Introduction

Diabetes is a disorder of chronic uncontrolled hyperglycemic state. Uncontrolled, hyperglycemic state leads to generation of reactive oxygen species (ROS) through various mechanisms leading to a condition known as oxidative stress. Oxidative stress is known to play important role in pathogenesis of diabetic retinopathy (DR). During DR, there is a state of crisis at cellular level due to decreased levels of antioxidant enzymes stores resulting in insufficient neutralization of ROS (Baynes and Thorpe, 1999; Hartnett et al., 2000; Kowluru and Chan, 2007; Madsen-Bouterse and Kowluru, 2008). This leads to activation of various inflammatory pathways leading to increased levels of major inflammatory cytokines (TNF-α and IL-1β) in serum and retinae of diabetic humans and animals (Demircan et al., 2006; Vincent et al., 2007; Kowluru and Odenbach, 2004a). Cytokines are key mediators in the pathogenesis of DR and activation of various apoptotic pathways in diabetic retinae. TNF-α and IL-1β have important role in basement membrane thickness and alternation of blood retinal barrier (BRB). Apart from this, VEGF has been shown to get activated very early during DR (Hammes et al., 1998), which is major growth factor mediating vascular leakage and proliferation. Also, Oxidative stress has been reported to result in enhanced generation of diacylglycerol, a physiologic activator of the protein kinase C (PKC) pathway. VEGF has been studied to perform its molecular action via stimulating PKC-β activity (Aiello et al., 1997; Aiello, 2002). Both angiogenic factors are highly implicated in the breakdown of BRB, increased BM thickness and formation of acellular capillary due to pericyte loss (Hammes et al., 1998; Aiello et al., 1997; Aiello, 2002).

Trigonella foenum-graceum (Fenugreek) is an annual herb belongs to the leguminosae family and, is used both as a vegetable and as a spice in Indian subcontinent. Fenugreek has high content of saponins, 4-hydroxyisoleucine, and trigonelline, an
alkaloid and fiber. Fenugreek has been very well known for its anti-diabetic

![Fundus photographs from different study groups. (A). Normal group rat fundus showing normal vessel caliber, (B). Diabetic group rat fundus showing dilation and leakage in retinal vessel (arrow), (C). Fenugreek-100 treated rat fundus showing normal vessel calibre as compared to diabetic group, (D). Fenugreek -200 treated rat fundus showing normal vessel caliber as compared to diabetic group.](image)

(Raghuram et al., 1994; Marzouk et al., 2013; Ajabnoor and Tilmisany, 1988), anti-oxidant (Ravikumar and Anuradha,1999; Middha et al., 2011), anti-inflammatory (Sindhu et al., 2012) anti-hyperlipdemic (Chaturvedi et al., 2013) and neuroprotective (Kumar et al., 2012) properties. Therefore, Dietary anti-oxidants play considerably important role in diabetic complications and are being used as daily supplements in the ayurvedic system of medicines from ages.

To the best of our knowledge this is the first study showing beneficial effects of fenugreek in experimental DR. In the present study, hydroalcoholic extract of fenugreek has been evaluated for its anti-oxidant, anti-inflammatory and anti-
Fenugreek angiogenic activities.

Figure 2. Effect of Fenugreek on retinal arteriolar and venular diameter after 24 weeks of diabetes. Values are mean ± SD, n=12. *p < 0.001 compared with normal; #p < 0.05 compared with Fenugreek -treated (Fenugreek -100 and Fenugreek -200) diabetic; $ = differences were insignificant between Fenugreek -100 and Fenugreek -200.

Apart from this, electron microscopic evaluation of basement membrane thickness has been done and anti-apoptotic activity was evaluated by caspase-3 expression.

Materials and Methods

Plant Material – Hydroalcoholic extract of fenugreek (seeds) was obtained from Sanat Products Ltd., New Delhi, India. The aqueous extract of fenugreek was prepared as per GMP compliance.

Study Design - Diabetes was induced in Wistar albino rats (either Sex; 200 to 250 g) with streptozotocin (STZ, 45 mg/kg body weight). Blood glucose was measured prior to the induction of diabetes and 48 hours post STZ/vehicle injection in all groups. STZ was prepared by dissolving in ice cold 50 mM citrate buffer (pH 4.5) and immediately injected intraperitoneally within 5 min of preparation. The rats showing a blood glucose concentration greater than 300 mg/dl were considered diabetic. Age-matched normal rats served as control. Diabetic rats were divided into 3 groups of 15 rats each: the rats in group 1 received normal diet without fenugreek, group 2 received oral fenugreek in a dose of 100 mg/kg body weight (BW) and group 3 received fenugreek in a dose of 200 mg/kg BW by oral gavage soon after establishment of
diabetes (48hr after administration of STZ). The rats were monitored throughout the

Figure 3. Fundus fluorescein angiograms from different study groups. (A). Normal Group fundus fluorescein angiogram not showing any vascular leakage, (B). Diabetic Group fundus fluorescein angiogram showing diffused and leaky vessels (arrows), (C and D). Fenugreek-treated (Fenugreek-100 and Fenugreek-200) fundus fluorescein angiogram not showing any vascular leakage.

study for body weight and blood glucose. After 24 weeks of diabetes, the rats were euthanized by an overdose of pentobarbital, the eyes removed, and the retinæ were isolated. Treatment of the animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, and prior approval was taken from Institutional Animal Ethics Committee.

Materials and methods have been described in earlier section (Moringa Oleiferae).

Results

Glycemic Parameters and Body Weight

Blood glucose levels in the diabetic group (544.71± 35.75 mg/dl) were significantly higher than in the normal rats (104.43±7.74 mg/dl) (p<0.001) at the end of 24 week
Table 1. Effects of Fenugreek on body weight and glycemic parameters

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>Fenugreek -100</th>
<th>Fenugreek - 200</th>
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</thead>
<tbody>
<tr>
<td><strong>Body Weight</strong></td>
<td>408.43±18.56</td>
<td>312.86± 17.23*#</td>
<td>356.88± 24.99NS</td>
<td>364.22 ± 32.49</td>
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<tr>
<td><strong>(gms)</strong></td>
<td></td>
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<tr>
<td><strong>Blood Glucose</strong></td>
<td>104.43±7.74</td>
<td>544.71± 35.75*#</td>
<td>373.75± 45.91NS</td>
<td>360.29 ± 44.48</td>
</tr>
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<td><strong>(mg/dl)</strong></td>
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<tr>
<td><strong>%HBA1C</strong></td>
<td>3.69 ±0.51</td>
<td>9.15 ± 2.04*@</td>
<td>6.41 ± 0.33NS</td>
<td>5.98 ± 0.52</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, * P<0.001 (Normal Vs Diabetic); # P<0.001 (Diabetic Vs Fenugreek-100 and Fenugreek -200); @ P<0.05 (Diabetic Vs Fenugreek -100 and Fenugreek -200). Differences in body weight and blood glucose were analysed by Kruskal wallis test. One way ANOVA was used for %HBA1C. NS = Difference between Fenugreek -100 and Fenugreek -200 were insignificant.

Table 2. Effects of Fenugreek on anti-oxidant, anti-inflammatory and anti-angiogenic parameters

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>Fenugreek -100</th>
<th>Fenugreek - 200</th>
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<tr>
<td><strong>VEGF</strong></td>
<td>9.16±1.11</td>
<td>22.83±1.73**%&amp;</td>
<td>15.76±3.20$</td>
<td>13.08±1.97</td>
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<tr>
<td><strong>(pg/mg protein)</strong></td>
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<tr>
<td><strong>PKC-beta</strong></td>
<td>24.50± 4.97</td>
<td>142.60± 31.78*#</td>
<td>98.16± 13.26$</td>
<td>79.41 ± 14.56</td>
</tr>
<tr>
<td><strong>(pg/mg protein)</strong></td>
<td></td>
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<tr>
<td><strong>TNF-alpha</strong></td>
<td>12.96± 1.40</td>
<td>39.97± 4.51*%&amp;</td>
<td>32.97± 4.45$</td>
<td>28.49± 3.85</td>
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<tr>
<td><strong>(pg/mg protein)</strong></td>
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<tr>
<td><strong>IL-1beta</strong></td>
<td>30.42± 3.98</td>
<td>98.11± 13.77*#</td>
<td>72.77± 7.89$</td>
<td>79.41 ± 14.56</td>
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<tr>
<td><strong>(pg/mg protein)</strong></td>
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<tr>
<td><strong>GSH</strong></td>
<td>16.58± 1.87</td>
<td>4.71±0.66*@</td>
<td>6.89±0.48NS</td>
<td>8.14± 1.61</td>
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<tr>
<td><strong>(nM/mg protein)</strong></td>
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<tr>
<td><strong>SOD</strong></td>
<td>8.82±1.57</td>
<td>2.81±0.48*@</td>
<td>4.86±0.82NS</td>
<td>5.94±0.72</td>
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<td><strong>(IU/mg protein)</strong></td>
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<tr>
<td><strong>CATALASE</strong></td>
<td>11.31±0.90</td>
<td>3.60±0.50*@</td>
<td>6.92±0.92NS</td>
<td>7.55±0.85</td>
</tr>
<tr>
<td><strong>(IU/mg protein)</strong></td>
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Values are Mean ± S.D, n=6. * P<0.001 (Diabetic Vs Normal); ** P<0.001 (Diabetic Vs Fenugreek-100 and Fenugreek-200); % P<0.05 (Diabetic Vs Fenugreek -100); & P<0.001 (Diabetic Vs Fenugreek-200); @ P<0.05 (Diabetic Vs Fenugreek -100 and Fenugreek-200); $ P<0.05 (Fenugreek-100 Vs Fenugreek-200). Differences were analyzed by one way ANOVA followed by post hoc tukey test. NS = Difference between Fenugreek -100 and Fenugreek -200 were insignificant.

period. In Fenugreek-treated (100 and 200 mg/kg BW) rats the blood glucose levels (373.75± 45.91 and 360.29± 44.48 mg/dl) were significantly lower than in the diabetic group (p<0.001), though remained higher than normal (p<0.001) (Table-1).
Similarly, %HBA1C in diabetic group (9.15 ± 2.04) was significantly higher than normal group (3.69 ±0.51) (p< 0.001). However, %HBA1C in Fenugreek-treated (100 and 200 mg/kg BW) groups (6.41 ± 0.33 and 5.98 ± 0.52 ) was found to be significantly lower than diabetic group (p< 0.05) (Table-1).

Body weight in normal group was found to be increased by 49.38 % as compared to diabetic group with a weight gain of 30.55 %. Rats in Fenugreek-treated (100 and 200 mg/kg BW) group gained 40.25 % and 41.98 %, respectively.

**Fundus Photographs and Microvasculature Diameter**
Fundus photographs from diabetic group showed dilated retinal and leaky vessels compared to normal group retinae. On the other hand, Fenugreek-treated (100 and 200 mg/kg BW) group retinae has not shown any such vascular dilatation (Fig.1). Retinal blood Vessels (arterioles and venules) in diabetic group were estimated to be dilated than normal group (p<0.001) (Fig. 2). However, Fenugreek-treated (100 and 200 mg/kg BW) rats showed significantly lesser dilated vessels (arterioles and venules) as compared to diabetic group (p< 0.05) (Fig. 2).

**Fluorescein Angiography**
Normal rat angiograms showed no vascular leakage at the end of six months (Fig.3A). Diabetic rat angiograms showed diffused retinal vasculature and leaky vessels (Fig. 3B). Fenugreek-treated (100 and 200 mg/kg BW) rat retinal angiograms showed lesser degree of vascular dysfunction compared to untreated rats (Fig.3C and D).

**Antioxidant parameters**
Retinal GSH levels were more than three times lower in diabetic rats as compared to normal rats (p<0.001). However, in Fenugreek-treated rats, retinal GSH levels were significantly higher than diabetic retinae (p<0.05) (Fig. 4A). The antioxidant enzymes SOD and CAT showed more than three fold decrease in activity in diabetic retinae as compared to normal retinae (p<0.001). Both SOD and CAT activities were restored close to normal in Fenugreek–treated (100 and 200 mg/kg BW) diabetic retinae (p<0.05) (Fig. 4B) (Table-2).

**Inflammatory parameters**
TNF-α levels in diabetic retinas were found to be more than three folds higher than normal retinas (p<0.001). On the other hand, TNF-α levels in Fenugreek-treated (100 and 200 mg/kg BW) retinas were found to be significantly lower than diabetic retinas (p<0.05 & p<0.001, respectively) (Fig. 5A) (Table-2).

Similarly, IL-1β levels in diabetic retinas were found to be more than three folds higher than normal retinas (p<0.001). However, IL-1β levels in Fenugreek-treated (100 and 200 mg/kg BW) retinas were significantly lower than diabetic retinas (p<0.001) (Fig. 5B) (Table-2).

**Angiogenic Parameters**

VEGF concentration in diabetic retinas were found to be more than 2.5 folds higher than normal retinas (p<0.001). On the other hand, VEGF concentration in Fenugreek-treated (100 and 200 mg/kg BW) retinas were found to be significantly lower than the diabetic retinas ((p<0.05 & p<0.001, respectively) (Fig. 6A) (Table-2).

Similarly, PKC-β concentration in diabetic retinas were found to be significantly higher than normal retinas (p<0.001). However, PKC-β concentration in Fenugreek-treated (100 and 200 mg/kg BW) retinas were found to be significantly lower than diabetic retinas (p<0.001) (Fig. 6B) (Table-2).
Figure 5. (A). Effect of Fenugreek on retinal TNF-α levels in different study groups after 24 weeks of diabetes in rats. Values are presented as mean ± SD, n=6. *p < 0.001 compared with normal; #p< 0.05 compared with Fenugreek-100 treated diabetic; $p< 0.001 compared with Fenugreek-200 treated diabetic; ^p<0.05 compared with Fenugreek-200. (B). Effect of Fenugreek on retinal IL-1β levels in different study groups after 24 weeks of diabetes in rats. Values are presented as mean ± SD, n=6. *p < 0.001 compared with normal; #p< 0.001 compared with Fenugreek-treated diabetic; $p<0.05 compared with Fenugreek-200.

**BM Thickness**

Electron microscopic observations of normal rat retinae clearly showed thin BM as compared to diabetic group. However, treatment with Fenugreek (100 and 200 mg/kg BW) in diabetic rats prevented thickening of BM as compared to diabetic rats (Fig. 7 and 8).

**Discussion**

The present study has shown interesting effects of fenugreek for preventing experimental DR via its anti-oxidant, anti-inflammatory and anti-angiogenic mechanisms. This is the first study showing beneficial effects of fenugreek in streptozotocin-induced diabetic rats after 24 weeks of diabetes.

Hyperglycemia is major contributor for generation of ROS, which ultimately leads to retinal oxidative stress. The possible reason of oxidative stress in diabetes retina is auto-oxidation of glucose (formation of polyols), shifts in redox balances, low antioxidants (reduced glutathione (GSH) and vitamin E), and impaired activities and low levels of antioxidant defense enzymes (SOD and CAT). However, exact molecular mechanisms are still unknown (Baynes and Thorpe, 1999; Hartnett et al., 2000; Kowluru and Chan, 2007; Madsen-Bouterse and Kowluru, 2008). Earlier studies conducted on diabetic rats have shown that there is tremendous fall in retinal
anti-oxidant enzymes activity (SOD and CAT) and glutathione levels (Kowluru and Kanwar, 2007; Kumar et al., 2012; Kumar et al., 2013). Similarly, diabetic rats in the present study showed reduced levels of anti-oxidant enzymes and GSH. However, fenugreek treated diabetic rats showed improved activity of retinal anti-oxidant enzymes and GSH. It has been already studied that anti-oxidants can effectively reverse oxidative stress (Ravikumar and Anuradha, 1999; Middha et al., 2011).

DR has been considered as a chronic inflammatory disorder. Various cytokines have been estimated to be elevated in retina and serum of diabetic patients and experimental rats (Abu el Asrar, et al., 1992; Yuuki et al., 2001; Demircan et al., 2006; Vincent et al., 2007; Kowluru and Odenbach, 2004a; Kowluru and Odenbach, 2004b). Therefore, cytokines have been studied to play important roles in various cellular and molecular mechanisms like retinal leakage, neovascularisation, pericyte death, basement membrane thickness, etc (Demircan et al., 2006; Vincent et al., 2007; Kowluru and Odenbach, 2004a; Kowluru and Odenbach, 2004b). Similarly, we have found raised retinal levels of cytokines in diabetic rats as compared to normal rats. However, fenugreek treated retinae showed lower levels of cytokines as compared to diabetic rats. Further, anti-inflammatory activity of fenugreek has been
very well reported in earlier studies (Sindhu et al., 2012). Moreover, Kowluru and

Figure 7. Retinal capillary BM thickness in different groups. (A and B). Capillary from normal group showing a thin BM (0.07 µm, arrowheads), (C and D). Capillary from diabetic group showing a thick BM (0.21 µm), (E and F), Capillary from Fenugreek-100 group, showing a relatively thin BM (0.15 µm), (G and H), Capillary from Fenugreek-200 group showing relatively thin BM (0.12 µm). l, lumen of capillary; e, Capillary endothelium; arrows shows BM. Scale Bar-500 nm for lower magnification (common) and 200 nm for higher magnification (common).
Figure 8. Effect of Fenugreek on retinal capillary BM thickness after 24 weeks of diabetes. Values are mean ± SD, n=4. *p < 0.001 compared with normal; #p< 0.05 compared with Fenugreek-treated (Fenugreek-100 and Fenugreek-200) diabetic; $ = differences were insignificant between Fenugreek -100 and Fenugreek -200.

Odenbach, 2004b have also shown preventive effects of antioxidants on DR by inhibiting IL-1β.

VEGF (also known as vascular permeability factor) is the strongest angiogenic cytokine and a potent enhancer of vascular permeability. VEGF has been found to be secreted locally under hypoxic conditions by obstruction of retinal vessels (Ferrara et al., 1992; Poulaki et al., 2002). Further, effects of VEGF on retinal permeability and endothelial cell growth are mediated by the PKC pathway and can be suppressed using a PKC-β inhibitor (Aiello et al., 1997; Aiello, 2002). Similarly, we have found raised levels of VEGF and PKC-β in diabetic rat retinae as compared to normal retinae and dilated and perfused retinal vessels. However, fenugreek treated retinae showed inhibitory effects on retinal angiogenic growth factors and retinal vessels were found to be comparatively lesser dilated. Our results are in agreement with other studies showing potential beneficial effects of herbal drugs in the prevention of vascular permeability (Kumar et al., 2012; Gupta et al., 2013).

Retinal basement membrane thickness is one of the commonly reported outcome of long standing diabetes in rats and humans (Roy et al., 1994; Cherian, et al., 2009; Roy et al., 2011; Gupta, et al., 2011; Kumar et al., 2012). Both inflammatory cytokines
(IL-1β and TNF-α) and PKC-β have been implicated in the thickening of BM in diabetic retinae by MMP-9 mediated increased synthesis of extracellular matrix proteins (Gardiner et al., 2003; Giebel et al., 2005). Similarly, we have found thickened BM in diabetic retinae. However, fenugreek treated retinae showed significantly lesser thickened capillary BM compared to diabetic retinae. Earlier we have also found inhibitory effects of herbal drugs on increased BM thickness (Gupta et al., 2011; Kumar et al., 2012; Kumar et al., 2013).

In the present study, Fenugreek has shown efficacy against DR at 100 mg/kg and 200 mg/kg doses. However, 100 mg/kg can be further considered in polyherbal combination as many of its effects are not significantly different than 200 mg/kg BW. In conclusion, it may be postulated that fenugreek has great potential in preventing diabetes induced retinal degeneration in humans after regular consumption in the specified dose.