Evaluation of *Moringa oleifera* for the Prevention of Diabetic Retinopathy in STZ-Induced Type-1 Diabetic Rats.

**Introduction**

Diabetes is the disorder of metabolism and characterized by elevated blood glucose levels. This chronic uncontrolled hyperglycemic state leading to diabetic complications *viz* Diabetic Neuropathy, Nephropathy and Retinopathy. Diabetic retinopathy (DR) is one of the complication, which is characterized by morphological (leaky vessels) and structural alterations (capillary Basement membrane (BM) thickening, apoptosis, etc) of retinal microvasculature. Chronic hyperglycemic state is the major culprit which leads to over-production of reactive oxygen species (ROS) causing retinal oxidative stress (Arden et al., 2011). Further, impaired retinal anti-oxidant status leading to over expression of pro-inflammatory (TNF-α & IL-1β) and angiogenic (VEGF & PKC- β) parameters, which are primarily responsible for the injured retinal microvasculature. Pro-inflammatory parameters are highly responsible for capillary endothelial cell apoptosis and increased BM thickness resulting in loss of pericytes (Kumar et al., 2012a; Kumar et al., 2012b; Wang et al., 2010). As a result of all these pathological abnormal alterations leading to hyperperfused retina (Kumar et al., 2012b) and appearance of new vessel in later stage.

*Moringa oleifera* (MO) Lam (Family – Moringacea, also known as drumstick or horse radish tree) is a highly nutritious plant and consumed in many countries, particularly Asian region (India, Pakistan), Hawaii and many parts of Africa. MO leaves have been reported to be a rich source of β-carotene, protein, minerals and flavanoids (zeatin, rutin, quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol). On the basis of scientific literature, MO has been found to possess hypoglycemic (Gupta et al., 2012), anti-inflammatory (Muangnoi et al., 2012), anti-oxidant (Gupta et al., 2012) and hypolipidemic (Adisakwattana and Chanathong, 2011) properties. The aim of the present study was to investigate the therapeutic potential of aqueous extract of MO on diabetes induced vascular damage to retina. To the best of our knowledge, no scientific study has been conducted earlier to study its direct effect on
Moringa oleifera

retina. Therefore, present study is an attempt to scientifically prove the indigenous claims of MO.

**Materials & Methods**

**Plant Material**

Aqueous extract of *Moringa oleifera* (leaves) was obtained from Sanat Products Ltd., New Delhi, India. The aqueous extract of *Moringa oleifera* 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 three groups of 20 rats each: the rats in group 1 received normal diet without MO, group 2 received oral MO in a dose of 100 mg/kg body weight (BW) (MO-100) and group 3 received oral MO in a dose of 200 mg/kg BW (MO-200) by oral gavage soon after establishment of diabetes (48hr after administration of STZ). The rats were monitored throughout the 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 retinas 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.
**Glycemic parameters**

Blood glucose was estimated with the help of Accu-Chek® Active Glucose Test Strips using an Accu-Chek® meter (Roche Diagnostics India Pvt. Ltd). Accu-chek active device was calibrated before every use to check accuracy and sensitivity. Glycosylated hemoglobin (HbA1C) was estimated at 24 weeks by ion exchange resin kit (Biosystems, S.A.Costa Brava 30, Barcelona, Spain).

![Fundus photographs](image)

Figure 1. Fundus photographs from different study groups. (A). Normal group rat fundus showing normal vessel caliber, (B). Diabetic group rat fundus showing dilated retinal vessels, (C). MO-100 treated rat fundus showing normal vessel calibre as compared to diabetic group, (D). MO-200 treated rat fundus showing normal vessel caliber as compared to diabetic group.

**Fundus photography and Vessel Diameter** - Animals were trained before start of the study so that they become accustomed to the fundus photography procedure. KOWA Handheld Digital Retinal Camera (Genesis – Df, Kowa Company Ltd., Japan, Tokyo) was used to photograph rat fundi. Photographs were taken using conscious rats. Rest of the procedure was performed as described in earlier section on hesperetin.
Final fundus photographs were used for estimating arteriolar and venular diameter. Arteriolar and venular diameters were estimated as described in chapter 5.

**Fluorescein angiography**

For retinal angiography the same general fundus photography procedure was opted except that barrier filter is used for fluorescein angiography, and the illumination & strobe of the camera were adjusted for angiography. Rats were intraperitoneally injected with 20% sodium fluorescein Injection USP (Samarth Life Sciences Pvt. Ltd., India) at a dose of 0.012 ml per 6 gm body weight. Soon after injecting the dye angiograms were captured without any delay at regular intervals.

**Anti-oxidant Parameters**

Superoxide dismutase (SOD), Catalase (CAT) and Glutathione (GSH) were estimated using commercially available assay kits from Cayman Chemicals Company, USA.

**Inflammatory Parameters**

TNF-α and IL-1β were estimated using commercially available kits from Gen-Probe Inc., USA and RayBiotech, Inc., USA., as per manufacturers instructions.
Moringa oleifera

**Angiogenic parameters**

VEGF and PKC-β levels in retinae were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit from RayBiotech, Inc, USA and USCN Life Science, Wuhan, China, respectively as per the manufacturer’s instructions. Protein estimation in each sample was done by Lowry’s method (Lowry et al., 1951).

**Transmission Electron Microscopy**

Retinal tissues were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 6 h at 4 °C. After fixation, the retina was circumcised.
Moringa oleifera

around 2mm around the optic nerve head and further trimmed into 1 mm² pieces.

Further, it is osmicated, dehydrated and embedded in araldite CY212. After selecting the areas of interest, the blocks (70nm) were cut on an ultra-microtome contrasted with uranyl acetate and lead citrate and viewed under TECNAI G20 transmission electron microscope (FEI Company, Netherlands). A mean BM thickness of capillaries from four retinae was reported.
Statistical analysis

The results are expressed as mean ± standard deviation (SD). The unpaired Student’s t-test and one way ANOVA with post hoc tukey test were used for statistical analysis. P values <0.05 were considered statistically significant.

Results

Glycemic Parameters and Body Weight

Blood glucose levels in the diabetic group (491.17 ± 14.75 mg/dl) were significantly higher than in the normal rats (95.67 ± 7.31 mg/dl) (p<0.001) at the end of 24 week period. In MO-treated (MO-100 and MO-200) rats the blood glucose levels (329.88 ± 42.34 & 287.13 ± 19.28 mg/dl) were significantly lower than in the diabetic group (p<0.001), though remained higher than normal (p<0.001). Similarly, % HBA1C in diabetic group (9.94±0.64) was significantly higher than normal group (3.66±0.40) (p< 0.001). However, % HBA1C in MO-treated (MO-100 and MO-200) groups (6.01±1.45 & 5.89±1.03) was found to be significantly lower than diabetic group (p< 0.05).

Body weight in normal group was found to be increased by 50.39 % as compared to diabetic group with a weight gain of 28.43 %. Rats in MO-treated (MO-100 and MO-) groups gained 39.91 % and 37.72 % weight.

Fundus Photographs and Microvasculature Diameter

Fundus photographs from diabetic group showed dilated retinal vasculature compared to normal group retinae. On the other hand, MO-treated 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, MO-treated (MO-100 and MO-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. 5B). MO-treated (MO-100 and MO-200 mg/kg BW) rat retinal angiograms showed lesser degree of vascular dysfunction compared to untreated rats (Fig.3C and D).
Table 1. Effects of MO on body weight and glycemic parameters.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>MO - 100</th>
<th>MO - 200</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (gms)</strong></td>
<td>403.83 ± 23.07</td>
<td>293.17 ± 13.35*#</td>
<td>342.00 ± 27.29NS</td>
<td>326.90±19.99</td>
</tr>
<tr>
<td><strong>Blood Glucose (mg/dl)</strong></td>
<td>95.67 ± 7.31</td>
<td>491.17 ± 14.75*#</td>
<td>329.88 ± 42.34NS</td>
<td>287.13±19.28</td>
</tr>
<tr>
<td><strong>%HBA1C</strong></td>
<td>3.66±0.40</td>
<td>9.94±0.64*@</td>
<td>6.01±1.45NS</td>
<td>5.89±1.03</td>
</tr>
</tbody>
</table>

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

Antioxidant parameters.

Retinal GSH levels were two times lower in diabetic rats as compared to normal rats. However, in MO-treated (MO-100 and MO-200) rats, retinal GSH levels were significantly higher than diabetic retinas (p<0.05) (Fig. 4A). The antioxidant enzymes SOD and CAT showed more than 1.5 fold decrease in activity in diabetic retinas as compared to normal retinas (p<0.001). Both SOD and CAT activities were restored close to normal in MO-treated (MO-100 and MO-200) diabetic retinas (Fig. 4B).

Inflammatory parameters

TNF-α levels in diabetic retinas were found to be more than 2.5 folds higher than normal retinas (p<0.001). On the other hand, TNF-α levels in MO-treated (MO-100 and MO-200) retinas were found to be more than 2 folds lower than diabetic retinas (p<0.001) (Fig. 5A).

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 MO-treated (MO-100 and MO-200) retinas were significantly lower than diabetic retinas (p<0.001) (Fig. 5B).

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 MO-treated (MO-100 and MO-200) retinas were found to be significantly lower than the diabetic retinas (p<0.001) (Fig. 6A).
Table 2. Effects of MO on anti-oxidant, inflammatory and angiogenic parameters

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>MO-100</th>
<th>MO-200</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/mg protein)</td>
<td>8.64±0.77</td>
<td>22.59±1.72*#</td>
<td>14.29±1.44$</td>
<td>11.03±.96</td>
</tr>
<tr>
<td>PKC-beta (pg/mg protein)</td>
<td>28.03±5.45</td>
<td>150.90±24.75*#</td>
<td>93.44±10.45$</td>
<td>71.86±7.18</td>
</tr>
<tr>
<td>TNF-alpha (pg/mg protein)</td>
<td>15.62±1.44</td>
<td>47.18±4.10*#</td>
<td>30.86±2.36$</td>
<td>26.18±2.80</td>
</tr>
<tr>
<td>IL-1beta (pg/mg protein)</td>
<td>33.94±3.74</td>
<td>97.23±7.85*#</td>
<td>70.48±7.63$</td>
<td>59.66±7.59</td>
</tr>
<tr>
<td>GSH (nM/mg protein)</td>
<td>17.20±2.02</td>
<td>5.23±0.64*@</td>
<td>7.85±0.55NS</td>
<td>8.38±1.18</td>
</tr>
<tr>
<td>SOD (IU/mg protein)</td>
<td>8.79±1.23</td>
<td>2.79±0.48*@</td>
<td>4.53±0.43NS</td>
<td>6.29±0.77</td>
</tr>
<tr>
<td>CATALASE (IU/mg protein)</td>
<td>11.98±0.87</td>
<td>3.65±0.34*#</td>
<td>8.16±0.43NS</td>
<td>9.65±1.28</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, n=6.  *P<0.001 (Diabetic Vs Normal);  †P<0.001 (Diabetic Vs MO-100 and MO-200);  ‡P<0.05 (Diabetic Vs MO-100 and MO-200);  §P<0.05 (MO-100 Vs MO-200). Differences were analyzed by one way ANOVA followed by post hoc tukey test. NS = Difference between MO-100 and MO-200 were insignificant.

Similarly, PKC-β concentration in diabetic retinas were found to be significantly higher than normal retinas (p<0.001). However, PKC-β concentration in MO-treated (MO-100 and MO-200) retinas were found to be significantly lower than diabetic retinas (p<0.001) (Fig.6B).

**BM Thickness**

Electron microscopic observations of normal rat retinas clearly showed thin BM as compared to diabetic group. However, treatment with MO in diabetic rats prevented thickening of BM as compared to diabetic rats (Fig. 7 and 8).

**Discussion**

The results of the present study demonstrate potential retinoprotective effects of MO in experimental DR in rats. MO has shown retinal antioxidant, anti-inflammatory and anti-angiogenic properties with additional systemic hypoglycemic effect. To the best of our knowledge, we are the first one to prove the beneficial retinal effects of MO at two dose levels (100 and 200 mg/kg BW) in experimental rat model.
In the present study, MO has shown slight hypoglycemic effect as compared to diabetic group. As MO contains a number of polyphenolic compounds (zeatin, rutin, quercetin, beta-sitosterol, caffeoylquinic acid and kaempferol), which could have played blood glucose lowering effects. Polyphenolic compounds are reported to increase glucose uptake in peripheral tissues by AMPK activation through stimulation of GLUT-4 gene expression apart from their \(\alpha\)-glucosidae inhibitory activity (Adisakwattana and Chanathong, 2011). However, specific molecular mechanisms still need to be investigated in this area. In the present study, significant lowering of blood glucose levels and %HbA1C as compared to diabetic group would provide additional benefits besides its strong anti-oxidant, anti-inflammatory and anti-angiogenic effects. Further, anti-diabetic activity of MO has been already reported in earlier studies (Gupta et al., 2011).

From last two decades animal models have been judiciously and extensively studied for characteristic features of DR which can be helpful in translational research. Therefore, vessel dilatation, vessel tortuosity and vascular leakage are certain classic features of DR in animal models as well as in diabetic patients (Tsai et al., 2011; Sasongko et al., 2012). Similarly, in the present study dilated arteriols and venules have been observed in diabetic retinæ as compared to normal retinæ. However, significantly lesser dilated arteriols and venules were seen in MO treated retinæ as compared to diabetic group (Fig.2 A & B). Apart from these subtle retinal changes...
Hyperglycemia leads to increased generation of free radicals through multiple mechanisms. Recent experimental studies suggest that over-production of ROS and lowered anti-oxidant defense may contribute to retinal oxidative stress (Arden et al., 2011). Although, retina contains robust anti-oxidant system in the form of anti-oxidant enzymes (SOD & CAT) and GSH to neutralize free radicals. However, chronic uncontrolled diabetes has been found to be associated with compromised retinal anti-oxidant status (Cai and Boulton, 2002). Similarly, in the present study we have found that diabetic retinae showed decreased levels of anti-oxidant enzymes (SOD & CAT) and GSH. Various polyphenolic compounds are proven to be potential anti-oxidants (Kamalakkannan and Prince, 2006). MO constitutes of a number of such polyphenolic compounds and in the present study we have found positive modulation of MO on retinal
Moringa oleifera

Figure 8. Effect of MO 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 MO-treated (MO-100 and MO-200) diabetic.

anti-oxidant status. Similarly, anti-oxidant effects of MO have been studied earlier as well (Sreelatha and Padma, 2011; Sasikala et al., 2010; Fakurazi et al., 2008).

Cytokines are polypeptides highly involved in the inter-cellular communication and over-expression of cytokines (TNF-α, IL-1β, IL-6) is strongly implicated in the pathogenesis of DR (Suzuki et al., 2011). Additionally, various studies have investigated the raised serum levels of cytokines in diabetic patients with different grades of DR. Therefore, these molecules may serve as therapeutic targets for the treatment and/or prevention of diabetes induced ocular microvascular complication like DR (Doganay et al., 2002). In the present study, we have also found high levels of TNF-α and IL-1β in diabetic retinas as compared to normal group retinas. However, MO treated retinas shows marked reduction in the expression of pro-inflammatory markers (TNF-α and IL-1β). Similarly, various studies conducted earlier have also shown potential anti-inflammatory effect of MO via inhibiting TNF-α and IL-1β (Muangnoi et al., 2012; Mahajan et al., 2007).

During diabetes, hyperglycemia and oxidative stress upregulates vascular endothelial growth factor (VEGF), a major angiogenic growth factor, which induces retinal neovascularization, vascular leakage, and formation of macular edema (Wang et al., 2010; Adamis et al., 1994; Miyamoto et al., 2000). These are certain early retinal pathologic changes which are associated with upregulation of VEGF, and anti-VEGF
Moringa oleifera treatments options are major therapeutic strategy for the treatment of DR (Waisbourd et al., 2011). Earlier studies have demonstrated that VEGF can increase intra-ocular vascular permeability through activation of PKC in vivo and suggest that oral pharmacological therapies involving PKC beta-isoform-selective inhibitors may prove efficacious for the treatment of VEGF-associated ocular disorders such as DR (Aiello et al., 1997; Xu et al., 2004). Therefore, role of VEGF and PKC-β in retinal pathology is inter-related (Xu et al., 2004; Harhaj et al., 2006). In the present study, we have also found increased levels of angiogenic parameters and their impact on increased retinal permeability. However, retinæ from MO-treated rats showed significantly lower expression of anti-angiogenic activity as compared to diabetic rats. These findings are inconsistent with fact that polyphenolic compounds present in MO are reported to have potential anti-angiogenic activity (Chen et al., 2008; Fan et al., 2003).

BM are integral vascular structures comprising of extracellular matrix primarily composed of laminins, type IV collagen, fibronectin, etc (Chronopoulos et al., 2011). One of the early hallmark of DR is thickened BM. Normally there is a careful balance maintained between synthesis of BM components and degradation. During hyperglycemic condition this balance is disrupted as a result there is increased synthesis of BM components resulting in accumulation of matrix and thickened BM (Chronopoulos et al., 2011; Roy et al., 2003). Further, it has been studied that growth factors (VEGF & PKC-β) and cytokines (TNF-α & IL-1β) leads to thickening of BM (Tsilibary, 2003; Geraldes et al., 2010; Roy et al., 2010). In the present study, we have found thickened BM in diabetic retinae as compared to normal retinae. This observation was in consistent with earlier studies (Kumar et al., 2012a; Kumar et al., 2012b). However, MO has been found to prevent thickening of BM in treated group (Fig.7). The mechanistic of this effect can be explained on the basis of its inhibitory effects of MO polyphenolic compounds on growth factors and cytokines, which are highly involved in the over-expression of matrix components. Therefore, MO has been found to ameliorate hyperglycemia induced vascular dysfunctions in diabetic retinae through its potential anti-oxidant, anti-inflammatory and anti-angiogenic effects. The desired beneficial effects of MO are due to its pool of polyphenolic constituents. In conclusion, it may be postulated that MO can play significant role in preventing and treating diabetes induced retinal dysfunctions.