6. Discussion

Diabetes mellitus (DM), a condition primarily defined by the level of hyperglycemia and insufficiency of secretion or action of endogenous insulin, represents a group of metabolic disorder in which there is impaired glucose utilization. While exogenous insulin and other medications can control many aspects of diabetes, numerous complications affecting the vascular system, kidney, retina, lens, peripheral nerves, and skin are common and are extremely costly in terms of longevity and quality of life. Diabetes Control and Complications Trial results have firmly established that chronic hyperglycemia is the primary causal factor underlying the development of various microvascular damage including retinopathy, nephropathy and neuropathy (DCCT Research Group, 1993).

Diabetic retinopathy is a chronic metabolic disorder affecting a major proportion of the population worldwide and is the most frequent diabetic microvascular complication. The prevalence of diabetic retinopathy increases with duration of diabetes. It is the most common microvascular diabetic complication and remains one of the leading causes of blindness worldwide among adults aged 20–74 years (Ahmed and Hani, 2011).

The high prevalence and severity of DR suggest the need for screening program able to recognize it as early as possible; this recommendation becomes even more important since DR may be asymptomatic even in its more advanced stages (Semeraro et al., 2011).

Diabetic retinopathy (DR) usually does not occur during the first five years from the diagnosis of diabetes mellitus, whilst after 10 years of diabetes it is present in over 50% of patients, being the most common cause of loss of vision (Schram et al., 2003; Zorena et al., 2007).

Advanced stages of DR are characterized by the growth of abnormal retinal blood vessels secondary to ischemia. These blood vessels grow in an attempt to supply oxygenated blood to the hypoxic retina. At any time during the progression of DR, patients with diabetes can also develop diabetic macular edema (DME), which involves retinal
thickening in the macular area. DME occurs after breakdown of the blood-retinal barrier because of leakage of dilated hyperpermeable capillaries and microaneurysms. The loss of vision has been linked to macular edema and vitreoretinal neovascularization secondary to vascular dysfunction, which results from the release of growth factors in response to retinal ischemia. The two most important visual complications of diabetic retinopathy are DME and proliferative diabetic retinopathy (PDR). Currently there is no drug regimen available for the treatment of DR and laser therapy remains to be the only treatment option, and that too, does not prevent but just limits the damage.

The current management strategy for DR/DME requires early detection and optimal glycemic control to slow the disease progression. Adherence to these recommendations is hampered as the condition is generally asymptomatic at early stages. Glycemic control (UKPDS, 1998) and photocoagulation (ETDRS Group, 1991) have been standard treatments for both DME and PDR. However, controlling blood glucose within the normal range has its own limitations as perfect glycemic control is not always possible, and the memory of glucose toxicity and the persistent progression of hyperglycemia-induced complications during subsequent period of normal glucose homeostasis (Yamagishi et al. 2004; Engerman et al. 1987; The Diabetes Control and Complications Trial Research group, 2000) suggest that exclusive management of glucose can no longer be viewed as sufficient for the control of long-term complications. Hence, agents that can prevent diabetic complications, irrespective of glycemic control, would have advantages in the management of secondary complications. Although photocoagulation and vitrectomy are effective, sight saving interventions, they are invasive, associated with destruction of neural retina, leading to decreased contrast sensitivity & loss of peripheral vision and only treat the late stages of the disease. Some patients suffer permanent visual loss despite prompt and appropriate therapy. Therefore, the development of noninvasive therapies to prevent and treat diabetic retinopathy is necessary and remains a priority for eye research.

Due to the limitations of the current treatments of DR, new drug regimens are the need of the hour. WHO recommends the evaluation of the effectiveness of medicinal plants in
conditions where we lack the conventional allopathic treatment of diabetes (WHO, 1980; Upathaya et al., 1984). Based on the literature survey for established antihyperglycemic, anti-inflammatory, antioxidant and/or antiangiogenic activities, we selected four drugs (Boerhaavia diffusa, Tinospora cordifolia, Eugenia jambolana and Momordica charantia) to evaluate their potential in the management of diabetic retinopathy. Combination of these drugs (polyherbal combination) was also studied to explore their synergistic efficacy in management of DR.

**Induction of Diabetes**

Chemical induction for diabetes is the most commonly used animal model to study the pathogenesis of diabetic retinopathy. STZ is a potent methylating agent for DNA and acts as a nitric oxide donor in pancreatic cells. β-cells are particularly sensitive to damage by NO and free radicals owing to their low levels of free radical scavenging enzymes (Ganda et al., 1976; Like et al., 1978). Standardization of models is required to check the biological variation and also to observe the reproducibility in the experimental animals. The streptozotocin induced rat diabetic retinopathy model was standardized at dose of 45mg/kg (i.p) in wistar rats weighing 200-250 gm for type I diabetes (Naqvi et al., 2012; Agrawal et al., 2012) and 90 mg/kg i.p. in two days old pubs for type II diabetes to achieve the desired diabetic state of animals for longer duration of period i.e.24 weeks. This model is used widely to see the earlier pathological changes in the rat retina (Halim & Mukhopadhyay, 2006; Martin et al., 2004; Wei et al., 2003). Rats were given a dose of 1 unit NPH insulin (s.c) without affecting the hyperglycemic state.

**Blood Glucose**

Diabetes Control and Complications Trial considers hyperglycemia as the primary factor in development of diabetic retinopathy (DCCT Research Group, 1993). Higher risk of increased severity of retinopathy has been associated with the severity and duration of the inadequate glycemic control (Semeraro et al., 2011, American Diabetes Association, 2010). This agrees with the risk factors for DR reported in various literatures (American Diabetes Association, 2010; Klein, 2007; Leske et al., 2005).
In the present investigation the extracts of *M. charantia* (MC), *B. diffusa* (BD), *E. jambolana* (EJ) and *T. cordifolia* (TC) produced a significant fall in the blood glucose of diabetic rats of type 1 (Annexure 1) as well as type-2 models (Annexure 2). This glucose uptake and antihyperglycemic activity of the drugs may attribute to their stimulating effect on the remnant β-cells or improvement in insulin action at cellular level or it could also be due to the insulin like effect of the active principle(s) present in the individual extracts. Reports by several authors who worked on these drugs support our findings. Phytochemical compounds such as charantin, steroid, glycosides, flavonoid and their derivatives have been implicated in hypoglycemic activity (Anandharajan et al., 2005) and these compounds which have been found in fruit extracts of MC may in part have been responsible for the observed up-regulatory activities of glucose uptake (Naqvi et. al, 2012; Kumar et al 2009). Chude et al., (2001) and Pari and Satheesh (2004) have revealed an insulin releasing mechanism by the aqueous leaf extract of BD. Sharma et al., 2008; Sharma et al., 2003; Sridhar et al., 2005 and Grover et al., 2002 also reported significant reduction of sugar level due to flavonoid rich extract of EJ. Grover, 2000; Rajalakshmi, 2009; Stanely, 2000 and Stanely 2003 have studied TC for its hypoglycemic/ antihyperglycemic effects. It is suggested that the action of this drug is due to its favorable effects on the endogenous insulin secretion and glucose uptake inhibition of peripheral glucose release (Agrawal et al., 2012; Sinha, 2004). Highest reduction in blood sugar level was found by polyherbal combination of these drugs.

**HbA1c**

There is a strong and consistent relationship between hyperglycemia and incidence and progression of DR. The higher probability for DR is associated with longer duration of diabetes and high HbA1c values (American Diabetes Association, 2010). The Early Treatment Diabetic Retinopathy Study (ETDRS) identified HbA1c as one of the most important risk factors for the progression of DR (Raman et al., 2011). AGEs including HbA1c are known to produce the micro-vascular complications in DR (Ciulla et al., 2003). Compared to the measurement of glucose levels, HbA1c assay is at least as good in defining the level of hyperglycemia at which the prevalence of DR increases.
DISCUSSION

(Sabanayagam et al., 2009). A sustained reduction in HbA1c will thus, decrease the risk of developing microvascular diseases and reduce their complications. Diabetes Control and Complications Trial (DCCT), the Stockholm Interventional Study and the United Kingdom Prospective Diabetes Study (UKPDS) have explored the long-term benefits of improving the glycemic control on DR (Rodriquez et al., 2009). In the present study high level of HbA1c in diabetic control group was seen in both type-1 and type-2 models, which reduced significantly on treatment with all the four drugs, thereby proving that they can decrease the probability for DR. However, in both the models of diabetes, reduction in HbA1c due to polyherbal extract (PHC) was maximum and least due to E. jambolana (Annexure 1 and 2).

**Body Weight and Food & Water Consumption**

Polyphagia, polydipsia and polyuria are the important indications of diabetes. In our study, average food and water consumption per day per rat was measured along with the body weight of animals. High consumption was observed in diabetic control group, but their body weight reduced significantly as compared to other groups. All treated groups of type-1 and type-2 models of DR showed a significant decrease in food and water intake (Tables 5.1 and 5.11). Induction of diabetes by STZ leads to loss of body weight due to increased muscle wasting and loss of tissue proteins. Also in diabetic condition the body is either not able to produce insulin (Type-1) or is resistant to it (Type-2), so the glucose cannot be processed. In either case the cells do not get enough glucose (blood sugar) and become exhausted. Essentially body expresses its need for glucose causing hunger and thus polyphagia. Glucose build up in the blood stream is expelled in the form of frequent urination dehydrating the body, which leads to polydipsia.

**Lenticular Changes**

In our study, lens and retinal photographs of animals were taken using slit lamp and fundus camera respectively to observe anterior and posterior segment changes in rat eye. The observations revealed that the experimental diabetes increased the incidence of cataract. This might be a result of diabetic oxidative stress causing necrosis of lenticular
lens fibers. Cataract seemed to invade the lens, causing lenticular necrosis and development of opacity. Protection with the drug extracts resulted in dramatic alterations in the experimental groups of type 1 (Figure 5.1.3 a-c) and type 2 (Figure 5.2.3 a-c) models of DR. Lens opacity has been detected in diabetic patients (Esteves et al, 2008; Kumamoto et al, 2007) and in animal models of diabetes (El-Sayyad et al, 2011) that exhibit intracellular accumulation of sugar alcohol and morphological changes including swelling, rounding of nuclei and vacuole formation in peripheral lens epithelial cells of cataract lenses (Takamura et al, 2003). The authors attributed the development of cataract to the disturbance of osmosis in the lens fibers, probably related with an accumulation of some polyols. Vinson (Vinson, 2006) reported that the opacity of the lens may be related to oxidative stress and generation of superoxide as a result of diabetes.

**Fundus Changes**

Retinal photographs showed development of tortuosity and increase in vessel diameter in diabetic control group. This is in agreement with the fact that hyperglycaemia leads to formation of microaneurysms which causes increase in vascular transmural pressure, hence causing retinal vein tortuosity (Patasius et al., 2007; Kifley et al., 2007). These signs were however either absent or reduced in normal and all the treated groups of type 1 and 2 models of diabetes (Figures 5.1.3 and 5.2.3 d-f), which provides an evidence of efficacy of the drug extracts in delaying the progression of early stages of DR. It is reported that growth factors like basic fibroblast growth factor, insulin-like growth factor and VEGF all are elevated in eyes of patients with proliferative DR (Aiello et al., 1994; Meyer et al., 1993). Extravasated plasma proteins may induce retinal neovascularization in conjunction with an increased VEGF mediated mitogenic effect on endothelial cells (Aiello and Wong, 2000). However, the diabetic rat model reportedly never develops preretinal neovascularization (Naqvi et al, 2012; Engerman et al., 1982), the reason being that the diabetic rodents do not live long enough to experience advanced stages of retinopathy. It is also possible that intrinsic anti-angiogenic factors that counteract angiogenic stimuli are highly active in the rat eye.
**Vascular Endothelial Growth Factor**

During the past few years, rapid advancement has been made in understanding of the mechanisms and molecules involved in the pathogenesis of DR. This is particularly true with regard to the role of the angiogenesis- and vasopermeability-inducing molecule, vascular endothelial growth factor. VEGF has been identified as a primary initiator of proliferative DR, and as a potential mediator of nonproliferative retinopathy (Aiello & Wong, 2000). It is a key mediator of vascular hyperpermeability and neovascularization, and plays a crucial role in normal and pathological angiogenesis (Pe’er et al., 1995, Dvorak, 2000). A marked increase in vitreous and plasma VEGF levels in patients with PDR has been observed (Yan et al, 2012). This implies with several clinical studies that have confirmed the correlation between ischemic retinopathies and intraocular VEGF concentrations (Carmaliet et al., 1996; Aiello et al., 1994; Keck et al., 1989). It is reported that ocular VEGF levels are elevated in diabetic rats before proliferative changes in retina (Sone et al., 1997). Similar finding was observed in our study where VEGF expression markedly elevated in the retina of diabetic animals when compared with normal. In the early stage of STZ-induced diabetes, significant increases in retinal VEGF levels have been found to correlate with retinal vascular permeability (Antonetti et al., 1998). From the experimental data by Hans et al. (1998), it can be ruled out that increase in VEGF expression was caused by the injection of STZ, as no increase in VEGF was observed in the rats that remained normoglycemic despite administration of high dosages of STZ. However, significant decrease in VEGF level was observed in the diabetic animals in both the models of diabetic retinopathy, which were treated with extracts of *Momordica charantia, Boerhaavia diffusa, Eugenia jambolana* and *Tinospora cordifolia* and their combination (PHC) (Annexure 1 and 2). Although all the drugs provided significant protection, but *E. jambolana* showed only mild action in both the models. The results suggest that the drugs may contain some active principle(s) which may downregulate VEGF gene expression. It is reported that *T. cordifolia* contains an active principle, Octacosanol which is known to downregulate VEGF gene expression by inhibiting matrix metalloproteinases and nuclear translocation of NF-κB and its DNA
binding activity (Aggarwal et al., 2011; Thippswamy et al., 2008). However, exact mechanism for all these drugs needs to be explored.

**Protein Kinase C**

The expression of VEGF in diabetic retina can be regulated by Protein Kinase C (PKC) (Ye, et al., 2010; Clarke & Dodson, 2007), whose activation is related to many vascular abnormalities (Das & King, 2007). Numbers of studies have reported about the activation of an important protein kinase, PKC in the framework of diabetes and retinopathy (Rohilla et al, 2012). Modulatory role played by protein kinases in transducing the adverse effects of hyperglycemia in the retinal vasculature, were proved as the exposure of cultured endothelial cells to high levels of glucose led to the rapid induction of protein kinase family members (Xin et al, 2004). PKC isoforms which show significant activation in animal models of chronic diabetes include PKC-α, β, γ, and δ among which PKC-β shows the highest level of induction in the retina of the diabetic animals (Amadio et al., 2010). Its activation causes a number of vascular effects leading to the progression of DR. PKC-b, phosphorylates proteins involved in endothelial function and neovascularisation. Excessive PKC activation therefore underlies the triad of microvascular ischaemia, leakage and angiogenesis in DR. The modulatory role of PKC in the pathogenesis of DR has further been proved by several experimental and clinical studies which have been carried out with selective PKC-β inhibitor, ruboxistaurin mesylate (Aiello et al, 2006). The present investigation also reflected a marked upregulation in PKC-β expression in diabetic control animals, which may be associated with vascular dysfunction due to hyperglycemia. The drug extracts administration resulted in inhibition of PKC expression in treatment group. Polyherbal combination (PHC) was found to be most effective to reduce PKC level among all the treatment groups, which reached closer to normal level in both type-1 (Figure 5.1.69) and type-2 models (Figure 5.2.50) of DR. Among individual drugs, *M. charantia* (MC) showed greatest reduction in PKC level, followed by *B. diffusa* (BD) > *T. cordifolia* (TC) > *E. jambolana* (EJ) (Annexure 1 and 2). Although there was a mild significant difference due to EJ in type-1 model (Figure 5.1.37), however it did not show any significant change in
PKC level \( (p>0.05) \) as compared to diabetic control in type-2 model of retinopathy (Figure 5.2.28). Other drugs which have shown action might have done so due to the possibility of presence of one or more of the biologically active constituents in different extracts which inhibit the PKC isozymes receptors. However, exact mechanism is yet to be established.

**Tumor Necrosis Factor**

Many of the molecular and functional changes that are characteristics of inflammation have also been detected in retinas of diabetic animals and humans. The role of inflammation in the development and progression of DR has been studied for a long time. It is important to note that inflammation starts very early and within one week of experimental diabetes, leukocytes accumulate in the vasculature of retina (Rangasamy et al, 2012; Adamis, 2002). Tumor necrotic factor-alpha (TNF-\( \alpha \)), one of the inflammatory marker, acts as local intensification signals in pathological processes associated with chronic eye inflammation. It plays a significant role in diabetes enhanced microvascular cell apoptosis (Małgorzata et al., 2010; Behl, 2008). Several authors have reported that the retinal levels of TNF-\( \alpha \) are significantly greater than normal in diabetic rats (Timothy SK., 2007; Joussen, et al., 2002; El-Remessy, et al., 2006) as was also observed in the present study. Oral administration of herb extracts significantly reduced the expression of this cytokine level in retina of type-1 and type-2 diabetic rats (Annexure 1 and 2). However, *T. cordifolia* could not provide significant protection \( (p>0.05) \) in expression of TNF-\( \alpha \) levels compared to the diabetic control group in type-2 model (Figure 5.2.40). All other extracts were found to suppress diabetes-induced elevation in rat retinal TNF-\( \alpha \) significantly, with PHC showing the best result (Figures 5.1.70 and 5.2.51). We predicted that these extracts are the strong inhibitors of TNF-\( \alpha \)-induced inflammation in DR. The mechanism behind this could be probably related to the ability of the drug to prevent the production of pro-inflammatory mediator, TNF-\( \alpha \). These drugs may act by binding to TNF-\( \alpha \) and thus, preventing it from signaling the TNF-\( \alpha \) receptors on the surface of cells. These drugs contain many pharmacologically active ingredients. A variety of these constituents belong to different classes of alkaloids, diterpenoid lactones, glycosides,
steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharide (Singh, et al., 2003). The inhibition of TNF-α induced inflammation by these extracts in diabetic retinopathy may be attributed to the action of one or more of these compounds by blocking the binding of cytokines to their specific cell surface receptors. However, the exact mechanisms are yet to be explored. Other studies have also reported anti-inflammatory activities of the drugs used in the present investigation, and hence support our findings. Aggarwal et al., 2011; Manu and Kuttan, 2008 and Taylor, 2005 have identified B. diffusa as an anti-inflammatory agent. Anti-inflammatory activity of EJ has been confirmed by Aggarwal et al., 2011; Kota et al., 2010 and Kumar et al., 2008. Antiangiogenic and anti-inflammatory activities of T. cordifolia has been established by Leyon & Kuttan, 2004. But the reason for its failure to suppress cytokine level in type-2 model could not be explored.

**Interleukins-1-Beta**

Another cytokine interleukins-1-beta (IL-1β) is also released on activation of proinflammatory mediators (Adamiec et al., 2010). In diabetic retinopathy, production of IL-1β in retina induces vascular dysfunction and cell death. (Jason and Susanne, 2007; Célia, 2010). Its concentration is increased in the vitreous fluid of DR patients (Abu et al., 1992; Yuuki et al. 2001) and in the retina of diabetic rats (Carmo et al., 1999; Krady et al., 2005) and is associated with angiogenesis and increased vascular permeability, leading to ischemia and apoptosis of retinal capillary cells (Kowluru and Odenbach, 2004a; Kowluru and Odenbach, 2004b). This contention has been supported by the fact that an intravitreal injection of IL-1β or the exposure of retinal endothelial cells to the cytokine in vitro has shown to cause degeneration of retinal capillary endothelial cells. Retinal capillary cell apoptosis in diabetes is considered to be a predictor of the development of retinopathy, and it occurs in retinas of rats diabetic for 6 to 8 months (Kern et al., 2000). IL-1β is also involved in the expression of the inducible nitric oxide synthase (iNOS) and BRB breakdown. This altered integrity of BRB is correlated with an increased level of IL-1β in retinas from STZ-diabetic rats (Carmo et al., 2000), which leads to visual loss in DR. These proinflammatory changes suggest that IL-1β might play
an important role in the pathogenesis of diabetic retinopathy. Span of 24 weeks in our investigation led to steep rise in IL-1β in diabetic animals in both the study models (Annexure 1 and 2) which is in agreement with many studies reported earlier. Elevations of these cytokines from the normal levels are strictly proangiogenic. However, the treatment of STZ-induced type-1 and type-2 diabetic rats with different herbal extracts and their combination significantly reduced the IL-1β expression in retinas, maximum reduction being in PHC treated groups (Figures 5.1.71 and 5.2.52). This suppression of cytokine level seemed to be associated with inhibition of proinflammatory mediators’ activation. The drug might have reduced the number of cells expressing iNOS, which is accompanied by a significant reduction of the BRB permeability or it might have blocked the binding of IL-1β to its specific cell surface receptors. The exact mechanism yet remains to be clarified.

**Oxidative Stress**

The oxidative stress associated with diabetes mellitus also plays a very important role in the initiation and progression of diabetic complications. It is a major pathogenic factor contributing to retinal dysfunction in DR (Madsen and Kowluru, 2008; Pazdro and Burgess, 2010; Katja et al., 2011). Oxidative stress is caused by excessively high levels of free radicals which damage the cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals. These mechanisms may include enhancement of glucose auto-oxidation, stimulation of the polyol pathway, production of advanced glycation products, and reduction in antioxidant defenses, such as depletion of cellular antioxidant levels and decreased antioxidative enzyme activity (Al-Rawi, 2011). Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Jiang et al. 1990; Wolff et
Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Halliwell et al. 1990; Hogg et al. 1993).

The cytotoxic action of STZ is also associated with the generation of reactive oxygen species (ROS) causing oxidative damage (Szkudelski, 2001). ROS form a link between elevated glucose and metabolic abnormalities important in the development of diabetic complications (Brownlee, 2005). Retina and capillary cells experience increased oxidative damage and the antioxidant defense mechanism is impaired (Kowluru & Kanwar, 2009).

Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Tsai et al. 1994; Kawamura et al. 1994). Persistent hyperglycemia in diabetes causes increased production of free radicals (ROS). These free oxygen radicals can trigger cataract (Ozmen et al., 2002), as also observed in lens examination in the present study. Efforts to understand cataract formation has provoked various hypotheses. In the aldose reductase osmotic hypothesis, accumulation of polyols initiates lenticular osmotic changes. It implicates activation of the sorbitol pathway by glucose as a component in the pathogenesis of diabetic complications. In addition, oxidative stress is linked to decreased glutathione levels and depletion of NADPH levels (Gonzalez et al. 1986; Cheng et al. 1986).

Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of Amadori product followed by advanced glycation endproducts (AGEs) (Hori et al. 1996; Mullarkey et al. 1990). These AGEs act on their receptors RAGEs, inactivate and alter structures and functions of enzymes and promote free radical formation (Baynes et al. 1999; Baynes et al. 1991). AGEs quench and block antiproliferative effects of nitric oxide (Vlassara et al. 1997; Wautier et al. 1994). If intracellular oxidative stress is increased, AGEs also activate the transcription factor NF-κB and promote up-regulation of various NF-κB controlled target genes. It further enhances production of nitric oxide, which mediates islet beta cell damage.
Hyperglycemia not only engenders free radicals, but also impairs the endogenous antioxidant defense system in diabetes (Saxena et al. 1993). Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. Others include lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoids, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, B12). These antioxidants work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation (Feher et al. 1987) and inhibit Fenton and Haber-Weiss-type reactions; vitamins A and E scavenge free radicals (Baynes et al. 1991; Halliwell et al. 1990).

It is reported that enzymatic mechanisms involved in the protection against oxidative stress are impaired in retina of diabetic rats (Renu et al., 1996). Enzymes of the glutathione redox cycle (glutathione reductase and glutathione peroxidase) and antioxidant enzyme catalase become subnormal in diabetic retina, as was also observed in STZ induced type-1 and type-2 diabetic groups in the present investigation (Annexure 1 and 2). In the present investigation, reduction in antioxidant enzymes GSH and CAT in diabetic groups further confirmed that there is a strong correlation between oxidative stress and DR. GSH is one of the major defense systems in the retina. Nonenzymatic antioxidant GSH plays an excellent role in preventing the oxidative cellular damage (Ahmed et al., 2011). In humans, (Puertas et al. 1993) it protects the retina from toxic effects of reactive oxygen species and helps maintain normal cellular redox potential. The retina has a very active system for maintaining glutathione in its reduced form (Winkler et al. 1983) The normal level of GSH within cells is maintained by a balance between its synthesis and utilization by the glutathione redox cycle (Meister et al. 1988), and alteration of either or both of these processes can result in an abnormal GSH level. Alterations in the glutathione redox cycle in hyperglycemia, might contribute to
subnormal retinal GSH levels in diabetes. Activity of glutathione reductase, which regenerates cellular glutathione, is reduced in retina. Activity of γ-glutamyl transpeptidase, an enzyme involved in both degradation and synthesis of glutathione, has been found to be subnormal in retina in diabetes (Kowluru et al. 1994).

Catalase, located in peroxisomes, decomposes hydrogen peroxide to water and oxygen, thereby forming free radicals.

The decreased level of these enzymes in type-1 and type-2 diabetic groups of our study may be due to their increased utilization in trapping the oxyradicals. Other possibility for this decline in the activities of antioxidant defense enzymes in the retina in hyperglycemia may also be attributed to the formation of ROS, which are known to contribute toward reduction in activities of antioxidant enzymes (Obrosova et al., 2003; Kumawat et al., 2009; Pigeolet et al. 1990).

Agents with antioxidant or free radical scavenging power have been shown to inhibit oxidative reactions associated with glycation (Ahmed et al., 2011). The drugs studied in this investigation are known to possess antioxidant activities. Dhar et al., 1999 has shown that *M. charantia* is a good antioxidant. Chaudhary and Dantu (2011) have shown antioxidant activity of *B. diffusa*. Antioxidant action of *E. jambolana* has been well established by Kasi et al (2004a, b). Prince et al (2004 a; 2004 b), Rawal et al, 2004; Goel et al., 2002; Subramanian et al., 2002 and Prince & Menon 1999 have shown restoration of GSH and CAT by *T. cordifolia* in diabetic rats. In support of these findings, a significant increase in the levels of GSH and catalase in *M. charantia* (MC), *B. diffusa* (BD), *E. jambolana* (EJ), *T. cordifolia* (TC) and PHC treatment groups was observed as compared to diabetic control in type-1 diabetic retinopathy proving the efficacy of the selected drugs as good antioxidants (Annexure 1).

Antioxidant defense due to GSH and catalase was also analyzed in type-2 model and their depletion was observed in diabetic retina. Both the enzyme levels were significantly restored in the treatment groups, with the exception of *E. Jambolana* treated animals (Annexure 2). *E. jambolana* could not provide significant protection (p>0.05) against
depletion of catalase enzyme (Figure 5.2.32); however it prevented depletion of GSH (Figure 5.2.31). The reason behind insignificant increase of catalase in type-2 diabetes by *E. jambolana* could not be explored.

**Light Microscopy and Immunohistochemistry**

It is clear from this study that diabetes exposes retinal cells and its microvasculature to sustained hyperglycemia-related biochemical stress, and as a result, they suffer progressive dysfunction leading to premature death. Although the pathogenic basis of this vasodegenerative phase of diabetic retinopathy remains equivocal, the histopathology is well characterized and includes thickening of vascular basement membrane of retina (Gooyer et al., 2006). Retinal histological findings in the present study were in agreement with the same, where increase in thickness of basement membrane was depicted in diabetic control retina in both the study models. Evaluation of histological findings was done by semi-quantitative histological grading method. Based on the severity of abnormal changes, grading was done as nil, mild, moderate and severe. Increase in retinal membrane thickening of the diabetic control group was graded as severe in type-1 as well as type-2 models of retinopathy (Annexure 3-A & B). This increase in thickness of basement membrane may be attributed to the destruction of pericytes or endothelial cells of retina. It has been reported earlier that pericytes of diabetic retinas undergo changes consistent with apoptosis (Alikhani et al., 2010). However, damage to endothelial cells and thus membrane thickening was reduced to some extent in all the treatment groups of type-1 and 2 models. Treatment with PHC and *M. charantia* provided a good control in type-1 study, as no appreciable widening of the retinal vessel was observed (Annexure 3A). These drugs however showed mild increase in membrane thickness in type-2 study (Annexure 3-B). Also, groups treated with *B. diffusa* and *T. cordifolia* showed only mild dilation in both the studies. *E. jambolana* showed comparatively thicker vessel membrane as compared to all other drugs in both the study models (Annexure 3-A & B), hence was least preventive.

In type-1 model of retinopathy, we also performed histopathology of other organs, i.e., kidney, pancreas, heart and liver to see if there were any abnormal changes due to
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hyperglycemia. Semi-quantitative grading was done to evaluate the histological findings. Light microscopy revealed that kidneys and pancreas were affected but no significant changes were observed in liver and heart sections. Kidney of diabetic control group was severely affected by apoptotic mesangium cells in the glomerulus. PHC provided a good control against increase in number of cells, while only mild protection was seen in *E. jambolana* group. *M. charantia, B. diffusa,* and *T. cordifolia* showed better effect than *E. jambolana* (Annexure 3-A).

Pancreas sections were evaluated for change in size of islet and presence of inflammatory cells. It was observed that none of the study drugs was able to prevent the reduction of islet structure, but could protect the increase of lymphocytes and other apoptotic cells (Annexure 3-A).

Retinal apoptosis of diabetic group was further confirmed by immunohistology of the retina in type-1 model. It has been demonstrated that hyperglycemia plays a role in inducing apoptosis mediated by increased Bax and BCL-2 levels (Podesta et al., 2000). These increased expressions assist in the apoptosis of retinal cells by tilting the cellular balance of apoptosis regulators in a direction that increases susceptibility to stressful stimuli, and may also be sufficient to kill the cells directly (Adams and Cory, 1998; Xiang et al., 1996). Immunohistochemical analysis of non-diabetic animal in this study exhibited normal arrangement of retinal cell layers. On the other hand, untreated diabetic retina revealed massive degeneration of nerve fiber, ganglion cells and nuclear cells. Layers of nerve fibers, ganglion cells and inner plexiform attained considerable atrophy in untreated diabetic rats. However, retina of diabetic rats protected with herbal drugs and their combination ameliorated the dramatic histological alterations and reduced Bax and BCL-2 immunoreactivity but did not reach the normal structural pattern.

The study conducted for evaluation of potential of plant extracts, clearly demonstrate that the study drugs *Momordica charantia, Boerhaavia diffusa, Eugenia jambolana* and *Tinospora cordifolia* and their combination (PHC) may have beneficial effects for prevention and management of diabetic retinopathy in type-1 and type-2 diabetes. The reason may be attributed to their antihyperglycemic, anti-angiogenic, anti-inflammatory
and antioxidant properties. Physical, biochemical and histological results confirmed that PHC was most potential and possessed synergistic actions to control retinopathy in prevention and management of diabetic retinopathy. Among the individual drugs studied, *M. charantia* proved to be most effective followed by *B. diffusa* and *T. cordifolia*. Efficacy of *E. jambolana* was least as compared to others. It can thus be used as an adjuvant drug in combination with other therapeutically active drugs.