To Study Protective Effects of Quercetin on Retinal Neurovascular Degeneration in Streptozotocin-Induced Type-1 Diabetic Rats

Introduction

Diabetic retinopathy (DR) is the leading cause of vision loss among working group. The most complicated and single cause of vision loss is because of diabetic macular edema, which is a direct complication of breakdown of Blood Retinal Barrier (BRB). An effective and safe therapy to treat this devastating disease is under rigorous research all over the world. Meanwhile, anti-VEGF and corticosteroids along with laser photoagulation are considered standard gold therapy, which can reduce progression of DR to some extent in the present time (Kumar et al., 2012). VEGF is a main vasopermeability factor in DR and plays central role in the breakdown of BRB (Qaum et al., 2001). The expression of VEGF in diabetic retina has been highly regulated by Protein Kinase C (PKC)-β dependent pathway. PKC-β has been considered to play major role in various diabetes induced functional and structural abnormalities. PKC-β activation leads to thickening of basement membrane, loss of pericytes, etc. At the cellular levels, BRB is composed of tight junction (TJ) between endothelial cells and disruption of TJs leads to vascular leakage. Earlier studies suggest that VEGF stimulated PKC-β in diabetic retina increases vascular permeability by downregulating occluding content. Occludin has been considered key tight junction protein highly expressed in endothelial cells of retina and decreased expression causes increased vascular permeability (Antonetti et al., 1998).

DR has been considered as disorder of retinal microvascularture. However, neuroinflammation has also been considered to be an early event in the pathogenesis of DR (Cabrera DeBuc and Somfai, 2010; Hernández and Simó, 2012; Stem and Gardner, 2013; Lasta et al., 2013; Simó et al., 2012; Zhang et al, 2013). Impaired retinal electrophysiology and neurodegeneration have also been recorded in diabetic patients (Kizawa et al., 2006). Therefore, hyperglycemia is the main culprit for neurovascular inflammation in DR.
Under routine metabolic processes, reactive oxygen species (ROS) are produced continuously by various catalytic enzyme (Superoxide dismutase; SOD and Catalase; CAT) to support normal cellular functions. During long standing hyperglycemic conditions excessive production of ROS and inefficient scavenging mechanisms (decreased glutathione levels; GSH) lead to various pathological conditions like DR. This imbalance between increased production of ROS and inefficient neutralization leads to a phenomena known as oxidative stress (Brownlee, 2001; Kowluru and Chan, 2007; Baynes and Thorpe, 1999). Chronic oxidative stress leads to oxidative damage to biological macromolecules such as DNA, lipids, proteins, carbohydrates, etc. and membrane lipid peroxidation (Kowluru and Chan, 2007). The resulted oxidation of cellular components leads to generation of important inflammatory cytokines (Tumour Necrosis Factor-α; TNF-α and Interleukin-1β; IL-Iβ). Both TNF-α and IL-1β induces the expression of various genes and promoters of these genes are primarily regulated through complex interactions with NF-kB (Nuclear Factor- kB) subunit (Kowluru and Odenbach, 2004; Demircan et al., 2006; Huang et al., 2011). NF-kB, a redox sensitive factor and a key regulator of antioxidant enzymes, can initiate transcription of many genes involved in apoptosis (Kowluru and Koppolu, 2003). NF-kB has been found to be a direct or indirect activator of caspase-3 expression in retinal cell (Kowluru and Koppolu, 2002). Further, NF-kB and Caspase-3 are highly indicated in the apoptosis of retinal pericytes, ganglion cells and neuroglial cells in diabetic retinae. It has been studied that Müller cells have important role in retinal water/fluid homeostasis (via Aquaporin water channels) (Newman., 1996; Newman and Reichenbach., 1996), regulation of neurotransmitter release and degradation (Matsui et al., 1999), and production of various bio-factors (VEGF) ( Eichler et al., 2000). Further, Müller cells are highly responsive to oxidative stress and inflammation in diabetic retinae, which can be seen by upregulated glial fibrillary acidic protein
Aquaporin-4 (AQP4) is the most abundant osmotically driven transmembrane water channel, which is essential in normal retinal fluid and water homeostasis (Nagelhus, et al 1998; Nagelhus et al., 1999). However, during reactive muller cells as a result of diabetic condition, there is redistribution of AQP4 expression. Altered AQP4 expression leads to retinal edema (Bringmann et al., 2005). Therefore, AQP4 has been suggested as the novel therapeutic target for the treatment of DR (Nagelhus et al., 1998; Bringmann et al., 2005; Da and Verkman, 2004).

Quercetin (Qtcn; 3,3',4',5,7-pentahydroxyflavone) is common flavanol found in vegetables and fruits, and major bioflavonoid in the human diet (Fig.1). Quercetin is abundantly found in red wine, onions, green tea, apples, berries, Ginkgo biloba, St. John's wort, and others. Quercetin has been found to possess strong antioxidant (Anjaneyulu and Chopra, 2004; Gitika et al., 2006;), anti-inflammatory (Lee, et al., 2013; Cho et al., 2003), anti-angiogenic (Zhuang et al., 2011), neuroprotective (Pandey et al., 2012; Sharma et al., 2007; Nakayama et al., 2011) and anti-apoptotic (Ishikawa et al., 2000; Choi et al., 2005) properties. Earlier, Qtcn have been found to be efficacious in cataract prevention (Cornish et al), Oxidative damage in RPE cell (in-vivo and in-vitro) (Cao et al., 2010), inhibition of choroidal and retinal angiogenesis (Chen et al., 2008).
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As neurovascular degeneration has been implicated in the pathogenesis of DR. In this study, we evaluated effects of Qctn on various angiogenic, neuroinflammatory markers and cytokines, apoptotic markers with supporting biochemical, immunohistochemical, structural and ultra-structural details. Apart from this a special emphasis has been given on effects of Qctn on breakdown of blood retinal barrier. Therefore, present study is first of its kind to show effects of Qctn on retinal vasculopathy, oxidative stress, neuroinflammation and apoptosis.

Materials & Methods

Study Design

Diabetes was induced in Wistar albino rats (200 to 250 g, either sex) 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. The rats showing a blood glucose concentration greater than 300 mg/dl were considered diabetic. Age-matched normal rats served as control. Study I - Diabetic rats were divided into 3 groups of 20 rats each: the rats in group 1 received normal diet without Qctn, group 2 received oral Qctn in a dose of 25 mg/kg body weight (BW) and group 3 received oral Qctn in a dose of 50 mg/kg BW by oral gavage soon after establishment of diabetes (48hr after administration of STZ). Study II- Diabetic rats were divided into three groups with 20 rats in each: group I received no treatment, group II received oral Qctn at 25 mg/kg BW and group III received oral Qctn at 50 mg/kg BW. Study III- Diabetic rats were divided into three groups with 15 rats in each: group I received no treatment and group II received oral Qctn at 25 mg/kg BW. The rats were weighed and their blood sugar levels were measured regularly. After 24 weeks of diabetes, the rats were euthanized by an overdose of pentobarbital, the eyes removed, and the retinae were isolated and and homogenised in phosphate buffer (50 mM, with added Protease inhibitor). 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.

Blood Glucose
Blood glucose levels were 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.

**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. This avoids complications of anesthesia such as clouding of the ocular media and the poor tolerance of rats to anesthesia. The vibrissae were trimmed to prevent them from obscuring the photograph. Eyes were dilated with a drop of 1 % tropicamide (Sunways India Pvt. Ltd.). Fundus photography was done regularly till 24 weeks to monitor the fundus changes. Final fundus photographs were used for estimating arteriolar and venular diameter. Arteriolar and venular diameters were estimated as described in previous study (Hesperetin). Diameter measurements were obtained at three different locations along each vessel near optic disk, with an average of three measurements reported.

**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 5-6 gm body weight. Soon after injecting the die angiograms were captured without any delay at regular intervals.

**Antioxidant Parameters**

Estimation of anti-oxidant parameter such as GSH, SOD and CAT were performed using commercially available kits from Cayman Chemicals Ltd. All estimations were done as per manufacturer's instructions.

**Inflammatory Parameters**

TNF-α and IL-1β levels in retinae were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit from Diaclone, France, and Ray Biotech, Inc., respectively as per the manufacturer's instructions.
Figure 2. Fundus photographs from different study groups. (A). Normal rat fundus not showing any vascular dysfunction, (B). Diabetic rat fundus showing leaky vessel and dilated retinal vessels, (C). Magnified view of leaky vessel as shown in fig.B, (D). Qctn-treated (25 mg/kg BW) rat fundus not showing any vascular dysfunction, and (E). Qctn-treated (50 mg/kg BW) rat fundus not showing any vascular dysfunction.
Angiogenesis parameters

VEGF and PKC-β levels in retinae were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit from Ray Biotech, Inc, USA and USCN Life Science, Wuhan, China, respectively as per the manufacturer’s instructions.

Light Microscopy and Morphometric Analysis

Cryostat sections (4 μm) were stained with Haemotoxylin & Eosin (H&E) for ganglion cell counting and histopathology. Ganglion cells were counted in central and peripheral retina (both peripheral sides, nasal and temporal), and average ganglion cell number was reported per 100 μm length in total six retinas from each group. Thickness of total retina, outer nuclear and inner nuclear layer measurements were made in the central and peripheral retina (both peripheral sides, nasal and temporal). The respective measurements were then averaged to report the values. All measurements were performed on microscope with attached digital camera (Leica DM6000 B microscope).

Immunofluorescence

Cryostat retinal sections (14 μm) were rehydrated in phosphate buffered saline (PBS) for 20 min, blocked with 10% normal goat serum in PBS for one hour, and incubated overnight in a moist chamber with the primary antibody diluted in PBS containing 3% normal goat serum and 0.5% Triton X-100. Following primary antibodies were used - rabbit polyclonal anti-AQP4, GFAP (1:1000 dilution, Abcam Plc., UK) Occludin (Invitrogen., UK, 1:500), Collagen IV (1:500 dilution, Santacruz) and fibronectin (1:1000 dilution, Santacruz). After that sections were washed and incubated in respective secondary antibody conjugated to FITC (Molecular Probes, USA) or rhodamine to identify the localization of AQP4, GFAP, Occludin, Collagen IV and Fibronectin in the retinal sections. Slides were mounted in anti-fade medium.
Figure 3. Effect of Qctn on retinal arteriolar and venular diameter after 24 weeks of diabetes. Values are mean ± SD, n=12. *p<0.001 compared with normal arterioles and venules; #p<0.05 compared with Diab.+Qctn-25 and Diab.+Qctn-50 arterioles. $=Differences are insignificant between Diab.+Qctn-25 and Diab.+Qctn-50 groups.

Laboratories, CA, USA) to counterstain the nuclei, and images were obtained with microscope (Leica DM6000 B fluorescence microscope).

**Immunohistochemistry**

Cryostat retinal sections (14 μm) were rehydrated in phosphate buffered saline (PBS) for 20 min, blocked with 10% normal goat serum in PBS for one hour, and incubated overnight in a moist chamber with the primary antibody diluted in PBS containing 3% normal goat serum and 0.5% Triton X-100. For rabbit polyclonal anti-Caspase-3 (1:2000 dilution, Abcam Plc., UK) and goat anti-NF-κB detection (1:500 dilution, Abcam Plc., UK), the sections underwent heat-induced antigen retrieval with a microwave oven (three 5-minute cycles in 10 mM Tris–EDTA buffer [pH 9] at 650 W). Further, the sections were incubated with the biotinylated secondary antibody and reacted with the avidin–biotinylated peroxidase complex. The reaction product was visualized by incubation in development solution (containing 3, 3-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide). The slides were lightly counterstained with hematoxylin in case of caspase-3. Finally, the sections were rinsed with distilled water, cleared, dehydrated in ethanol, mounted and cover slipped.
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Figure 4. (A). Normal Group fundus fluorescein angiogram not showing any vascular leakage, (B). Diabetic Group fundus fluorescein angiogram showing vascular leakage (arrow), (C). Qctn-treated (25 mg/kg BW) fundus fluorescein angiogram not showing any vascular leakage, and (D). Qctn-treated (50 mg/kg BW) fundus fluorescein angiogram not showing any vascular leakage.

RT-PCR Analysis

cDNA was synthesised using a kit (QuantiTect Reverse Transcription Kit, Valencia, CA, USA) as per manufacturer’s protocol and stored at −20°C until use. Real-time PCR was performed with a sequence detection system (ABI Step One) and a Quantitect Sybr Green PCR Kit (Qaigen, Valencia, CA, USA). Primers (Table 5) were designed using NCBI Primer Blast software. cDNA was diluted to 8 ng/μl. The PCR reaction mixture contained 10 μl of Sybr PCR master mix, 8 μl RNase-free water, 1 μl (18μM) of forward and reverse primers, and 1 μl cDNA constituting a total volume of reaction mixture to 20 μl. Reactions were performed in triplicate at 95°C for 15 min; then 95°C for 15 s, 58°C for 30 s and 30 s at 72°C for 40 cycles, followed
by a melt cycle consisting of stepwise increase in temperature from 72 to 99°C. The

Figure 5. (A). Effect of Qctn 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< 0.05 compared with Qctn-25; $p< 0.001 compared with Qctn-50; \$p<0.05 compared with Qctn-50. (B). Effects of Qctn 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< 0.05 compared with Qctn-25; $p< 0.001 compared with Qctn-50; \$p<0.05 compared with Qctn-50.

Figure 6. (A). Effect of Qctn 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 Qctn-25; $p< 0.001 compared with Qctn-50; \$p<0.05 compared with Qctn-50. (B). Effect of Qctn 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< 0.05 compared with Qctn-25; $p< 0.001 compared with Qctn-50; \$p<0.05 compared with Qctn-50.

fold change values were calculated using Livak Method and values are normalized in response to GAPDH.

**Flat Mount Immunolabeling**

Immunolabeling of tight junction proteins was performed on whole mounted retinas prepared and processed as described by Barber et al. 1998 with some modifications. Eyes fixed in formalin for two hours were washed in PBS, and the retina was isolated, mounted nerve fibre layer side up on glass slides. The whole mounts were dehydrated through graded alcohols, defatted in xylene overnight at 4°C, then rehydrated,
permeabilized with 0.3% Triton X-100 for 15 min, and treated with proteinase K (20 µg/ml) for 30 min at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol water solution for 10 min at room temperature. The preparations were blocked with 5% BSA in PBS and incubated for 2 h at room temperature. The flat mount sections were incubated overnight with the primary antibodies. The primary antibodies were polyclonal rabbit anti-Occludin (1:500; Invitrogen), mouse monoclonal anti-Claudin-5 (1:1000; Invitrogen), and rabbit polyclonal anti-ZO-1 (1:500; Invitrogen).

After incubation with primary antibody, retinal flat mounts were washed and incubated with secondary antibody (tagged with FITC or Rhodamine conjugates). Finally, washed and mounted with an anti-fade medium (Vectashield) and sealed with white nail polish. The preparations were observed under the fluorescence microscope, and multiple fields were photographed (Asnaghi et al., 2003).

Western blot analysis
The retinae were isolated and homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 1% Nonidet P-40, with a protease inhibitor cock-tail. The protein concentrations of the samples were measured using

Figure 7. (A). VEGF levels in Qctn-treated rats after 24 weeks of diabetes. Values are presented as mean ± SD, n=6. *p < 0.001 compared with normal; $p < 0.001 compared with Diab.+Qctn-25 and Diab.+Qctn-50 groups; $p < 0.05 compared with Diab.+Qctn-50 groups. (B). PKC-β levels in Qctn-treated rats after 24 weeks of diabetes. Values are presented as mean ± SD, n=6. *p <0.001 compared with normal; $p < 0.001 compared with Diab.+Qctn-25 and Diab.+Qctn-50 groups; $p < 0.05 compared with Diab.+Qctn-50 groups.
the Bradford protein assay. Each protein sample (25 µg) was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for Occludin (10%), Caspase-3 (10%), NF-Kb (12%), AQP4 (12%) and GFAP (12%), Collage IV (5%) and Fibronectin (5%), and then transferred onto nitrocellulose membranes. Immunoblotting was performed using rabbit anti-occludin (Invitrogen; 1:500), rabbit anti-Caspase-3 (1:1000), goat anti-NF-Kb (1:500), anti-mouse GFAP (1:1000), rabbit anti-AQP4 (1:1000), rabbit anti-Collagen IV (1:500 dilution, Santacruz) and rabbit anti-NF-kB (1:500) antibodies. Values are presented as mean ± SD, n=6. *p < 0.05 compared with normal; #p< 0.05 compared with Qctn-treated (25 and 50 mg/kg BW) diabetic.

Figure 8. (A). H&E stained retinal sections showing loss of ganglion cells in diabetic retina compared to normal retina, Qctn-treated retinal showed significantly higher ganglion cells numbers (Fig.B), (C). Showing decreased total retinal thickness in diabetic retina as compared to normal, Qctn-treated retina showing significantly more thickened retina compared to diabetic retina, (D-E). Showing decreased thickness of ONL and INL in diabetic retina compared to normal retina, on the other side significantly thickened ONL and INL were recorded in Qctn-treated retinas. Values are presented as mean ± SD, n=6. *p < 0.05 compared with normal; #p< 0.05 compared with Qctn-treated (25 and 50 mg/kg BW) diabetic.
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anti-fibronectin (1:1000 dilution, Santacruz) as the primary antibodies. Further, the membranes were incubated with the biotinylated secondary antibody and reacted with the avidin–biotinylated peroxidase complex. The protein bands were visualized by incubation in development solution (containing 3, 3-diaminobenzidinetetrahydrochloride (DAB) and hydrogen peroxide) (Fig.9).

![Figure 9](image)

Figure 9. (A). Normal retina showing expression of occludin in capillaries of NFL, INL and OPL (arrows), Fig. (B). Diabetic retina without recognizable occludin expression. Fig. (C and D). Qctn-treated (Qctn-25 and Qctn-50 groups) retina showing similar expression pattern of occludin as shown in normal retina (A). NFL-Nerve Fibre Layer, INL- Inner Nuclear Layer, OPL- Outer Plexiform Layer.

**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 central retina was into 1
mm² pieces. After fixation in 1% osmium tetroxide, tissue is dehydrated, and embedded in araldite CY 212. Semithin section (500 nm thick) were stained with 0.5% toluidine blue and examined under a light microscope. 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 six retinae was reported. Detailed procedure has been already described in our earlier studies.

**Isolation of Retinal Vasculature by Trypsin Digestion**

Retinal tissues were fixed overnight in 4% formalin in phosphate buffer saline. Next morning the retinae were washed in milli-Q water for 6-8 hours. After that retinae were digested in 3% trypsin (prepared Tris-Hcl Buffer,pH-7.4) for around 1.5 hours with gentle shaking at 37°C. Finally, the tissues were processed under dissecting microscope. Isolated retinal vasculature was stained with PAS and Hematoxylin for clear identification of pericytes and endothelium.

**Statistical analysis**

The results are expressed as mean ± standard deviation (SD). The one way ANOVA followed by post hoc tukey test were used for statistical analysis. P values <0.05 were considered statistically significant.

**Results**

**Blood Glucose and Body Weight (Study I)**

Blood glucose levels in the diabetic group (101.31±10.14 mg/dl) were significantly higher than in the normal rats (513.83±36.64 mg/dl) (p<0.001) at the end of 16 week period. In Qctn-25 and Qctn-50 mg/kg BW groups the blood glucose levels (478.92±39.01 and 470.69±38.73 mg/dl, respectively) were significantly lower than in the diabetic group (p<0.001) (Table -1).

Body weight in normal group was found to be increased by 44.80 % as compared to diabetic group with a weight gain of 31.57 %. In Qctn-25 and Qctn-50 mg/kg BW groups gained was 35.98 % and 38.83 %, respectively (Table -1). Diabetic rats showed all three cardinal signs i.e. Polyuria, polyphagia and polydipsia.
Table 1. Effects of Qctn on body weight and blood glucose

<table>
<thead>
<tr>
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<th>Diabetic</th>
<th>Qctn-25</th>
<th>Qctn-50</th>
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<tr>
<td><strong>Body Weight</strong></td>
<td>376.5±7.74</td>
<td>312.0±5.54*%</td>
<td>323.7±7.86NS</td>
<td>339.3±15.54</td>
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<td>(gms)</td>
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<tr>
<td><strong>Blood</strong></td>
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<tr>
<td><strong>Glucose</strong></td>
<td>101.3±10.14</td>
<td>513.8±36.64#</td>
<td>478.9±39.01NS</td>
<td>470.6±38.73</td>
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<tr>
<td>(mg/dl)</td>
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Values are Mean ± S.D, * P < 0.001 (Normal Vs Diabetic); % P < 0.001 (Diabetic Vs Qctn -25 and Qctn-50); @ P > 0.05 (Diabetic Vs Qctn -25); # P < 0.05 (Diabetic Vs Qctn-50). Differences in body weight and blood glucose were analysed by Kruskal Wallis test. NS = Difference between Qctn -25 and Qctn-50 were insignificant.

Table 2. Effects of Qctn on angiogenic parameters

<table>
<thead>
<tr>
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<tr>
<td><strong>VEGF</strong></td>
<td>7.3±1.08</td>
<td>25.1±4.26#</td>
<td>15.2±2.01$</td>
<td>10.13±1.35</td>
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<tr>
<td>(pg/mg protein)</td>
<td></td>
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<tr>
<td><strong>PKC-β</strong></td>
<td>34.19±5.26</td>
<td>131.0±21.42#</td>
<td>73.5±11.57$</td>
<td>47.10±10.05</td>
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<td>(pg/mg protein)</td>
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Values are Mean ± S.D, n=6. * P < 0.001 (Diabetic Vs Normal); # P < 0.001 (Diabetic Vs Qctn-25 and Qctn-50); $ P < 0.05 (Qctn-25 Vs Qctn-50). Differences were analyzed by one way ANOVA followed by post hoc tukey test.

Blood glucose and body weight (Study II)

Blood glucose levels in the diabetic group (538.5±36.06 mg/dl) were significantly higher than in the normal rats (99.7±7.99 mg/dl) (P < 0.001) at the end of 24 week period. In Qctn-treated (Qctn-25 and Qctn-50) rats the blood glucose levels (495.54±30.20 mg/dl) and (482.62±39.82 mg/dl) were significantly lower than in the diabetic group (P < 0.001) (Table-3).
Body weight in normal group was found to be increased by 54.32% as compared to diabetic group with a weight gain of 20.86%. Rats in Qctn-treated (Qctn-25 and Qctn-50) group gained 35.63%.

Table 3. Effects of Qctn on body weight and blood glucose

<table>
<thead>
<tr>
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<th>Qctn-50</th>
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<tr>
<td>Body Weight (gms)</td>
<td>382.08±17.73</td>
<td>307.25±4.18*##</td>
<td>338.46±9.96NS</td>
<td>346.31±18.20</td>
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<tr>
<td>Blood Glucose (mg/dl)</td>
<td>99.75±7.99</td>
<td>538.50±36.06*#</td>
<td>495.54±30.20NS</td>
<td>482.62±39.82</td>
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</table>

Values are Mean ± S.D, *P<0.001 (Normal Vs Diabetic); #P<0.001 (Diabetic Vs Qctn-25 and Qctn-50). Differences in body weight and blood glucose were analysed by Kruskal wallis test. One way ANOVA was used for %HBA1C. NS = Difference between Qctn-25 and Qctn-50 were insignificant

Table 4. Effects of Qctn on inflammatory and anti-oxidant parameters.

<table>
<thead>
<tr>
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<th>Qctn-50</th>
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<tr>
<td>TNF-α (pg/mg protein)</td>
<td>17.66±2.95</td>
<td>48.92±8.99*#@</td>
<td>31.28±3.97$</td>
<td>22.13±3.40</td>
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<tr>
<td>IL-1¿ (pg/mg protein)</td>
<td>27.41±5.19</td>
<td>89.67±12.12*#@</td>
<td>59.48±6.35$</td>
<td>44.69±5.79</td>
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<td>GSH (nM/mg protein)</td>
<td>16.52±1.53</td>
<td>3.97±0.92*#@</td>
<td>8.62±1.06$</td>
<td>11.28±2.41</td>
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<tr>
<td>SOD (IU/mg protein)</td>
<td>7.98±1.31</td>
<td>2.46±0.11*#@</td>
<td>5.12±0.46$</td>
<td>6.54±1.06</td>
</tr>
<tr>
<td>CAT (IU/mg protein)</td>
<td>11.97±1.77</td>
<td>2.26±0.41*#@</td>
<td>7.42±1.56$</td>
<td>9.58±1.06</td>
</tr>
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</table>

Values are Mean ± S.D, n=6. *P<0.001 (Diabetic Vs Normal); #P<0.05 (Diabetic Vs Qctn-25); @P<0.05 (Diabetic Vs Qctn-50) $P<0.05 (Qctn-25 Vs Qctn-50). Differences were analyzed by one way ANOVA followed by post hoc tukey test.
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**Fundus Photographs and Microvasculature Diameter.**

Fundus photographs from diabetic group showed leaky vessels as compared to normal group retinae (Fig.2A and D). On the other hand, Qctn-treated groups (Qctn-25 mg/kg and Qctn-50 mg/kg BW) retinae do not show any vascular leakage (Fig.2C and D). Retinal blood vessels (arterioles and venules) in diabetic retinae were measured to be dilated than normal group (p<0.001). However, Qctn-treated rats showed significantly lesser dilated vessels (arterioles and venules) as compared to diabetic group (p< 0.05) (Fig. 3).

![Image of DAPI, GFAP-FITC, and DAPI + FITC](image)

Figure 10. GFAP expression in normal, diabetic and Qctn-treated retina. It is prominent in Müller cell inner processes in diabetic retina (D-F), but insignificant in Qctn-treated (25 mg/kg BW) (G-I) and Qctn-treated (50 mg/kg BW) (J-L) retina. Normal retina is GFAP negative (A-C).
Fluorescein Angiography
Normal rat angiograms showed no dysfunction of either kind at the end of six months (Fig. 4A). Diabetic rat angiograms showed vascular leakages in the form of hyperfluorescent areas around vessels in the retinae (Fig. 4B). Qctn-treated rats (Qctn-25 mg/kg and Qctn-50 mg/kg BW) retinal angiograms showed lesser degree of vascular dysfunction compared to untreated rats (Fig. 4C and D).

Antioxidant parameters
Retinal GSH levels were almost five fold lower in diabetic rats as compared to normal rats (P<0.001). However, in Qctn-treated (Qctn-25 and Qctn-50) rats, retinal GSH...
levels were significantly higher than diabetic group (P<0.05 and P<0.001, respectively) (Fig. 5A). 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 Qctn-treated (Qctn-25 and Qctn-50) diabetic rats (P<0.05 and P<0.001, respectively) (Fig. 5B) (Table-4).

Inflammatory parameters

TNF-α estimations in untreated diabetic retinae were more than 2.5-fold higher levels than in the normal retinae (P<0.001). The TNF-α levels in the retinae from Qctn – treated (Qctn-25 and Qctn-50) rats were 1.5-fold lower than untreated diabetic retinae (P<0.05 and P<0.001, respectively) (Fig. 6A).

Mean IL-1β value in normal rat retinae was found to be more than 3-fold lower than the untreated diabetic retinae (P<0.001). Mean IL-1β values in Qctn-treated (Qctn-25
and Qctn-50) rats were significantly lower than untreated diabetics (P<0.05 and P<0.001, respectively) (Fig. 6B) (Table-4).

Angiogenic Parameters

VEGF estimations in untreated diabetic retinas showed more than three folds higher levels than in the normal retinae (p<0.001). The VEGF levels in the retinas from Qctn-treated rats (Qctn-25 mg/kg and Qctn-50 mg/kg BW) were more than 1.5 folds and 2 folds lower than the untreated diabetic retinae (p<0.001), but remained significantly higher than normal retinae (p<0.05). There was significant difference between Qctn-25 and Qctn-50 mg/kg BW groups (p<0.05) (Fig.7A).

Similarly, PKC-β levels in normal rat retinae were found to be more than 3.5 folds lower than the untreated diabetic retinae (p<0.001). PKC-β values in Qctn-treated rats (Qctn-25 mg/kg and Qctn-50 mg/kg BW) were significantly lower than the untreated
diabetics (p<0.001) There was significant difference between Qctn-25 and Qctn-50 mg/kg BW groups (p<0.05) (Fig. 7B)(Table-2).

**Light Microscopy and Morphometric Analysis**

H&E stained retinal sections showed loss of ganglion cells in diabetic retina compared to normal retina. However, Qctn-treated retinae (25 mg/kg and 50 mg/kg BW) showed significantly higher ganglion cells numbers (Fig.8). Diabetic retinae showed decreased total retinal thickness in as compared to normal. On the other side, Qctn-treated groups showed significantly more thickened retina compared to diabetic

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Figure 14. NFκB expression in normal, diabetic and Qctn-treated retinae. (A). Normal retina shows normal pattern of NFκB expression in all layers. Counter stained with hematoxylin. (B). Diabetic retina showing increased expression of NFκB in NFL and, endothelium and pericytes of blood vessels, increased expression also seen IPL and INL. (C). Qctn-treated (25 mg/kg BW) retina shows relatively lesser expression of NFκB in NFL, IPL and INL. D. Qctn-treated (50 mg/kg BW) retina also shows relatively lesser expression of NFκB in NFL, IPL and INL. Counterstained with hematoxylin. NFL-nerve fibre layer, INL-inner nuclear layer, IPL-inner plexiform layer, ONL-outer nuclear layer, PRL-photoreceptor layer.
Quercetin

rats. Further, diabetic retinala showed decreased thickness of ONL and INL compared to normal retina; on the other side significantly thickened ONL and INL were recorded in Qctn-treated retinalae (25 mg/kg and 50 mg/kg BW) (Fig.8).

Figure 15. Caspase-3 expression in normal, diabetic and Qctn-treated retinalae. (A). Normal retina shows normal pattern of Caspase-3 expression. Counter stained with hematoxylin, (B). Diabetic retina showing increased expression of caspase-3 in astrocytes (NFL, arrow) and along Müller cell fibres (IPL, arrow head), INL and outer nuclear layer, (C). Qctn-treated (25 mg/kg BW) retina shows slightly more expression in NFL, but lesser expression of caspase-3 in NFL, IPL and INL, (D). Qctn-treated (50 mg/kg BW) retina showing overall insignificant expression. Counterstained with hematoxylin. NFL-nerve fibre layer, INL-inner nuclear layer, IPL-inner plexiform layer, ONL-outer nuclear layer, PRL-photoreceptor layer.

**Immunofluorescence**

Occludin immunofluorescence data showed insignificant expression of occludin in diabetic retinalae as compared to normal retinalae. As we can see in Fig.9B that undetectable fluorescence is noticed in diabetic retina as compared to normal retinalae.
Quercetin (Fig. 9A) with bright intensity on the retinal capillaries in NFL, INL and OPL layers. However, Qctn-treated retina showed similar fluorescence of occluding expression in NFL, INL and OPL layers (Fig. 9C and D).

Figure 16. (A). Occludin gene expression in Qctn and Hsp treated retinae. Normal vs Diabetic, *p< 0.001; Diabetic vs Qctn and Hsp, *p< 0.05. (B). Claudin-5 gene expression in Qctn and Hsp treated retinae. Normal vs Diabetic, *p< 0.001; Diabetic vs Qctn and Hsp, *p< 0.05. (C).ZO-1 gene expression in Qctn and Hsp treated retinae. Normal vs Diabetic, *p< 0.001; Diabetic vs Qctn and Hsp, *p< 0.01.

Normal retina showed insignificant GFAP expression. However, It is prominent in Müller cell inner processes in diabetic retina (Fig.10D-F), but GFAP expression was restricted to astrocytes in Qctn-treated (25 mg/kg BW) (Fig.10G-I) and Qctn-treated (50 mg/kg BW) (Fig. 10J-L) retina.

AQP4 immunoreactivity was moderately present in Müller cell endfeet of normal retina; (Fig.11D–F) In diabetic retina, AQP4 was strongly expressed in Müller cell endfeet and in perivascular space (Fig.11G–I). However, Qctn-treated (25 mg/kg BW) and Qctn-treated (50 mg/kg BW) (Fig.11J-K) retina showing lesser immunoreactivity compared to that in diabetic retina and less staining in Müller cell endfeet.
Diabetic rats showed strong expression of Collagen IV in inner retina as compared to normal rats. However, there was relatively lesser expression of Collagen IV expression in Qctn-treated group (Fig.12).

![Image of immunohistochemical staining](image-url)

**Figure 17.** (A). Blood vessel from normal retina showing uniform expression of occludin, B-C. blood vessels showing normal pattern of claudin-5 and ZO-1 expression in normal retina, D. showing overlay of claudin-5 (B) and ZO-1 (C) expression, E. Blood vessel from diabetic retina showing reduced occludin immunofluorescence at some areas on vessel, (F-G). blood vessels from diabetic retina showing lesser immunofluorescence of claudin-5 (F) and ZO-1(G) respectively, (H). Showing overlay of claudin-5 (F) and ZO-1(G), (I). blood vessel from Qctn-treated retina showing improved expression of Occludin , J-K. blood vessel from Qctn-treated retina showing improved immunofluorescence of claudin-5 and ZO-1 respectively, (L). Showing overlay of claudin-5 (F) and ZO-1(G).

Similarly, normal retinas showed normal pattern of fibronectin expression in and around blood vessels in NFL and OPL. However, diabetic retinas showed overexpression of fibronectin in blood vessels of NFL and OPL. On the other side, Qctn treated retina showed lower expression of fibronectin in NFL and OPL (Fig.13).

**Immunohistochemistry**

Overall lower expression of NF-κB expression observed in normal retina (Fig.14A). However, Diabetic retina showed increased expression of NF-κB in NFL IPL and
INL. Also, NF-κB expression was recorded in endothelium and pericytes of blood vessels in NFL. Qctn (Fig. 14B). Qctn-treated (25 mg/kg and 50 mg/kg BW) retina showed relatively lesser expression of NF-κB in NFL, IPL and INL. (Fig. 14C and D). Normal pattern of Caspase-3 expression was recorded in normal retina (Fig. 15A). However, Diabetic retina showed increased expression of caspase-3 in astrocytes (NFL) and along Müller cell fibres (IPL). Also, increased caspase-3 expression was recorded in INL and ONL (Fig. 15B). Qctn-treated (25 mg/kg BW) retina shows slightly more expression in NFL, but lesser expression of caspase-3 in NFL, IPL and INL (Fig. 15C). Qctn-treated (50 mg/kg BW) retina showed overall insignificant caspase-3 expression (Fig. 15D).

Figure 18. (A). Retinal proteins were extracted using RIPA buffer and separated by SDS-PAGE. Fig. (B). showing relative density of immunoblot. Normal vs Diabetic, *P< 0.001; Diabetic vs Qctn-Treated, *P< 0.05.
RT-PCR Analysis

Occludin gene expression has been significantly reduced in diabetic retinae. However, Occludin gene expression was significantly increased in Qctn-treated group (Fig. 16).

Similarly, in case of Claudin-5 and ZO-1 genes expression have been significantly reduced in diabetic retinae. However, Claudin-5 and ZO-1 genes expression were significantly increased in Qctn-treated group (Fig. 16).

Flat Mount Immunolabeling

Uniform expression of tight junction proteins was observed in flat mount retinal sections of normal rats compared to diabetic rats showed reduced expression of tight
junction proteins. However, Qctn treated retina showed similar expression pattern of tight junction proteins as compared to normal retinae (Fig.17).

Western Blot Analysis
Western blot analysis for occludin from diabetic retinas showed reduced protein expression as compared to normal retinas (Fig.18). However, Qctn-treated retinas showed improved occluding protein expression (Fig.18).

Figure 20. (A). Retinal proteins were extracted using RIPA buffer and separated by SDS-PAGE. Fig. (B). showing relative expression of collagen-IV and fibronectin in normal, diabetic and Qctn-treated group. Normal vs Diabetic, *p< 0.001; Diabetic vs Qctn, #p< 0.05.
Table 5. Gene nomenclature, GenBank accession code, primer sequences and predicted size of the amplified product for rat genes.

<table>
<thead>
<tr>
<th>Unigene Symbol</th>
<th>Description</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>TAGTGGTCTGGGTCTGTCCT</td>
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<tr>
<td>Cldn5</td>
<td>Claudin 5</td>
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<td>TAACAAAGAGTGCCACAAAGC</td>
<td>191</td>
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<tr>
<td>Tjp1</td>
<td>Zonula Occludens 1 (ZO1)</td>
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<td>GCCTTGGATATGTATGTTGAG</td>
<td>150</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>TTGCCATCAATGACCCCTCCA</td>
<td>CGCCCACTTTGATTTTGGGA</td>
<td>185</td>
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</tbody>
</table>

Western blot showed lesser expression of caspase-3 and NF-κB in normal retina compared to diabetic retina. However, Qctn-treated (25 mg/kg and 50 mg/kg BW) retina showed lesser expression of caspase-3 and NF-κB (Fig.19).

Lower expression of APQ4 was seen in normal retina compared to diabetic retina showing higher expression. On the other hand, Qctn-treated (25 mg/kg and 50 mg/kg BW) retina showed lower APQ4 expression. In case of GFAP, undetectable expression was recorded in normal retina. However, significantly higher GFAP expression was observed in diabetic retina. Qctn-treated (25 mg/kg and 50 mg/kg BW) retina showed inhibitory effects on AQP4 expression (Fig. 19).

Collagen IV band was localized at 170 kDa and fibronectin band was localized at 220 kDa. We found that protein expression of collagen IV and fibronectin were significantly increased in diabetic retinas. However, Qctn inhibited protein expression of collagen-IV and fibronectin to a significant extent (Fig. 20).

**Ultra-structural Vascular Changes**

**a. BM Thickness** - Diabetic rats showed significantly thickened retinal capillary BM thickness compared to normal rats (p<0.001). However, Qctn-25 and Qctn-50 mg/kg BW groups showed inhibitory effects on BM thickness relative to normal group (p<0.001). Differences were insignificant between Qctn-25 and Qctn-25 mg/kg BW groups (Fig. 21 and 22).
Figure 21. (A). Capillary from normal group, showing a thin BM (0.06 µm, arrowheads), (B). Capillary from diabetic group, showing a thick BM (0.19 µm, arrowheads) and endothelium hypertrophy, (C). Retinal capillary from Qctn-treated (25 mg/kg BW) group, showing a relatively thin BM (0.13 µm, arrowheads), (D). Retinal capillary from Qctn-treated (50 mg/kg BW) group, showing a relatively thin BM (0.12 µm, arrowheads). l, lumen of capillary; n, nucleus of endothelial cell; e, endothelium.

Figure 22. Effect of Qctn on retinal capillary BM thickness after 24 weeks of diabetes. Values are mean ± SD, n=6. *p<0.001 compared with normal; *p < 0.05 compared with Diab.+Qctn-25 and Diab.+Qctn-50 groups. Differences are insignificant between Diab.+Qctn-25 and Diab.+Qctn-50 groups.
Figure 23. (A). Capillary from normal retina showing intact TJ (arrow, TJ) and endothelium (e), (B). Magnified view of TJ (arrow) as shown in Fig. A, (C). Capillary from diabetic retina showing vacuolated (arrowhead) and widened tight junction (arrow). Also, note appearance of abluminal pinocytotic vesicles, (D). Magnified view of the TJ from Fig. C, (E) Capillary from Qctn–treated (25 mg/kg BW) retina showing intact TJ (arrow) and endothelium (e) without pinocytotic vesicles, and magnified view has been shown in Fig. F, (G). Capillary from Qctn–treated (50 mg/kg BW) retina
Quercetin showing intact TJ (arrow) and endothelium (e) without pinocytotic vesicles, and magnified view has been shown in Fig. H. bm, basal lamina of capillary endothelium (in A, C, E and G).

**b. TJ Structures** - Higher magnification electron micrographs showed vacuolated and widened TJ structures in diabetic rats compared to relatively less widened TJs. On the other side, TJs were intact and less widened in Qctn-25 mg/kg and Qctn-50 mg/kg BW groups (Fig. 23).

**c. Vascular endothelium** – Capillary vascular endothelium hypertrophy was found in diabetic rat retinae compared to normal rat retinae. However, Qctn-25 and Qctn-50 mg/kg BW groups showed protective effect on endothelium hypertrophy. Apart from this, capillary endothelium from diabetic rats showed increased percentage of abluminal pinocytotic vesicles suggesting increase active uptake of fluids from luminal side to extraluminal side causing vascular hyperpermeability in comparison normal rats. In contrary, Qctn-25 and Qctn-50 mg/kg BW groups showed significantly lower pinocytotic vesicles towards abluminal side of capillary (Fig. 21 and 22).

**Trypsin Digestion of Retina**

Trypsin digested retinal vasculature of diabetic rat showed significant appearance of acellular retinal capillaries as compared to normal retinae. On the other side, Qctn-25 and Qctn-50 treated retinae showed insignificant appearance of acellular capillaries (Fig. 24 and 25).

**Discussion**

This is the first of its kind study where a range of neuroinflammatory markers were targeted along with anti-oxidant and anti-apoptotic parameters to prevent diabetes induced neurodegeneration in rats. Apart from this effects of Qctn were also evaluated on breakdown of BRB in diabetic rats. Present study showed potential protective effects of Qctn via its anti-oxidant, anti-inflammatory, anti-angiogenic and anti-apoptotic effects.

Hyperglycemia leads to increased generation of ROS in retinae. Although, retinae contains robust anti-oxidant defense system in the antioxidant molecules (GSH,
Figure 24. Showing trypsin digested retinal vasculature, (A). Normal retinal vasculature with sufficient ratio of endothelium/pericyte ratio and without any acellular capillary, (B). Diabetic retinal vasculature showing acellular capillary (arrow) and lesser endothelium/pericyte ratio can be seen, (C and D). Qctn-25 and Qctn-50 group (respectively) rat retinal vasculatures not showing any vascular abnormality.

Vitamin C and Vitamin E) and enzymes (SOD, CAT and Glutathione Peroxidase). However, an imbalance between pro-oxidant and anti-oxidants leads to oxidative stress (Baynes and Thorpe, 1999; Brownlee, 2001; Kowluru and Chan, 2007). Oxidative stress has been associated with cellular inflammation and release of inflammatory cytokines (Kowluru and Chan, 2007). Similarly, in the present study diabetic retinæ showed decreased levels of GSH and subnormal activity of antioxidant enzymes (SOD and CAT) (Kumar et al., 2012, Kumar et al., 2013). However, Qctn-treated retinæ showed improved levels of GSH and positive modulation of enzyme activities. Earlier, Qctn has been reported to possess strong
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free radical scavenging activity and potential antioxidant activity in in-vivo and in-

vitro conditions (Anjaneyulu and Chopra, 2004; Gitika et al., 2006).

TNF-α and IL-1β are most important contributor factors in diabetes induced retinal neuroinflammation/neurodegeneration. Increased levels of interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-α) have been estimated in the vitreous of diabetic patients with proliferative DR (El-Asrar et al., 1992; Demircan et al 2006) as well as in diabetic rat retinas (Carmo et al., 1999; Joussen et al., 2002; Krady et al., 2005). Increased levels of cytokines (TNF-α and IL-1β) in diabetic retinæ activates NF-Kb (Kowluru and Odenbach, 2004), which further amplifies inflammatory stage and caspase-3 expression leading to increased apoptosis of endothelium cell, pericytes and glial cells. Similarly, in the present study we have estimated raised levels of inflammatory cytokines in diabetic retinæ (Fig.6). However, Qctn-treated retinæ showed significantly lower levels of cytokines compared to diabetic retinæ. Qctn has been widely studied for its strong anti-inflammatory properties in rats (Cho et al., 2003; Lee et al., 2013).

Figure 25. Effect of Qctn on retinal microvasculature (isolated after digestion with 3% trypsin) after 24 weeks of diabetes. Values are mean ± SD, n=6. *p≤0.001 compared with normal; *p ≤ 0.001 compared with Diab.+Qctn-25 and Diab.+Qctn-50 groups. $=Differences are insignificant between Diab.+Qctn-25 and Diab.+Qctn-50 groups.
Figure 26. Flow chart showing pathways inhibited by Qctn in prevention of breakdown of blood retinal barrier.

VEGF activates important cellular processes of angiogenesis, such as activation of endothelial cells, breakdown of BRB, and migration, loss of pericytes and proliferation of endothelial cells following hypoxic insult (Murata et al., 1995). Therefore, VEGF plays central role in the development and progression of DR and diabetic macular edema. Pathological responses of VEGF are selectively mediated by activation of PKC-β isoform (Aiello et al., 1997; Xu et al., 2004). Also, in the present study we have found significantly higher levels of VEGF and PKC-β in diabetic retina as compared to normal rat retina (Fig. 7). Earlier studies have found that anti-angiogenic compounds are most suitable for the prevention of diabetes induced retinal complications. Similarly, we have found significantly lower VEGF and PKC-β levels in Qctn-treated diabetic retinae as compared to untreated diabetic retinae. Anti-angiogenic effects of Qctn have been very well documented in many earlier reports (Tan et al., 2003; Lin et al., 2012).
Increased vascular permeability and dilated retinal vessels are hallmarks of DR in humans as well as in rats. Increased levels of angiogenic (VEGF and PKC-β) markers and decrease expression of tight junction protein (Occludin) have been strongly implicated in the breakdown of BRB. As a result of decreased occluding expression and increased phosphorylation, we have found leaky blood vessels in fundus photographs and fluorescein angiographs of diabetic rat retina and dilated blood vessels (arterioles and venules). In the present study, fundus photographs from diabetic rats showed leaky blood vessels (Fig.2). Subtle retinal leakages can be detected by FA, therefore, Fluorescein angiograms from diabetic retina showed diffused sodium fluorescein. However, Qctn-treated retinas have not shown any detectable leakage either in fundus photographs or in fluorescein angiograms. These suggest protective effects of Qctn on BRB via anti-angiogenic effects. Anti-angiogenic effects of querectin and other polyphenolic compounds have been reported earlier by many studies (Tian et al., 2013; Bucolo et al., 2012; Chen et al., 2008).

Retinal BRB is maintained by various tight junction proteins between endothelium cells of inner retina and pigmented epithelium of outer retina. Occludin, a tight junction protein, which express specifically in endothelium of neuronal tissues in the brain and retina than non-neuronal tissues. Harhaj et al., 2006 have shown that VEGF activation of PKC-β results in occluding phosphorylation as a result contributes to increased retinal vascular permeability. Apart from this, there has been significantly reduced expression of tight junction proteins reported in diabetic retina. Similarly, we have found significantly lesser expression of occludin in diabetic retina as compared to normal retina. However, Qctn-treated rat retinae showed improved of occludin expression (Fig.18).

At the cellular level, BRB breakdown is due to disruption of TJ leads to flow of fluids across endothelial cells (Giebel et al., 2005; Klaassen et al., 2009) and increased uptake of pinocytotic vesicle (Vinores et al., 1998; Vinores et al., 1999, Hofman et al.,
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2000). The former is energy independent mechanism and later is energy dependent mechanism. Similarly, in the present study ultramicrographs from diabetic retinas showed widening of TJ and increased accumulation of pinocytotic vesicles along abluminal side of endothelium. However, protective effects of Qctn have been observed on these abnormalities.

Different members of the caspase protease family play crucial role in initiation and execution of cellular apoptosis. Caspase-3 is activated at very late stage of apoptosis and play crucial role in activating proteolytic cascade. Therefore, detection of caspase-3 is a reliable marker for cells which are destined to undergo apoptosis (Kowluru and Koppolu, 2002; El-Asrar.,et al., 2004). In the present study, activated caspase-3 over-expression has been observed in ganglion cells. Our results are

Figure 27. Figure 28. Flow chart showing pathways inhibited by Qctn in prevention of retinal neurodegeneration.
consistent with earlier studies showing caspase-3 activation in diabetic rats (Kowluru and Koppolu, 2002; Mohr et al., 2002). However, Qctn-treated retinae showed relatively lesser expression of caspase-3 as compared to diabetic retinae.

As discussed, apoptotic retinal neural cell death leads to reduction in thickness of retinal layers i.e. NFL, INL, ONL, and leads to overall thinning of retina. This observation has been already reported in diabetic retina by many authors in experimental diabetic animal models and clinically using optical coherence tomography (Barber et al., 1998; Park et al., 2003; Martin et al., 2004; Cabrera DeBuc and Somfai, 2010). Similarly, we have seen decreased thickness of retinal layers in diabetic retina. On the other hand, Qctn-treated retinae showed lesser thinning of diabetic retinae compared to diabetic retinae as a result of reduced apoptotic cell death (Fig. 8).

Muller cells expand through entire length of retinal tissue and muller cell processes enseath retinal blood vessels and neuronal tissues. Muller cells performs various beneficial actives like maintenance of fluid homeostasis, secretion of anti-oxidants (GSH), regulations of neurotransmitters (release, uptake and synthesis), release of neurotrophic growth factors and most importantly in neural regeneration. In normal retinae, muller cells do not express GFAP. However, as a result of oxidative stress or/and retinal enjury to the retinal GFAP expression has been found to be drastically increased (Barber et al., 2000, Kumar et al., 2013). Various studies have shown that upregulated GFAP in diabetic retina. Similarly, in the present study we have found increased expression of GFAP in diabetic retinae as compared to normal retina. However, Qctn-treated retina also does not show any upregulated GFAP expression due to its protective effects (Fig.10). Similarly, others have also shown that bioflavanoids inhibits GFAP expression (Mizutani et al., 1998; Nakayama et al., 2011).

AQP4 is the most abundant transmembrane water channel found in the brain and retina ((Nagelhus et al 1998; Nagelhus et al., 1999). In the retina, AQP4 distribution has been seen in a polarized fashion in the Müller cell endfeet, which facilitates the water flux at the glial-vascular and glial-vitreal interfaces (Nagelhus et al 1998; Nagelhus et al., 1999; Bringmann et al., 2005). AQP4 has been suggested to be critically important in retinal and brain edema in various pathological disease
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conditions. Da and Verkman, 2004 have shown the deletion of AQP4 gene in mice significantly reduces retinal edema and preserves the retinal function and architecture after retinal ischemia. We have also that AQP4 expression has been found to be increased in Muller cell end feet and perivascular space. Similarly, in the present study we have found increased AQP4 expression in Muller cell endfeet and along perivascular space. However, Qctn treated retinae showed inhibitory effects on AQP4 expression in Muller cell end feet and perivascular space (Fig.11).

Earlier studies have shown that VEGF stimulates growth factors and extracellular matrix gene expression in the retina. Therefore, increased VEGF expression has been implicated in the thickening of BM by up-regulating extracellular matrix proteins (Kuiper et al., 2007). Also, PKC activation has been studied to play key role in the increased synthesis of type IV collagen, fibronectin, and laminin (Roy and Sato, 2000; Studer et al., 1993). Similarly, in the present study diabetic retinae showed thickened capillary BM as compared to normal. However, Qctn-treated retinae showed significantly less thickened capillary BM. Anti-fibrotic effects of Qctn are postulated due to its strong inhibitory effects on MMP-2 and MMP-9 enzymes (Vijayababu et al., 2006; Saragusti et al., 2010).

Appearance of acellular capillaries is hallmark of DR. Pericytes helps in maintaining retinal microvascular structural stability and control endothelial proliferation. These structural alternations occur long before proliferative DR (Hammes et al., 2002; Cai and Boulton, 2002). Similarly, we have found acellular capillaries in diabetic retinae as compared to normal retinae. However, retinal microvasculature in Qctn treated retinae showed insignificant appearance of acellular capillaries as compared to diabetic retinae (Fig.24 and 25).

Qctn has been found effective at two dose levels @ 25 and 50 mg/kg BW. Consequently, the therapeutic efficacy between Qctn-25 and Qctn-50 was not significant in all the parameters. Therefore, Qctn @ 25 mg/kg BW may be further considered for future interventions. The present study showed significance of plant based polyphenolic compounds in prevention and management of complicated disorder like DR, which has no specific therapy available in the present time. In present study, Qctn has shown potential anti-oxidant, anti-inflammatory , anti-angiogenic and anti-apoptotic. Qctn has shown prevention in breakdown of BRB and
thickening of BM. Based on the results it can be concluded that Qctn has great potential in prevention of retinal vascular and neural degenerative effects in diabetic patients.

Summary

Querectin was the second bioflavanoid evaluated in the present dissertation. In the first part, we evaluated effects of quercetin on angiogenic factors, expression of occludin, retinal vessel diameter, formation of acellular capillary, vascular leakage, basement membrane thickness, endothelium hypertrophy and integrity of tight junctions in diabetic rat retinae. As evident from results, we found that quercetin inhibited over-expression of angiogenic factors, dilatation of retinal vessels, breakdown of blood retinal barrier and thickening of capillary basement membrane. Further, we found that quercetin inhibits endothelium hypertrophy, formation of acellular capillary, widening of tight junction and increased uptake of pinocytotic vesicles towards apical side via its anti-angiogenic effects. Apart from this, we found that quercetin inhibits PKC-β mediated retinal occluding phosphorylation as shown in the results of western blot and immunofluorescence studies.

In the later part, we evaluated neuroprotective effects of quercetin in diabetic rat retina. Quercetin showed marker improvement in oxidative stress and neuroinflammation. Quercetin was effective in preventing ganglion cell death via inhibitory effects on caspase-3 activity and muller end feet edema via inhibiting aquaporin-4 expression.

We also evaluated effects of quercetin on gene expression of tight junction proteins (Occludin, Claudin-5 and ZO-1) and protein expression of extracellular matrix (Collagen-IV and Fibronectin) in diabetic rat retina. We found that quercetin prevents inhibition of gene expression of tight junction proteins and inhibits increase expression of collagen-IV and fibronectin in diabetic retinae as evident from real time-PCR, western blot and immunofluorescence studies.