina of HF group rats indicate the association of glycation and oxidative stress, respectively. Early intervention of garlic to HF+G group rats attenuated retinal functional, structural, and molecular abnormalities.

Keywords: *Allium sativum*, Glycation, Impaired glucose tolerance, Insulin resistance, Oxidative stress, Polyol pathway, Retinopathy

Pre-diabetes is an early stage of diabetes associated with impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or both. IGT and IFG are generally associated with insulin resistance (IR), obesity and metabolic syndrome. The prevalence of pre-diabetes in Indian adults varies from 5.7 to 14.7%1,2 which increases the burden of type-2 diabetes (T2D) and subsequent short and long-term complications. Diabetic retinopathy (DR) is one of the important long-term micro vascular complications of diabetes, also occurs in pre-diabetic individuals3,4, which is one of the leading causes of blindness globally. Some of the epidemiological and clinical studies had reported the prevalence of DR in IGT/IFG or pre-diabetic subjects to be 2.5 to 20.91%3,5,6. However, pathophysiology and early molecular events associated with pre-diabetes induced retinal abnormalities in these subjects are not clear.

Though, the development of retinopathy was not clear in pre-diabetic state, earlier studies with experimental animals7,8 and humans9 indicates that retinal functional abnormalities develop at an early stage of diabetes/diabetic retinopathy. Apart from functional abnormalities, up regulation of GFAP is considered as an early feature of diabetic retinopathy10 and a marker for retinal damage11. In addition, upregulation of GFAP was also observed among humans with non-proliferative retinopathy12 and in photoreceptor degeneration of mutant rat model13. Vascular endothelial growth factor (VEGF) is an angiogenic molecule known to be involved in the pathogenesis of diabetic retinopathy14 and its elevated expression was also reported in non-fasting hyperglycemic7 as well as in obesity associated retinal degeneration in WNIN-Ob rat model15. Although, enhanced AR activity and glycation are known to associate with diabetic retinopathy15, involvement of these changes in pre-diabetes induced retinal abnormalities is not clear.
There are several diet induced animal models available for understanding pathophysiology of pre-diabetes induced complications including retinopathy. High fructose (HF) induced pre-diabetic model is one among them and also used as metabolic syndrome model. This model was earlier used to study retinal abnormalities/retinopathy with different experimental durations of 3-8 days, 4 months and 1-6 months. However, preventive strategies on pre-diabetes induced retinal abnormalities are not attempted earlier. Hence, halting or slowing down the progressive insulin resistance associated with dysglycemia is the key stage not only to delay or prevent the conversion of pre-diabetes to clinical T2D, but also its complications.

There are several natural dietary agents including spices which have therapeutic potentials. Garlic (Allium sativum L.) is one of the spices used all over the world which has hypoglycemic, antioxidant, insulin sensitizing, antiglycating and very low in vitro to significant in vivo AR inhibitory properties. However, beneficiary outcome of garlic on retina in pre-diabetic state has not been studied. Hence, in the current investigation we have explored the protective role of dietary garlic on long term pre-diabetes induced retinal abnormalities using HF induced pre-diabetes in Wistar NIN (WNIN) rats.

Materials and Methods

Materials

Fructose was purchased from SRL Company, whereas cellulose, vitamin and mineral mixture were obtained from MP Biomedicals. Other dietary ingredients were obtained from the National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition-Hyderabad. VEGF (PA547021), GFAP (PA3-16727), Rhodopsin (MA1-722), Beta Actin (MA 1-91399) primary antibodies and HRP conjugated anti-goat (A24452) secondary antibodies were obtained from Thermo Scientific, USA; 4-HNE primary antibody (Ab46545) was purchased from Abcam, USA; TRI-reagent, HRP conjugated anti-mouse (A9044) and anti-rabbit (A6154) secondary antibodies were procured from Sigma Chemicals, St. Louis, MO, USA; Alexafluor-488 conjugated anti-rabbit, Alexafluor-555 conjugated anti-mouse and AlexaFluor-594 conjugated anti-goat antibodies were obtained from Molecular Probes (Eugene, OR, USA); DAPI antifade mounting medium was obtained from Vectashield, USA; SYBR Green Master Mix was obtained from Applied Biosystems, UK and other regular chemicals were AR grade and procured from local companies.

Experimental design

Male WNIN inbred rats (6-8 weeks old) with an average body weight of 195±21 g received from the National Center for Laboratory Animal Science (NCLAS) were kept on AIN-93 diet (control group; n=9) or AIN-93 diet with 56% fructose (HF group, n=9); or AIN-93 diet with 56% fructose and 3% freeze-dried garlic powder (HF+G group n=9). Animals were maintained with their respective diets and water ad libitum for a period of ten months in individual cages with a 12 h light-dark cycle. This study was approved by the Institutional Animal Ethics Committee (P29/IAEC/NIN/2012/7/PS/71) and followed the guidelines of ARVO for use of animals in ophthalmic and vision research.

Oral glucose tolerance test (OGTT) and homeostasis model assessment (HOMA) for insulin resistance (IR)

OGTT was performed at three and ten months on overnight fasted rats by administering the glucose solution, at a dose of 2.0 g/kg body wt. Blood samples were collected at 0 min, (before OGTT), 30, 60 and 120 min after OGTT. Plasma glucose and insulin levels were estimated by the glucose oxidase-peroxidase kit (Ozone Biomedicals Pvt. Ltd., New Delhi, India) and RIA kit (BRIT-DAE, Mumbai, India) methods respectively. Area under curve (AUC) for glucose and insulin was calculated. IGT and HOMA-IR was also calculated as reported earlier.

Electroretinography (ERG)

ERG is a non-invasive method, commonly used for studying visual functions in humans and experimental animal models. Alteration in the retinal function was assessed by ERG at the end of the experiment as described earlier31. Animals were anesthetized under dim red light illumination after overnight dark-adaptation and pupils were dilated with atropine eye drops. ERG-positive electrode was placed on the cornea of the eye, reference electrode on the ear and a ground electrode on a tail. Standard ERG was performed by a UTAS Visual Diagnostic System. Scotopic and photopic responses were recorded using a series of flash stimuli of −3.0 to 1.19 log cd·s/m2 using dim white LED with Ganzfeld (BigShot) (LKC Technologies; Gaithersburg, MD, USA). Recordings were amplified with UBA-4204 amplifier and analyzed with EM for windows software. We used the same
photopic and scotopic amplitudes recorded between 0.19 to 1.19 log cd-s/m² for calculation of Oscillatory potentials (OPs). Mean ERG values of each eye were calculated, and this resultant value was used to compute the group means of a- and b-wave amplitudes as well as for OPs.

Histology and Immunohistochemistry

At the end of the experiment animals were sacrificed by CO₂ asphyxiation and eye balls were collected proceeded for embedding and sectioning using standard protocols for histology and immunohistochemistry, as described earlier13,32. A small cut was made on ora serrata and kept in 4% paraformaldehyde in phosphate-buffer (pH 7.2) for 24 h. The eyeballs were embedded in paraffin blocks and cut into the sections of 5 µm thickness followed by their mounting on slides. These sections were then used for staining with Hematoxylin & Eosin (H&E) and were observed under light microscope.

Eye ball sections used for IHC were deparaffinized followed by antigen retrieval and blocking. Further, these sections were incubated with polyclonal primary antibodies (VEGF, GFAP, rhodopsin, AR, CML-KLH or 4-HNE) followed by incubation with secondary antibody conjugated with alexafluor 488 (green)/594 (red). It was then mounted with DAPI antifade mounting medium (Vectashield). These sections were examined for specific staining for above molecules using a Leica laser microscope (LMD6000, Leica microsystems, Germany). Images were represented in the form of merge with that of counterstain DAPI.

SDS-PAGE and Immunoblot (IB)

Retinas were dissected from a set of eye balls, snap frozen immediately. qRT-PCR was performed as described earlier32. Total RNA was extracted from whole frozen retina by TRI reagent. The isolated RNA was purified using the RNeasy mini kit (Qiagen). The concentration of total RNA was quantified by Nanodrop spectrophotometer (ND1000) and the integrity was checked by running the RNA samples on denaturing MOPS-Formaldehyde gel. Known quantity (5 µg) of RNA was immediately subjected to reverse transcriptase using cDNA Synthesis Kit (bioline). qRT-PCR was performed using 25 ng of cDNA template and SYBR green master mix with the gene specific primers (Table 1) using real-time PCR (ABI 7500). The specificity of products generated for each set of primers was examined for each fragment with the use of a melting curve analysis at the end of the run. The relative expression levels of each targeted gene were normalized by subtracting the corresponding beta-actin threshold cycle (CT) values by using the ∆∆CT values comparative method. A total of three samples for each group were used, and each sample was run in triplicate for real-time PCR.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 and quantative data was presented as mean ± standard deviation (SD). One-way ANOVA, followed by Tukey HSD test was used to analyze the differences among groups. Significant level was set at $P < 0.05$.

Results

General characteristics

There was a significant ($P < 0.05$) increase in mean food intake (g/rat/day) in HF group animals when
compared to controls (Table 2). Similarly, there was a significant ($P < 0.05$) increase in mean body weights of HF group rats when compared to controls by the end of the experiment (Table 2). This increased body weights in HF group animals could be due to increased adiposity. However, feeding of garlic to HF+G group animals had no effect on food intake and body weights (Table 2).

**Fructose induced pre-diabetes**

Feeding of HF to WNIN rats for a period of three months developed IGT as evidenced by high levels of two hours glucose (Fig. 1A) and a significant ($P < 0.05$) increase in AUC for glucose (Table 2). These HF group animals also developed IR as there was a significant ($P < 0.01$) increase in HOMA-IR index and a significant ($P < 0.05$) increase in AUC for insulin (Table 2). Feeding of garlic to HF+G group rats had inhibited development of IGT and IR as there was a significant ($P < 0.01$) decrease in two hours glucose levels (Fig. 1A) and HOMA-IR index, respectively. Further, end point OGTT results of the experiment indicate that HF group animals maintained pre-diabetic state till the end of the experiment as the HOMA-IR index and AUC for insulin was higher when compared to controls (Table 2). Feeding of garlic to HF+G group animals by the end of the experiment had shown low HOMA-IR index and a significant ($P < 0.05$) reduction in AUC for insulin (Table 2) when compared to HF group rats (Table 2). However, these animals did not develop fasting hyperglycemia.

**Pre-diabetes induced retinal functional abnormalities**

ERG is a non-invasive method, commonly used for studying visual functions in humans and animal models. In the present study, ERG data indicate that HF group animals developed retinal functional abnormalities by the end of the experimental period ten months as there was a marked decrease in scotopic, photopic b-wave responses (Fig. 2 A & B) and OPs (Fig. 2 C & D) when compared to controls. Feeding of garlic to HF+G group animals had marginally prevented retinal functional abnormalities especially loss of scotopic, photopic b-wave responses when compared to HF group animals (Fig. 2 A-D). However, there was no difference in scotopic and photopic a-wave amplitudes between the groups (data not shown).

**Table 2 — General characteristics of control, HF and HF+G group rats**

<table>
<thead>
<tr>
<th>Parameter/group</th>
<th>Control</th>
<th>HF</th>
<th>HF+G</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-IR</td>
<td>0.89±0.1266</td>
<td>2.08±0.5469*</td>
<td>1.32±0.2171*</td>
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<tr>
<td>Glucose AUC (mmol/h)</td>
<td>16.06±2.285</td>
<td>18.44±3.179</td>
<td>16.52±2.204</td>
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<tr>
<td>Insulin AUC (μmol/h)</td>
<td>127.08±23.466</td>
<td>174.21±20.451*</td>
<td>132.88±15.290*</td>
</tr>
<tr>
<td>Body weight (g/rat)</td>
<td>496±44.62*</td>
<td>16.09±1.13</td>
<td>112.75±18.09</td>
</tr>
<tr>
<td>Food intake (g/day/rat)</td>
<td>14.98±1.60*</td>
<td>16.25±3.03</td>
<td>104.83±18.99</td>
</tr>
<tr>
<td>Insulin AUC (μmol/h)</td>
<td>154.83±26.22*</td>
<td>112.75±16.09</td>
<td>152.83±26.22*</td>
</tr>
</tbody>
</table>

[Values are expressed as mean ± SD. *significantly different from control group; significantly different from HF group; significantly different from control group]

![Fig. 1 — Glucose and insulin response during OGTT. OGTT was conducted in control, HF and HF+G group rats at third and tenth month after feeding of their respective diets. (A) Glucose; and (B) insulin levels were estimated and presented as third month; and (C & D) tenth month. [Values are mean ± SD, n=9; *P < 0.05 vs. control, **P < 0.05, vs. HF group](](image1)

![Fig. 2 — Scotopic and photopic responses of b-waves and Ops. ERG was recorded after ten months in control, HF and HF+G group rats. (A) ERG responses presented as Scotopic b-wave (light intensity from -3.0 to 1.19 cd-s/m²); (B) Photopic b-wave (light intensity from -0.6 to 1.19 cd-s/m²); (C) Scotopic; and (D) Photopic OPs (light intensity from 0.19 to 1.19 cd-s/m²)](image2)
Pre-diabetes altered retinal structure

There was a reduction in retinal thickness in HF group animals as compared to controls indicating the partial damage of the retina. Garlic feeding to HF+G group animals has partially prevented this structural alteration when compared to HF group animals (Fig. 3A).

Pre-diabetes altered protein expression in retina assessed by IHC

Expression of key molecular markers such as VEGF, GFAP, rhodopsin, AR, CML-KLH and 4-HNE associated with retinopathy were analyzed in the retinas of all the three groups. We had used the IHC method to study site specific expression of these molecules. There was a marked increase in intensity of immunofluorescence of VEGF in the ganglion cell layer (GCL) to inner nuclear layer (INL), GFAP in the INL and decrease of rhodospin in the photoreceptor layer of HF group rat retinas when compared to controls. This indicates initiation of angiogenesis, glial activation and retinal degeneration, respectively. In addition, there was an increased immunofluorescence signal for CML-KLH and 4-HNE in the outer plexiform layer (OPL) and ganglion cell layers (GCL) of HF group rat retinas indicate the association of glycation and oxidative stress in this group. Interestingly, feeding of garlic to HF+G group animals had prevented these alterations when compared to HF group rat retinas. However, there was no difference in AR immunoreactivity among these groups (Fig. 3B).

Pre-diabetes altered protein expression in retina assessed by IB

Further, expressions of the above molecules at protein levels were analyzed by IB in the retina of all the three groups. There was a marginal increase in GFAP with a significant \( P < 0.05 \) increase in CML-KLH, VEGF, AR, 4-HNE and also a significant \( P < 0.05 \) decrease in rhodopsin expression was observed in HF group rat retinas over to controls. Feeding of garlic to HF+G group animals had shown marginally reduced expression of VEGF, GFAP, 4-HNE and significantly \( P < 0.05 \) reduced expression of CML-KLH, AR and also marginally improved the rhodopsin expression compared to HF fed group animals (Fig. 4 A & B). Majority of these observations were well correlated with above IHC results.

![Fig. 3](image-url) — (A) Morphology of retina; and (B) Expression of key molecules in retinas of control, HF and HF+G group rats by IHC. [Morphology of retina is shown by H&E stained retinas of control, HF and HF+G groups (Panel A). White arrow indicates the changes in the retinal structure. Expression of VEGF, GFAP, rhodopsin, AR, CML-KLH and 4-HNE in control, HF and HF+G fed retinas by IHC. White arrow indicates respective protein expression in different retinal layers of all the three groups (Panel B)].

![Fig. 4](image-url) — (A) Protein expression by the Western blot; (B) Density by densitometry; and (C) Gene expressions by qRT-PCR. [Expression of VEGF, GFAP, Rhodopsin, AR, CML-KLH and 4-HNE in control, HF and HF+G group rat retinas by western blot (Panel 4A) and densitometry (Panel 4B). Fold change was calculated by normalizing these proteins with beta actin. Expression of mRNA encoding VEGF, GFAP, Rhodopsin and AR levels by qRT-PCR in control, HF and HF+G group rat retinas. Gene expression results for above genes were normalized with beta actin (Panel 4C). Results are mean ± SD for three independent mRNA preparations for each group. \( ^{a}P<0.05; \) vs. control; \( ^{b}P<0.05, \) vs. HF]
Pre-diabetes altered gene expression in retina assessed by qRT-PCR
To support the above protein expression pattern, we further analyzed some of the genes at mRNA levels by qRT-PCR. There was a significant ($P < 0.01$) increase in mRNA expression of VEGF, GFAP, AR and a significant ($P < 0.01$) decrease in the expression of rhodopsin in HF group rat retinas compared to controls. This indicates the development of retinal abnormalities (Fig. 4), which further supports IHC and WB data. Feeding of garlic to HF+G group animals had significantly ($P < 0.01$) prevented altered expressions of VEGF, GFAP, AR and rhodopsin (Fig. 4C).

Discussion
In this present study, we demonstrated a protective role of garlic in long-term pre-diabetes induced retinal abnormalities using HF induced pre-diabetic rat model. Though the earlier study did not find initiation of retinal functional abnormalities in HF fed rats over duration of 16 weeks$^{21}$, the other study demonstrated that, feeding of HF for period of six months resulted in reduction of b-wave amplitude in those animals$^{22}$. Further, a recent study on high fat diet induced mouse models of pre-diabetes had shown a reduction of both a- & b-wave amplitudes under scotopic and photopic conditions by six months$^{17}$.

In the present study, we conducted this experiment for a longer duration (ten months) for notable changes (if any) and also studied protective role of garlic (if any) by early intervention before development of pre-diabetes and retinal abnormalities. Feeding of HF to WNIN rats developed IR and IGT which are common conditions of pre-diabetes. Feeding of garlic to the HF+G group had delayed development of IR and IGT as evidenced by OGTT and HOMA-IR results at third and tenth month. This protective effect could be mainly through its hypoglycemic$^{23}$ and insulin sensitizing properties$^{24,25}$.

The ERG detects early retinal functional abnormalities before detectable visible alterations by ophthalmoscopy. Reduced ERG a- and b-wave amplitudes and OPs can be used as indicators for studying various types of retinal degeneration related to photoreceptor and/or inner retinal cells in animal studies$^{33}$. Muller cells are early effected cells of the retina during development of retinopathy and ERG b-wave response represents these cells function$^{34}$. In the present study, ERG data at the end of the experiment, showed a decrease in scotopic and photopic b-wave amplitudes and OPs in HF group animals when compared to controls. This indicates the initiation of retinal functional abnormalities at pre-diabetic state and it may be due to dysfunction of the inner retinal layers (On bipolar, Muller and amacrine cells)$^{35}$. These ERG observations were correlated with previous studies$^{17,22}$. Interestingly, garlic had shown a partial protective effect by preventing reduction in the scotopic and photopic b-wave amplitudes as well as OPs (Fig. 2).

Since HF group animals developed functional abnormalities, we additionally studied retinal morphology using H&E after termination of the experiment and there was a loss of the inner retinal layers in HF group rat retinas as compared to controls that correlates with ERG data. Nevertheless, loss of the inner retinal layers marginally prevented by the garlic in HF+G group rat retinas when compared to HF group rat retinas.

The retinal abnormalities at molecular level are extremely complex due to the involvement of various retinal cell types, pathways and key molecules$^{15}$. GFAP is one of the key molecules and its elevated expression or glial activation was associated with retinal damage$^{17}$ and retinal degeneration$^{13}$. Since in the present study, we observed a decrease in ERG b-wave which mainly comes from Muller and bipolar cells, we studied the expression of GFAP to link the association of glial activation with retinal functional abnormalities. As expected there was an increased expression of GFAP at mRNA and protein levels (IHC) in the INL of the retinas of HF group when compared to controls indicates retinal damage through glial activation. Feeding of garlic to HF+G group rats had prevented retinal damage through inhibiting glial activation as there was a significant ($P < 0.01$) inhibition of GFAP expression when compared to HF fed animals (Fig. 3B & Fig. 4). Similar findings were observed in a recent study were there was a reduction of scotopic and photopic b-wave amplitudes along with upregulation of GFAP in Mullar cells of high fat fed pre-diabetic mouse model$^{17}$.

VEGF is another molecule known to play a key role in diabetic retinopathy$^{35,36}$. Further, elevated expression of VEGF had been reported in HF fed normoglycemic rat retinas with choroidal neovascularization$^{22}$ and also in obesity & IGT associated mutant rat retinas with retinal degeneration$^{37}$. In the present study, we also observed elevated expression of VEGF in retinas of HF group rats indicating VEGF has a role in pre-diabetes induced
retinal abnormalities. These results were well correlated with the earlier studies with mutant rat models in which WNIN/GR-Ob mutant model has pre-diabetic characteristics like IR and IGT. In this study, garlic had prevented elevated expression of VEGF in HF+G group rat retinas probably through its VEGF inhibitory potential. This present observation on VEGF inhibitory property of the garlic is correlated with previous report on diabetic rats.

Rhodopsin is an important rod specific biological pigment molecule present in the photoreceptor cells of the retina may have role in diabetic retinopathy and its decreased expression had been reported in the retinas of experimental and in mutant rat models with retinal degeneration. In the present diet induced pre-diabetic model, we observed a decreased expression of rhodopsin along with elevated GFAP in HF group rat retinas indicates glial activation associated with retinal degeneration. Interestingly, garlic prevented retinal degeneration by preventing diminish rhodopsin levels in HF+G group rat retinas.

In addition to these, earlier studies have demonstrated activation of polyol pathway in prediabetic rat lens and also in metabolic syndrome model of Wistar rats. The other studies reported the presence of advanced glycation end products (AGEs) in non-diabetic rat and human retinas and its level increases in diabetes. It has also been reported that long-term fructose consumption accelerates glycation in rats. In the present prolonged pre-diabetic state, increased expression of AR and CML-KLH was noticed in the HF group rat retinas indicating activation of these pathways may initiate at pre-diabetic state in the HF rat model. Interestingly, a significant decrease in the AR expression (at mRNA and protein levels) and a significant decrease in CML-KLH at protein levels were observed in HF+G group rat retinas. This reflects the protective effect of garlic probably through its antiglycating and AR inhibiting properties. The activation of this enzyme in the high fructose model could be due to feedback mechanism of uric acid.

Further, the increased 4-HNE level in HF group rat retinas compared to controls, indicating the association of oxidative stress at pre-diabetic state (Figs 3 & 4). This increased oxidative stress could be due to a combination of IR state, activation of polyol pathway, glycation and glial activation. Garlic marginally protected pre-diabetes induced oxidative stress in HF+G group retinas due to its antioxidant property and this observation is well correlated with previous studies on fructose induced oxidative stress in rats.

Conclusion
In this study, feeding of high fructose (HF) to Wistar NIN rats had developed impaired glucose tolerance (IGT) and insulin resistance (IR) associated pre-diabetes as indicated by the increased oral glucose tolerance (OGT) at 2 h and homeostasis model assessment (HOMA)-IR index, respectively by 3 months. Further, these animals initiated retinal abnormalities by the end of the experiment duration of 10 months. Early intervention of garlic at 3% to HF+G group rats partially diminished retinal abnormalities/retinal degeneration by inhibiting development of IR, IGT. Results of this study indicate that Garlic delays longterm pre-diabetes induced retinal abnormalities probably through its hypoglycemic, antiglycating, aldose reductase (AR) inhibition and antioxidant potentials.

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Conflict of interest
Authors declare no conflict of interests.

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