Evaluation of Triphala for the Prevention of Diabetic Retinopathy in STZ-Induced Type-1 Diabetic Rats.

**Introduction**

Diabetic retinopathy is a disorder of microvasculature of the retina caused by various abnormal metabolic pathways as triggered by uncontrolled hyperglycemia. Hyperglycemia is the key abnormality in diabetes mellitus that promotes oxidative stress (Wolff et al., 1987; Baynes, 1991; Jennings et al., 1992) and it results in increased oxidative stress, and elevated oxidative stress plays an important role in the pathogenesis of diabetic retinopathy (Baynes, 1991). Further, Oxidative stress leads to the production of reactive oxygen species which is considered as a strong stimulus for the release of cytokines (IL-1β, TNF-α); they can damage endothelial cells, increase release of vascular permeability factor (VEGF) and Protein Kinase C-β (PKC-β), and finally leads to retinal neovascularisation (Kowluru and Odenbach, 2004; chang and LoCicero, 2004; Wilkinson-Berka, 2004; Aileo and Wong, 2000). Capillary BM thickening is the most widely reported lesion of diabetic microangiopathy, being observed consistently in human diabetes and experimental diabetes in several different animal models (Stitt, et al., 1994).

Triphala churna is a powdered preparation of three myrobalan fruits, Emblica officinalis Gaertn (Amla), Terminalia chebula Retz (Haritaki) and Terminalia belerica Roxb (Bibhitaki) in equal proportions (Singh et al., 2008; Pawar et al., 2009; Baliga et al., 2010). This fruit formulation has been commonly used in the traditional Indian system of medicine (Ayurveda) from ages. In ayurveda triphala is commonly used for the treatment of several disorders of the gastrointestinal and cardiovascular systems (Singh et al., 2008; Pawar et al., 2009; Baliga et al., 2010; Deep et al., 2005). In addition, triphala is also consumed by the people of Indian subcontinent for its high nutritional value (Deep et al., 2005). Recently, Gupta et al., 2010 demonstrated anti-cataract properties of triphala in-vivo and in-vitro studies via its anti-oxidant properties.
As oxidative stress and inflammation are the key underlying factors in the onset and progression of diabetic retinopathy, triphala is expected to provide therapeutic benefits. To the best of our knowledge no scientific studies has been conducted earlier to evaluate Preclinical efficacy of triphala in the DR and correlate them with antioxidant and inflammatory markers have not been done.

Materials & Methods

Plant Material
Aqueous extract of triphala (fruits) was obtained from Sanat Products Ltd., New Delhi, India. The aqueous extract of triphala 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

Figure 2. Effect of Triphala 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 Triphala-treated (Triphala-175 and Triphala-350) diabetic; $=differences were insignificant between Triphala-175 and Triphala-350.

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). Triphala-treated (Triphala-175 and Triphala-350) fundus fluorescein angiogram not showing any vascular leakage.
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 MO, group 2 received oral triphala in a dose of 175 mg/kg body weight (BW) (Triphala-175) and group 3 received triphala in a dose of 350 mg/kg BW (Triphala-350) 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 retinae 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.

**Results**

**Body Weight and Glycaemic parameters**

At the end of 24 weeks period the blood glucose level in Normal group was 97.33±5.72 mg/dl as compared to 517.75±17.85 mg/dl in diabetic group (p < 0.001). However, in Triphala treated groups (Triphala-175 and 350) the blood glucose levels, were 394.13±39.79 mg/dl and 358.86±33.09 mg/dl respectively, which were significantly lower compared to diabetic group (p < 0.001) (Table-1).

At the end of 24 weeks period the %HBAIC level in normal group was 3.70±0.40 as compared to 9.79±0.73 of diabetic group (p<0.001). In Triphala treated groups at 175 and 350 mg/kg doses the %HBAIC levels were 6.57 ± 0.51 and 6.12 ± 0.22 mg/dl respectively, being significantly lower when compared with diabetic group (p < 0.05) (Table-1).

Body weight gain in normal group was found to be increased by 46.89% as compared to diabetic group with a weight gain of 27.61%. Rats in Triphala-175 group gained 34.64% and in Triphala-350 it was 38.71%.
Table 1. Effects of triphala on body weight and glycemic parameters.

<table>
<thead>
<tr>
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<th>Normal</th>
<th>Diabetic</th>
<th>Triphala-175</th>
<th>Triphala-350</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (gms)</td>
<td>399.43±16.15</td>
<td>292.56±11.27</td>
<td>318.25±26.24</td>
<td>335.43±20.28</td>
</tr>
<tr>
<td>Blood Glucose (mg/dl)</td>
<td>97.33±5.72</td>
<td>517.75±17.85</td>
<td>394.13±39.79</td>
<td>358.86±33.09</td>
</tr>
<tr>
<td>%HBAIC</td>
<td>3.70 ±0.40</td>
<td>9.79 ±0.73</td>
<td>6.57 ± 0.51</td>
<td>6.12 ± 0.22</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D,  *P<0.001 (Normal Vs Diabetic);  $P<0.05 (Diabetic Vs Triphala-175);  &P<0.001 (Diabetic Vs Triphala-350);  %P<0.05 (Triphala -175 Vs Triphala-350). Differences in body weight and blood glucose were analysed by Kruskal wallis test. One way ANOVA was used for %HBAIC. NS = Difference between Triphala-175 and Triphala-350 were insignificant.

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

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>Triphala-175</th>
<th>Triphala-350</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF (pg/mg protein)</td>
<td>8.04±1.05</td>
<td>25.34±1.57</td>
<td>16.51±1.99</td>
<td>14.80±2.44</td>
</tr>
<tr>
<td>PKC-beta (pg/mg protein)</td>
<td>32.52±9.77</td>
<td>138.33±15.05</td>
<td>104.04±15.35</td>
<td>95.75±7.63</td>
</tr>
<tr>
<td>TNF-alpha (pg/mg protein)</td>
<td>14.06±1.47</td>
<td>46.47±4.17</td>
<td>30.12±3.32</td>
<td>28.26±3.19</td>
</tr>
<tr>
<td>IL-1beta (pg/mg protein)</td>
<td>36.54±4.33</td>
<td>91.21±6.66</td>
<td>82.44±15.32</td>
<td>67.34±21.89</td>
</tr>
<tr>
<td>GSH (nM/mg protein)</td>
<td>15.59±1.65</td>
<td>4.18±0.48</td>
<td>7.68±1.28</td>
<td>9.23±0.85</td>
</tr>
<tr>
<td>SOD (IU/mg protein)</td>
<td>9.14±0.50</td>
<td>2.59±0.28</td>
<td>4.53±0.43</td>
<td>6.29±0.77</td>
</tr>
<tr>
<td>CATALASE (IU/mg protein)</td>
<td>12.48±0.60</td>
<td>3.34±0.35</td>
<td>8.16±0.43</td>
<td>9.65±1.28</td>
</tr>
</tbody>
</table>

Values are Mean ± S.D, n=6.  *P<0.001 (Normal Vs Diabetic);  $P<0.05 (Diabetic Vs Triphala-175 and Triphala-350);  %P<0.05 (Triphala -175 Vs Triphala-350). Differences were analyzed by one way ANOVA followed by post hoc tukey test. NS = Difference between Triphala-175 and Triphala-350 were insignificant.

Fundus changes and microvascular diameter

Retinal photographs of some animals from diabetic control group showed hyper permeability around optic nerve head but in Normal group the vasculature was found to be normal in morphology and characteristics. Similarly, in Triphala treated groups
Triphala (175 mg/kg and 350 mg/kg) the vasculature did not show any abnormality (Fig. 1). The mean retinal vessel diameter in normal group were significantly less as compared to diabetic control group ($p < 0.001$). The mean retinal vessel diameters in Triphala and Triphala 350 mg/kg treatment groups were also significantly less 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). Triphala-treated (175 and 350 mg/kg BW) rat retinal angiograms showed lesser degree of vascular dysfunction compared to untreated rats (Fig.3C and D).

**Anti-oxidant Parameters**

Retinal GSH levels were significantly lower in diabetic rats as compared to normal rats ($p<0.001$). However, in Triphala-treated rats, retinal GSH levels were significantly higher than diabetic group ($p<0.001$) (Fig. 4 A). 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 enzymatic activities were restored close to normal in Triphala-treated diabetic rats ($p<0.001$) (Fig. 4B) (Table-2).

**Inflammatory Parameters**

TNF-α levels in untreated diabetic retinae were significantly higher than normal retinae ($p<0.001$). However, TNF-α levels in the retinae from Triphala-treated groups were significantly lower than the untreated diabetic retinae ($p<0.001$) (Fig. 6 A) (Table-2).

Similarly, IL-1β levels in untreated diabetic retinae were significantly higher than normal retinae ($p<0.001$). However, IL-1β levels in the retinae from Triphala-treated (Triphala-175 and 350) groups were significantly lower than the untreated diabetic retinae ($p<0.001$) (Fig. 6B) (Table-2).

**Angiogenesis Parameters**

VEGF levels in untreated diabetic retinae were significantly higher than normal retinae ($p<0.001$). On the other side, VEGF levels in the retinae from Triphala-treated groups were significantly lower than the untreated diabetic retinae ($p<0.001$) (Fig. 6 A) (Table-2).
Figure 4. (A). Effect of Triphala on retinal GSH 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 Triphala-treated (Triphala-175 and Triphala-350) diabetic; $ = differences were insignificant between Triphala-175 and Triphala-350. (B). Effects of Triphala on retinal SOD and CAT activities 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 Triphala–treated (Triphala-175 and 350) diabetic. $ = differences were insignificant between Triphala-175 and Triphala-350 in case of Catalase, but $p <0.05 compared with Triphala -350 in case of SOD.

Similarly, PKC-β levels in untreated diabetic retinae were significantly higher than normal retinae ($p<0.001). However, PKC-β levels in the retinae from Triphala-treated groups were significantly lower than the untreated diabetic retinae ($p<0.001) (Fig. 6 B) (Table-2).

**BM Thickness**

Electron microscopic observations of normal rat retinae clearly showed thin BM as compared to diabetic group. However, treatment with Triphala (175 and 350 mg/kg BW) in diabetic rats prevented thickening of BM as compared to diabetic rats (Fig. 7 and 8).

**Discussion**

Hyperglycemia is the key metabolic abnormality in diabetes mellitus. Hyperglycemia if prevented early in the disease course can help in preventing the onset or delaying the progression of microvascular disease (Engerman & Kern, 1987 Engerman, 1989). The Diabetes Control and Complications Trial (DCCT) demonstrated that the risk for the development and progression of diabetic retinopathy was significantly reduced in patients receiving intensive insulin therapy, however the significance was observed only after 6-7 years (DCCT research group, 1993). Nevertheless, the data clearly indicates that the early control of hyperglycemia may be instrumental in preventing the onset and progression of vascular changes associated
Triphala

Figure 5. (A). Effect of Triphala 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< 0.001 compared with Triphala-treated diabetic; $ = differences were insignificant between Triphala-175 and Triphala-350. (B). Effect of Triphala 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 Triphala-treated diabetic; $p<0.05 compared with Triphala-350.

with diabetic retinopathy. Treatment with triphala in our study significantly reduced the blood glucose levels as compared to untreated diabetic controls. However, the blood glucose failed to reach the normal control level. Similar results have also been reported previously (Rajan and Antony, 2008).

The vascular changes associated with diabetic retinopathy affect the microvasculature of retina early in the disease. Chronic hyperglycemia causes altered retinal metabolism and hypoxia leading to endothelium dysfunction and over-expression of vasorelaxants leading to autoregulatory dilatation and prolongation of retinal blood vessels (Kristinsson et al. 1997) as was observed in our study. Further, these mechanisms are explained on the basis of increased hydrostatic pressure and

Figure 6. (A). Effect of Triphala on retinal VEGF 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 Triphala-treated diabetic; $ = differences were insignificant between Triphala-175 and Triphala-350. (B). Effect of Triphala on retinal PKC-β 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 Triphala-treated diabetic; $p<0.05 compared with Triphala-350.
decreased osmotic pressure after a chronic hyperglycemic state leading to the formation of macular edema, vessel dilation, tortuosity, and elongation that are classical features of diabetic retinopathy. Indeed, the elongation and dilation of vessels increase prior to the onset of clinically significant macular edema (Kristinsson et al. 1997, Gardner et al., 2002). Results of the present study shows that there is marked prevention in the retinal vasodilatation in triphala treated group as compared to diabetic group.

The retinal metabolic abnormalities initiated by hyperglycemia lead to increased oxidative stress which might contribute to development of diabetic complications (Wolff et al. 1987, Baynes 1991, Jennings et al. 1992). The retinal oxidative stress might be due to excess production of reactive oxygen species or impaired antioxidant defence mechanisms within the retina or both. Retina has a highly efficient antioxidant defence mechanism comprising of free radical scavengers such as α-tocopherol, glutathione, and ascorbic acid and antioxidant enzymes such as glutathione peroxidase, SOD and CAT (Armstrong et al. 1981, Castorina et al. 1992). The diabetic rats in our study show subnormal levels of glutathione (GSH) and subnormal activity of antioxidant enzymes (SOD & CAT). These changes in antioxidant parameters are consistent with the previously reported results (Kowluru et al. 1997). Sandhya et al. (2006) have shown potential antioxidant activity of triphala in experimental diabetes. Similarly, in our study positive modulation of GSH and antioxidant enzymes was observed.

Vascular endothelial cell growth factor is an endothelial cell-specific angiogenic and permeability-inducing factor that has been implicated in the pathogenesis of diabetic retinopathy (Rajah and Grammas, 2002). Inhibition of VEGF function may control pathologic neovascularization such as in diabetic retinopathy and tumor growth, whereas enhancement of VEGF may stimulate new blood vessel growth in ischemic tissue (Robinson et al., 2001, Jung et al., 2001). Retinal expression of VEGF is elevated by ROS (Lu et al., 1998), and VEGF can also interact with other metabolic pathways important to the development of retinopathy such as PKC and the polyol pathway (Aiello et al., 1997; Frank et al., 1997). Both preclinical and clinical studies have shown that VEGF participated in the pathogenesis of proliferative diabetic
retinopathy (Adamis et al., 1994, Aiello et al., 1994). Similarly, in the present study

Figure 7. Retinal capillary endothelial BM thickness in different groups. (A) Capillary from normal group, showing a thin BM (0.07 µm, arrowheads), (B) Capillary from diabetic group, showing a thick BM (0.25 µm), (C) Retinal capillary from Triphala-175 group, showing a relatively thin BM (0.12 µm), (C) Retinal capillary from Triphala-350 group, showing a relatively thin BM (0.11 µm). l, lumen of capillary; e, Capillary endothelium; arrows shows BM.

expression of retinal VEGF has been found to be significantly increased in diabetic rats than the normal rats. On the other hand triphala treated group prevented the expression of VEGF. Lu et al.,2012 shown clinical significance of triphala as potential anti-angiogenic agent.

The contribution of TNF-α and IL-1β to the pathogenesis of DR is clearly supported by a number of reports (Smith.,1993 ; Joussen et al., 2002, Doganay et al., 2002, Armstrong et al., 1998; Kumaramanickavel et al., 2001; Limb et al.,1996; Slepova et al., 2001), and significantly higher levels of cytokines (TNF-α and IL-1β)
are found in the plasma of patients affected by either type 1 or type 2 diabetes versus age-matched healthy control subjects (Hussain et al., 1996, Lechleitner et al., 2000, Foss et al., 1992, Bertin et al., 2000). Similarly, in our study we have found that pro-inflammatory cytokines expressions were significantly more in diabetic group than normal group. In the present study that triphala treated group showed significantly lower levels of cytokines than diabetic group. Our results are in accordance earlier reports showing strong ant-inflammatory effects of triphala in animal studies (Sabina and Rasool, 2008).

The thickening of the vascular BM remains the most prominent early histological lesion of diabetic microangiopathy and occurs in multiple organs. Overexpression of extracellular matrix (ECM) components is closely associated with the development of vascular BM thickening, a histological hallmark of diabetic microangiopathy as observed in our study. Several studies have shown that ECM Proteins, fibronectin and collagen, synthesis is upregulated by high glucose or diabetes (Roy et al., 1996, Das et al., 1990, Ljubimov et al., 1996). Similarly, in present study increase in BM thickness was prevented in triphala treated group.

Current study was aimed for the development of plant based drug for the onset and progression of diabetic retinopathy. Plant based products are safe and economical. Multiple mechanism of action of plant based provides advantage over the current
drugs. Further, mechanistic based studies need to be carried out for further exploration of the active principles.

Therefore, the present study clearly demonstrates the therapeutic benefits of triphala in experimental DR at two dose levels i.e. 175 and 350 mg/kg BW. However, there were not significant differences in the activities between two doses except on few parameters. The beneficial effects of triphala polyphenols against development of DR can be attributed to their hypoglycemic, antioxidant anti-inflammatory and anti-angiogenic properties. In conclusion, it can be postulated that triphala could have potential benefits in the prevention of onset and progression of retinopathy in diabetic patients.