5.1. Type-I Diabetic Retinopathy

5.1.1. Momordica charantia

5.1.1.1. Effect of M. charantia on Glycemic Parameters

A. Blood glucose

The glucose level of the diabetic control group (423.15 ± 49.28 mg/dl) statistically increased (p<0.0001) as compared to the non-diabetic (normal control) group (96.07 ± 2.76 mg/dl). However, oral administration of M. charantia (MC) suppressed the elevation of blood glucose level to 337.23 ± 42.05 mg/dl, which was significantly lower (p<0.001) as compared to untreated diabetic rats (Fig 5.1.1).

![Blood Glucose Graph]

Figure 5.1.1. Effect of M. charantia on blood glucose. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).
B. Glycosylated hemoglobin (HbA1c)

Glycated hemoglobin (HbA1c) in healthy adult rats was in the normal range of 4.45±0.31%. However, HbA1c in diabetic control rats was significantly higher as compared to normal control group. Oral administration of M. charantia for 24 weeks brought down the level of HbA1c to 5.91 ± 0.90% in the treatment group, which was significantly lower (p<0.001) than untreated diabetic group (Fig 5.1.2).

![Glycated hemoglobin](image)

Figure 5.1.2. Effect of M. charantia on %HbA1c. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.1.2. Effect of M. charantia on body weight and food and water intake

A significant decrease in body weight was observed in diabetic rats compared with the normal rats (p<0.0001). However, treatment with M. charantia led to increase in body weight of rats as compared to that of untreated diabetic animals (p<0.001, Table- 5.1). Also increase in consumption of food and water was seen in diabetic group when compared with normal group (p<0.0001). However, increase in food and water
consumption due to hyperglycemia was not altered in diabetic rats on treatment with extract of *M. charantia* (Table-5.1).

Table 5.1 Effects of the oral administration of *M. charantia* extract on the body weight and food and water consumption of STZ-induced type 1 diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th><em>M. charantia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Change in BW (%)</strong></td>
<td>55.21±4.98*</td>
<td>25.34±4.87</td>
<td>40.49±6.28*</td>
</tr>
<tr>
<td><strong>Water intake (ml)</strong></td>
<td>33.08±4.56*</td>
<td>147.21±23.74</td>
<td>68.85±2.96*</td>
</tr>
<tr>
<td><strong>Food intake (gm)</strong></td>
<td>13.45±1.16*</td>
<td>35.81±1.24</td>
<td>24.36±2.60*</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

5.1.1.3. Clinical Ocular Examination

Normal group rat lenses had no signs and symptoms of cataract development. However, significant changes were observed in diabetic control group as compared to normal and treatment group of animals, when scored by 12-clock hour cycle method for cataract. Cataract score for normal group was 0 and 22 for diabetic group. *M. charantia* treated group was scored 6. The treatment group showed significantly less development of cataract as compared to diabetic control group (Fig 5.1.3, table 5.2).

Retinal photographs of some animals from diabetic control group showed tortuosity in the retinal vessels, but in non-diabetic healthy group, normal vascular architecture was present. Also, no tortuosity was seen in *M. charantia* treated group (Fig 5.1.3). After 24 weeks, the vessel diameter (measured using Adobe Photoshop CS3 version) in normal group was 18.75 ± 0.37 pixels. The retinal vessels of diabetic group became significantly
dilated (35.96 + 0.34 pixel) as compared to the normal and treatment group (24.94 + 0.27 pixel, p<0.0001) (Table 5.2).

Figure 5.1.3. Photographs showing lenticular and retinal changes at the end of 24 weeks of diabetes (a) Normal rat lens (b) Diabetic lens showing cataract (shown by arrow) (c) *M. charantia* treated lens showing no abnormality (d) Normal fundus (e) Diabetic rat fundus showing tortuosity (f) *M. charantia* treated rat fundus

Table 5.2 Effects of the oral administration of *M. charantia* extract on cataract score and vessel diameter of STZ-induced type 1 diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th><em>M. charantia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cataract Score</td>
<td>0*</td>
<td>22</td>
<td>6*</td>
</tr>
<tr>
<td>Vessel diameter</td>
<td>18.75±2.36*</td>
<td>35.96±1.05</td>
<td>24.94±1.15*</td>
</tr>
<tr>
<td>(Pixels)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control
**Fluorescein Angiography**

Fluorescein angiography of normal rat retina showed well defined vessel structure. In *M. charantia* treated group, fundus visibility was not as good as that of normal, but was far much better than that of diabetic group. Reason for this disorientated visibility in diabetic animal might be due to emerging opacity as a result of cataract formation. However, neovascularization was not observed in any of the groups (Figure 5.1.4).

![Fluorescein angiogram](image)

Figure 5.1.4. Fluorescein angiogram of the rats’ eyes at the end of 24 weeks of diabetes (a) normal (b) diabetic control (c) *M. charantia* treated

**5.1.1.4. Effect of *M. charantia* on Angiogenic Parameters**

**A. Vascular Endothelial Growth Factor (VEGF)**

Non-diabetic rats showed the levels of retinal VEGF to be 2.99 ± 0.94 pg/mg protein. However, the VEGF concentration was found to be significantly increased in diabetic retina (p<0.0001). Treatment with *M. charantia* was found to protect rise in the expression of VEGF levels significantly (p<0.0001). The angiogenic expressions in the treated group reduced to half of the level found in untreated group (Fig 5.1.5).
RESULTS - Type 1 DR

Figure 5.1. Effect of *M. charantia* on VEGF after 24 weeks. Each value represents mean±S.D (n=6). ***p<0.0001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Protein Kinase-C beta (PKC-β)**

A significant rise was observed in the expression of PKC-β levels in diabetic control group as compared to normal control group (p<0.0001). However, 24 weeks of oral administration of *Momordica charantia* led to about 60% reduction in the PKC level in treatment group as compared to that in diabetic control animals (p<0.0001; Figure 5.1.6).
5.1.1.5. Effect of *M. charantia* on Inflammatory Parameters

**A. Interleukin-1 beta (IL-1β)**

Cytokine marker IL-1β showed significant increase in diabetic control group in comparison to normal group (p<0.0001). In the *M. charantia* treated group, the level of IL-1β significantly reduced to 48.89 ±15.01 pg/mg protein (p<0.0001), which was in close proximity to normal levels (45.06 ±14.73 pg/mg protein) (Figure 5.1.7).
**B. Tumor Necrosis Factor-alpha (TNF-α)**

A significant rise (p<0.001) was observed in the expression of TNF-α levels in diabetic control group (58.90 ±12.22 pg/mg protein) as compared to normal group (26.94 ±15.15 pg/mg protein). However *M. charantia* showed significant protection (p<0.001) against the rise in inflammatory expression of TNF-α (35.96 ± 8.75 pg/mg protein) in the treatment group as compared to the untreated diabetic animals (Figure 5.1.8).
RESULTS

Type 1 DR

**Figure 5.1.8.** Effect of *M. charantia* on inflammatory TNF-a after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.1.6. Effect of *M. charantia* on Antioxidant Parameters

A. Reduced Glutathione (GSH)

Retina of normal animals had high antioxidant level. A significant fall was observed in GSH level in diabetic control group as compared to normal group (p<0.0001). However, treatment with *M. charantia* significantly (p<0.001) protected the depletion of retinal GSH levels as compared to diabetic control animals (Figure 5.1.9).
Figure 5.1.9. Effect of *M. charantia* on reduced glutathione concentration after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Catalase

Activity of catalase enzyme was measured in the experimental groups. It was observed that catalase activity in untreated diabetic rats reduced by more than one third as compared with normal control group (p<0.0001). This decrease was however attenuated and thus depletion of enzyme was significantly prevented (p<0.001) by *M. charantia* in the treatment group (Figure 5.1.10).

![Catalase Activity](image)

**Figure 5.1.10.** Effect of *M. charantia* on catalase enzyme after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.1.7. Histological Changes

Histological changes in retina, kidney, pancreas, heart and liver were observed to see the potential of *M. charantia* against diabetic complications. According to severity of the abnormal changes observed in the tissues, semi-quantitative histological grading was done as a) Nil (--) b) Mild (+) c) Moderate (+++) and d) Severe (+++). The observed changes are depicted in figures 5.1.11 – 5.1.15

**Retina:** High power photomicrograph (HE x 400) from each group showed a retinal blood vessel (Ret BV) from the retina (Figure 5.1.11). Semi-quantitative histological
grading was done based on the thickening of vascular basement membrane. Thin long endothelial cells were observed in the blood vessel wall. Normal vasculature was seen in retina of non-diabetic animal, whereas significant widening of vascular basement membrane was seen in diabetic rat and was graded as severe (+++). *M. charantia* treated animal showed no appreciable widening (Annexure 3-A).

![Figure 5.1.11. High power photomicrographs of retina of rats showing a retinal blood vessel (Ret BV) with thin long endothelial cells (HE x 400). (a) Normal (b) Diabetic control rat showing widening of vascular basement membrane (c) *M. charantia* treated](image)

**Kidney:** Renal parenchyma sections of kidney were stained with PAS. Changes in glomerulus were observed and were graded accordingly. Photomicrograph of renal parenchyma from normal group showed the glomerulus of normal size. There was no
increase in the mesangium as well as in the glomerular basement membrane (\( \rightarrow \)). On the other hand, abnormality in glomerular structure of diabetic control group was graded as severe (\( +++ \)) (Annexure 3-A). It represented a thick basement membrane and the glomerulus was filled with inflammatory mesangium cells (shown by arrow). However, section from the treatment group showed only mild increase in mesangium (\( + \)) in the glomerulus as compared to that in diabetic control (Figure 5.1.12, Annexure 3-A).

![Figure 5.1.12. High power photomicrographs of PAS stained section of kidney of rats (PAS x 400). (a) Normal (b) Diabetic control with glomerulus (G) loaded with mesangium cells represented by arrows (c) M. charantia treated](image)

**Pancreas:** HE stain was used for the section of pancreas. The changes were graded as nil, mild, moderate and severe on the basis of a) increase size of islet and b) presence of inflammatory cells which cause insulinitis (Annexure 3-A). Insulin-positive cells were
found in all three groups of animals. Non-diabetic rat showed a normal sized islet structure with sufficiently good number of beta cells. No lymphocytes or apoptotic bodies were seen in the pancreas of normal group (−). Diabetic rat on the other hand, showed a severe reduction in size of islet (+++). Beta cells were very few in number while inflammatory cells (lymphocytes) covered a major portion of the islet. A number of lymphocytes were found infiltrating in between the islet cells (+++). Treatment with *M. charantia* could not provide much protection to the pancreas. Size of islet in the treated group also reduced to a major extent (+++). However, it was able to protect beta cells and only a few lymphocytes were seen on the edge of the islet (+) as compared to that in diabetic control group (Figure 5.1.13).

![Figure 5.1.13](image)

**Figure 5.1.13.** High power photomicrographs of rat pancreas (HE x 400). Red boundary represents islet size (a) Normal control with large islet (b) Diabetic control with reduced islet structure and loaded with inflammatory cells (marked by arrows) (c) *M. charantia* treated
**Liver & Heart:** High power photomicrograph of liver and heart sections (stained with HE) showed the portal triad structures and coronary arteries respectively, in different groups (Figure 5.1.14 and 5.1.15). No significant changes were observed in liver or heart sections of any of the groups.

![Portal triad structures of rat liver](image1.png)

**Figure 5.1.14.** High power photomicrograph showing portal triad structures of rat liver. No significant changes were observed. (a) Normal (b) Diabetic control (c) *M. charantia* treated
5.1.1.8. Immunohistochemical Changes

Effect on Bax (proapoptotic) Protein Immunoreactivity

In order to establish a possible altered regulation of apoptotic gene transcription under various experimental conditions, immunocytochemistry was performed to localize pro-apoptotic protein Bax in retinas of normal, diabetic and treatment groups by using anti-Bax monoclonal antibody as shown in figure 5.1.16 a-c. The Bax protein’s immunoreactivity in the normal retina was found to be minimum. In diabetic control group the protein’s immunoreactivity was significantly enhanced as evidenced by intense staining of untreated diabetic retina. Ganglion cell positivity was mainly found in nuclear fiber layer (NFL) and outer nuclear layer (ONL), where maximum intensity of DAB (brown pigmentation) could be observed. However, with the treatment of M. charantia, Bax protein expression was significantly reduced.

Figure 5.1.15. High power photomicrograph of heart of rats showing normal cardiac muscle fibres and coronary artery (HE x 400). (a) Normal (b) Diabetic control (c) M. charantia treated
**Effect on Bcl-2 (antiapoptotic) Protein Immunoreactivity**

Bcl-2 protein was also localized in retinas by performing immunohistochemistry with the help of anti-Bcl-2 monoclonal antibody under defined experimental conditions. Similar to Bax, Bcl-2 positive retinas also showed brown stain mainly in nuclear fiber layer (NFL) and Inner nuclear layer (ONL). Whereas normal retina showed purpled coloured ONL of hematoxylin stain (Figure 5.1.6 d-f). However treatment with M. charantia resulted in increase of Bcl-2 expression.
Figure 5.1.16. High power photomicrograph of retina showing the pattern of staining for BCL-2 in the different layers of the retina (IHC x 40x). (a) Normal showing scanty expression of Bax (arrow) (b) Diabetic control showing intense retinal positivity for Bax (arrow) (c) M. charantia treated group showing occasional brown staining (arrow) for Bax positivity in retina (d) Normal retina showing strong positivity for Bcl-2 (arrow) (e) Diabetic control retina showing occasional positivity for Bcl-2 (arrow) (f) M. charantia treated group showing increased brown staining (arrow) for Bcl-2 positivity in retina.

NFL=Nerve Fibre Layer, IPL=Inner Plexiform layer, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.
5.1.2. Boerhavia diffusa

5.1.2.1. Effect of B. diffusa on Glycemic Parameters

A. Blood Glucose

At the end of 24 weeks study period, the blood glucose in normal group was 96.07±2.76 mg/dl. The blood glucose in diabetic control animals increased significantly (p<0.0001) to 423.15±49.28 mg/dl. However, oral administration of Boerhavia diffusa (BD) significantly suppressed the elevation of blood glucose level (p<0.001) by 15.20% (337.23 ± 42.05 mg/dl) in a period of 24 weeks as compared to untreated diabetic rats. (Figure 5.1.17).

![Blood Glucose Graph]

**Figure 5.1.16** Effect of Boerhavia diffusa on blood glucose. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Glycated Hemoglobin (HbA1c)

Percentage glycated hemoglobin level in the diabetic control group increased significantly as compared to that in the normal group (p<0.0001). However, %HbA1c was significantly reduced (p<0.001) in the diabetic rats treated with Boerhavia diffusa for a period of 24 weeks (Figure 5.1.18).
RESULTS - Type 1 DR

Figure 5.1.7. Effect of *B. diffusa* on %HbA1c. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.2.2. Effect of *B. diffusa* on Body Weight and Food and Water Intake

Table 5.3 shows the results of body weight and food and water intake. After 24 weeks of STZ induction, the percent change in body weight of the diabetic animals was significantly lower than that in the healthy animals (p<0.0001). However, change in body weight of diabetic animals treated with *B. diffusa* was significantly higher as compared to untreated animals (p<0.0001).

In spite of lesser weight gain due to STZ, diabetic control group exhibited higher consumption of food and water than that of other groups. BD treated diabetic animals presented significantly lesser intake of water and food (p<0.0001) than diabetic control animals.
Table 5.3 Effects of the oral administration of BD extract on the body weight and food and water consumption of STZ-induced type I diabetic rats

<table>
<thead>
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<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>B. diffusa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in BW (%)</td>
<td>55.21±4.98*</td>
<td>25.34±4.87</td>
<td>38.71±3.16*</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>33.08±4.56*</td>
<td>147.21±23.74</td>
<td>78.05±4.08*</td>
</tr>
<tr>
<td>Food intake (gm)</td>
<td>13.45±1.16*</td>
<td>35.81±1.24</td>
<td>26.75±2.07*</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

5.1.2.3. Clinical Ocular Examination

Normal group rat lenses had no signs and symptoms of cataract development. However, significant changes were observed in diabetic control group as compared to normal and treatment group of animals, when scored for cataract (Table 5.4; Fig. 5.1.19 a-c).

Normal retinal vessels are radial and almost straight (Fig. 5.1.19 d). After 24 weeks of diabetes, the retinal vessels of diabetic group became tortuous and dilated. Vessel diameter in diabetic group showed significant increase than that in normal group (Fig. 5.1.19 e; Table 5.4). The treatment group did not show any visible changes or tortuosity. The fundus in treatment group showed a normal structure and comparatively smaller diameter than diabetic group till the end of the study (Fig. 5.1.19 f; Table 5.4).
RESULTS - Type 1 DR

Figure 5.1.18. Photographs showing lenticular and retinal changes at the end of 24 weeks of diabetes

(a) Normal rat lens (b) Diabetic lens with cataract (indicated by arrow) (c) B. diffusa treated lens (d) Normal fundus (e) Tortuous Diabetic rat fundus (f) B. diffusa treated rat fundus

Table 5.4 Effects of the oral administration of B. diffusa extract on cataract score and vessel diameter of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>B. diffusa</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clock hours</td>
<td>0*</td>
<td>22*</td>
<td>8*</td>
</tr>
<tr>
<td>affected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vessel diameter</td>
<td>18.75±2.36*</td>
<td>35.96±1.05</td>
<td>26.75±0.99*</td>
</tr>
<tr>
<td>(Pixels)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

5.4.2.1 Fluorescein Angiography

Fluorescein angiography of normal rat retina showed well defined vessel structure. In BD treated group, fundus visibility was not as good as that of normal, but was far much better
than that of diabetic group. Reason for this disorientated visibility in diabetic animal might be due to emerging opacity as a result of cataract formation. However, neovascularization was not observed in any of the groups (Fig. 5.1.20).

Figure 5.1.19. Fluorescein angiogram of (a) Normal rat (b) Diabetic rat (c) B. diffusa treated rat

5.1.2.4. Effect of B. diffusa on Angiogenic Parameters

A. Vascular Endothelial Growth Factor (VEGF)

A significant increase of more than 4-folds (p<0.0001) in VEGF level of diabetic control group was observed in comparison with normal control animals. However, treatment with B. diffusa (BD) significantly (p<0.0001) reduced VEGF concentration approximately to half in the retina of the treated group as compared to that found in diabetic control group in a span of 24 weeks (Figure 5.1.21).
Figure 5.1.20. Effect of *B. diffusa* on VEGF after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. Diabetic control, One Way Analysis of Variance.

**B. Protein Kinase-C beta (PKC-β)**

Steep upregulation was observed in the concentration of protein kinase- C in diabetic control group as compared to normal group (p<0.0001). This upregulation was significantly attenuated by 57% reduction in retinal PKC level (p<0.0001) in the group treated for 24 weeks with *B. diffusa* (BD), as compared to that with untreated group (Figure 5.1.22).
5.1.2.5. Effect of *Boerhavia diffusa* on Inflammatory Parameters

**A. Tumor Necrosis Factor- alpha (TNF-α)**

The level of TNF-α exhibited more than 2-folds increase in the retina of diabetic control animals in comparison to that of normal control retinas (p<0.001). Treatment with *B. diffusa* significantly reduced the retinal TNF-α level by 34% (p<0.001) to that of untreated diabetic group (Figure 5.1.23).
Figure 5.1.22. Effect of *B. diffusa* on TNF-α after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Interleukin-1β**

The concentration of IL-1β in diabetic control group was found to be more than three times higher than that of normal control group (p<0.0001). However, in a span of 24 weeks, treatment with *B. diffusa* was able to prevent the increase in the level of cytokine significantly (p<0.0001) by about 57.4% as compared to that of diabetic control group (Figure 5.1.24).
Figure 5.1.23. Effect of *B. diffusa* on IL-1β after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.2.6. Effect of *B. diffusa* on Antioxidant Parameters

A. Reduced Glutathione (GSH)

A significant fall was observed in GSH level in the retina of diabetic control group as compared to normal control group (p<0.0001). However, *B. diffusa* was found to significantly (p<0.001) protect the depletion of the antioxidant GSH in retina of the treatment group (Figure 5.1.25).
RESULTS - Type 1 DR

Figure 5.1.24. Effect of *B. diffusa* on reduced glutathione (GSH) after 24 weeks. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Catalase

A significant depletion of catalase enzyme was observed in the retina of diabetic control animals as compared to that of normal control group (p<0.0001). Treatment with *B. diffusa* however provided a significant protection (p<0.001) to the enzyme activity and the enzyme level was restored by 73% in the treatment group as compared to diabetic control animals (Figure 5.1.26).
RESULTS - Type 1 DR

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>Boerhavia diffusa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase (IU/mg Protein)</td>
<td><strong>8.51</strong></td>
<td><strong>2.33</strong></td>
<td><strong>4.66</strong></td>
</tr>
</tbody>
</table>

Figure 5.1.25. Effect of B. diffusa on catalase enzyme activity after 24 weeks. Each value represents mean±S.D (n=6). ***p<0.0001 and **p<0.01 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.2.7. Histological Changes

Histological changes in retina, kidney, pancreas, heart and liver were observed to see the potential of Boerhavia diffusa against diabetic complications. The observed changes are depicted in figures 5.1.27 - 5.1.30.

Retina: High power photomicrographs (x 400) of HE stained retinal sections from each group were evaluated for semi-quantitative histological grading. Grading was done as nil (--), mild (+), moderate (++) or severe (+++) according to the increase in thickness of the retinal blood vessel (Ret BV). Non-diabetic rat showed vasculature with thin and long endothelial cells in the blood vessel wall with no abnormal increase in the basement membrane. The change in this group was graded as nil (--). In diabetic control retina appreciable widening (+++) of vascular basement membrane was seen and the abnormality was graded as severe (Annexure 3-A). However, retinal photograph of B. diffusa treated animal showed mild widening (+) in the basement membrane as compared to normal group, but was lesser than that in diabetic control group (Fig. 5.1.27).
Kidney: PAS stained sections of kidney of all three groups were observed for abnormal increase in inflammatory cells. Photomicrograph of renal parenchyma from normal group showed the glomerulus of normal size with no increase in mesangium cells and the abnormality was graded as nil (--) (Annexure 3-A). On the other hand, diabetic control group had thick glomerulus, loaded with sufficient number of mesangium cells (+++). Section from the *B. diffusa* treated group however showed only a mild increase in mesangium cells (+) as compared to that of diabetic control group. (Fig. 5.1.28).
**Pancreas**: Sections of pancreas were stained using hematoxylin and eosin (HE stain) and were observed for decrease in size of islets and increase in inflammatory cells. Section from non-diabetic rat showed a normal sized islet structure with a good number of beta cells (−−). Also no lymphocytes or apoptotic bodies were seen in the pancreas of normal rats (−−). On the other hand, size of islet sufficiently reduced in the diabetic control group and was graded as severe (+++). It showed very few beta cells but a large number of lymphocytes were found infiltrating in between the islet cells (+++). Even treatment with *B. diffusa* could not prevent the reduction in size of islet (+++). However, there was only a mild increase in presence of inflammatory cells in this group as compared to diabetic control group (Fig. 5.1.29, Annexure 3-A).
Figure 5.1.28. High power photomicrographs of rat pancreas (HE x 400). Red boundary represents islet size (a) Normal control with large islet (b) Diabetic control with reduced islet structure and loaded with inflammatory cells (marked by arrows) (c) B. diffusa treated
**Liver & Heart:** Hematoxylin and eosin (HE) was used to stain the sections of liver and heart. High power photomicrograph (400 X) of liver showed the portal triad structure and that of heart showed the coronary arteries in normal, diabetic control and treatment groups (Fig. 5.1.30 and 5.1.31). No appreciable changes were observed in sections of both the tissues in any of the groups.

![Liver and Heart photomicrographs](image)

Figure 5.1.29. Low power photomicrographs from section of liver from normal control group animal showing normal hepatic parenchyma. (HE x 40) (a) Normal control (b) Diabetic control (c) *B. diffusa* treated
Figure 5.1.30. High power photomicrograph of cardiac muscle and coronary artery (BV) of rat showing normal arterial wall and cardiac muscle fibres. (HE x 400) (a) Normal (b) Diabetic control (c) B. diffusa treated

5.1.2.8. Immunohistochemical Changes

Effect on Bax (proapoptotic) Protein Immunoreactivity

The apoptosis induced by high glucose implicates hyperglycemia as a causative factor in vivo, and thus, the role of Bax in the accelerated death of retinal cells induced by diabetes was established. Immunocytochemistry was performed using anti-Bax monoclonal antibody. The Bax protein’s immunoreactivity was minimum in the normal non-diabetic retina. However, the diabetic control group selectively altered the retinal BAX expression as it causes increased rates of apoptosis. The protein’s immunoreactivity was enhanced as shown by intense staining, and ganglion cell positivity was mainly found in nuclear fiber
layer (NFL) and outer nuclear layer (ONL). Bax protein expression was reduced in *B. diffusa* treated group (Figure 5.1.32 a-c).

**Effect on Bcl-2 (antiapoptotic) Protein Immunoreactivity**
Paraffin sections were immunostained with anti-Bcl-2 monoclonal antibody. Bcl-2 positive retinas also showed brown pigmentation mainly in nuclear fiber layer (NFL) and Outer nuclear layer (ONL). Purple coloured ONL was observed in normal retina which was due to hematoxylin (HE) stain (Figure 5.1.32 d-f). However treatment with *B. diffusa* resulted in comparatively increased Bcl-2 expression.
RESULTS

Figure 5.1.32. High power photomicrographs of retina showing the pattern of staining for BAX in the different layers of the retina (IHC x 40x). (a) Normal showing scanty expression of Bax (arrow) (b) Diabetic control showing intense retinal positivity for Bax (arrow) (c) *B. diffusa* treated group showing occasional brown staining (arrow) for Bax positivity in retina (d) Normal retina showing strong positivity for Bcl-2 (e) Diabetic control retina showing occasional positivity for Bcl-2 (f) *B. diffusa* treated group showing increased brown staining for Bcl-2 positivity in retina

NFL=Nerve Fibre Layer, IPL=Inner Plexiform layer, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer.
5.1.3. *Eugenia jambolana*

5.1.3.1. Effect of *Eugenia jambolana* on Glycemic Parameters

**A. Blood glucose**

Till the end of 24 weeks study period, the blood glucose in healthy non-diabetic rats did not deviate from the normal values (96.07±2.76 mg/dl). However it significantly increased by 4.7 folds in the untreated diabetic animals (p<0.0001). Treatment group showed a significant reduction in the blood glucose (p<0.01) as compared to diabetic control group on oral administration of *Eugenia jambolana* (Figure 5.1.33).

![Blood Glucose Graph](image)

*Figure 5.1. 33. Effect of *E. jambolana* on blood glucose. Each value represents mean ± S.D (n=6). **p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).*

**B. Glycated hemoglobin (HbA1c)**

As in case of blood glucose, %HbA1c in non-diabetic animals exhibited no attenuation from the normal range (4.45 ± 0.31). Glycated hemoglobin level in diabetic control group increased significantly (p<0.0001) as compared to normal group. However, investigational drug, *Eugenia jambolana* showed significant reduction in HbA1c (6.21 ± 0.56 %; p<0.01) in comparison to diabetic control group (Figure 5.1.34).
RESULTS - Type 1 DR

Figure 5.1.314. Effect of *E. jambolana* on %HbA1c. Each value represents mean±S.D (n=6).
***p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.3.2. Effect of *Eugenia jambolana* on body weight and food and water intake

Percent change in body weight over six months duration for all the groups is shown in table 5.5. Change in body weight of diabetic control rats was significantly lower than normal control group (p<0.0001). However the of EJ treated group showed significantly higher body weight than that of untreated rats (p<0.001).

Similarly, diabetic animals consumed lesser food and water as compared to normal group. However significantly higher consumption of food (p<0.05) and water (p<0.0001) was seen in treatment group as compared to diabetic control (table 5.5).

Table 5.5: Effects of the oral administration of *E. jambolana* extract on the body weight and food and water consumption of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th><em>E. jambolana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Change in BW (%)</strong></td>
<td>55.21±4.98***</td>
<td>25.34±4.87</td>
<td>35.16±2.93***</td>
</tr>
<tr>
<td><strong>Water intake (ml)</strong></td>
<td>33.08±4.56***</td>
<td>147.21±23.74</td>
<td>100.69±4.99***</td>
</tr>
<tr>
<td><strong>Food intake (gm)</strong></td>
<td>13.45±1.16***</td>
<td>35.81±1.24</td>
<td>33.97±2.49**</td>
</tr>
</tbody>
</table>
RESULTS

Type 1 DR

Each value represents mean ± S.D (n=6). ***p<0.0001; **p<0.001 vs Diabetic control

5.1.3.3. Clinical Ocular Examination

Rat from diabetic control group exhibited a strikingly increased incidence of cataract. Cataract originated from the lens epithelium and slowly occluded the whole lens causing opacity (Figure 5.1.35 b). However, no such signs developed in normal and EJ treated groups (Fig. 5.1.35 a & c).

Also a significant dilation of retinal vessels was seen in diabetic rats (Figure 5.1.35 e). There was a 48 % increase in vessel diameter of untreated rats (p<0.0001). Although, 38% increase in diameter of treated group was significantly lower than diabetic control (p<0.0001), but was more than that of normal animals (table 5.6).

Figure 5.1.35. Photographs showing lenticular and retinal changes at the end of 24 weeks of diabetes (a) Normal rat lens (b) Diabetic lens showing opacity due to cataract (c) E. jambolana treated lens (d) Normal fundus (e) Diabetic dilated fundus (f) E. jambolana treated rat fundus
Table 5.6 Effects of the oral administration of *E. jambolana* extract on cataract score and vessel diameter of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th><em>E. jambolana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of clock hours affected</strong></td>
<td>0*</td>
<td>22</td>
<td>12*</td>
</tr>
<tr>
<td><strong>Vessel diameter (Pixels)</strong></td>
<td>18.75±2.36*</td>
<td>35.96±1.05</td>
<td>30.19±1.37*</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

5.1.3.4. Effect of *Eugenia jambolana* on Retinal Angiogenic Parameters

A. Vascular Endothelial Growth Factors (VEGF)

The endothelial growth factor, VEGF was elevated by approximately 5-fold (p<0.0001) in the retina of diabetic rats in comparison to normal non diabetic animals. Supplementation with *E. jambolana* significantly prevented diabetes induced increase in the angiogenic factor (p<0.05) by 18% as compared to untreated diabetic animals (Fig. 5.1.36).
**Figure 5.1.36.** Effect of *E. jambolana* on VEGF after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and *p<0.005 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Protein Kinase-C (PKC)**

Significant elevated level of PKC-β (p<0.0001) was found in diabetic control group as compared to normal control group. However, treatment with *E. jambolana* showed prevention against increase in the cytokine level by 35.6% in the retina of diabetic animals of treated group (p<0.001; Fig. 5.1.37).
Figure 5.1.37. Effect of *E. jambolana* on PKC after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

### 5.1.3.5. Effect of *Eugenia jambolana* on Retinal Inflammatory Markers

**A. Tumor Necrosis Factor (TNF)-α**

More than 2-folds increase in the concentration of the inflammatory cytokine TNF- α was observed (p<0.001) in the retina of diabetic rats in comparison to non-diabetic animals. However, administration of *E. jambolana* inhibited diabetes-induced increase in the retinal level of TNF-α (p<0.05) in the treatment group over a period of 24 weeks of treatment (Figure 5.1.38) and the level reduced by about 27% as compared to untreated animals.
Figure 5.1.38. Effect of *E. jambolana* on TNF-α after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). **p<0.001 and *p<0.05 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Interleukin -1β (IL-1β)**

A highly significant rise was observed in the expression of IL-1β levels in the retina of diabetic control group as compared to normal control group (p<0.0001). The *Eugenia jambolana* treated group had shown more than 37% reduction in IL-1β expression as compared to untreated diabetic animals (p<0.001) and has produced an anti-inflammatory effect (Figure 5.1.39).
**RESULTS**

Type 1 DR

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-1β (pg/mg Protein)</th>
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</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>45.06</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>139.15</td>
</tr>
<tr>
<td>Eugenia jambolana</td>
<td>87.42</td>
</tr>
</tbody>
</table>

*Figure 5.1.39. Effect of *E. jambolana* on IL-1β after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001; and **p<0.001 vs. DC. One Way Analysis of Variance.*

5.1.3.6. Effects of *Eugenia jambolana* on Antioxidant Parameters

**A. Reduced Glutathione (GSH)**

Concentration of antioxidant glutathione determined the oxidative stress in the retina of rats diabetic for 24 weeks. The activity of glutathione enzyme was found to be significantly depleted in diabetic control group retinas in comparison to that of normal retinas (p<0.0001). However, *E. jambolana* was able to restore the level of GSH significantly in the treatment group (p<0.001) as compared to that in the diabetic control group (Figure 5.1.40).
### Figure 5.1.40. Effect of *E. jambolana* on reduced glutathione concentration after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and *p<0.05 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

#### B. Catalase enzyme

The retinal catalase activity of the diabetic control group was significantly reduced as compared to normal control group (p<0.0001). However, a significant defense against depletion of catalase enzyme was observed in the *E. jambolana* treatment group (p<0.01), and the enzyme activity was restored by 75.6% in comparison to diabetic control animals (Figure 5.1.41).
RESULTS - Type 1 DR

Figure 5.1.41. Effect of *E. jambolana* on catalase enzyme activity after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.3.7. Histological changes

*Retina:* High power photomicrograph (HE x 400) of retina of non-diabetic rats depicted normal vasculature of blood vessel with thin long endothelial cells. No widening of the vascular basement membrane was observed (−). However, an appreciable widening could be seen in retina of diabetic control animal as compared to that of normal control and this change was graded as severe (+++). In *E. jambolana* treated group, mild widening (+) was observed in retinal basement membrane (Figure 5.1.42; Annexure 3-A).
RESULTS - Type 1 DR

Figure 5.1.42. High power photomicrographs of retina of rats showing a retinal blood vessel (Ret BV) (HE x 400). (a) Normal group blood vessel (b) Diabetic control group shows significant widening of the vascular basement membrane (c) E. jambolana treated group shows blood vessel wall with mild widening.

**Kidney:** Sections of kidney were stained using Periodic Acid-Schiff (PAS stain) and observed under high magnification (PASx400) to observe and accordingly grade for any abnormal changes in glomerulus of normal, diabetic or E. jambolana treated animal. Non-diabetic group represented normal glomerular architecture (---). However, excessive presence of glomerular mesangium could be seen in diabetic control animal, and the abnormality was graded as severe (+++). The glomerulus from treatment group also showed moderate presence of mesangium cells (++) as compared to diabetic control glomerulus (Figure 5.1.43; Annexure 3-A).
Figure 5.1.43. High power photomicrographs of PAS stained section of renal parenchyma (PAS x 400) (a) Normal control group with glomerulus (G) of normal size and cellularity (b) Diabetic control group shows glomerulus loaded with mesangium cells (c) Glomerulus of E. jambolana treated group shows a moderate increase in mesangium

**Pancreas:** High Power photomicrograph (x 400) of hematoxylin and eosin stained rat pancreas from non-diabetic group showed a normal sized islet structure with a good number of beta cells. No lymphocytes or apoptotic bodies were observed (--). Whereas, pancreas of diabetic control animal presented sufficiently reduced islet structure with very few beta cells (+++). A large number of inflammatory cells were found infiltrating in between the islet cells (+++). Treatment with *E. jambolana* was not able to maintain normal size of islet (+++). However, comparatively fewer number of inflammatory cells were seen in the treatment group (++) as compared to diabetic control group (Figure 5.1.44, Annexure 3-A).
Figure 5.1.44. High power photomicrographs of pancreas of rat (HE x 400). (a) Normal islet (marked by red boundary) with no lymphocytes or apoptotic bodies present. (b) Diabetic group shows a reduction in islet structure with a very few beta cells. A No. of lymphocytes are seen infiltrating in between the islet cells. (c) *E. jambolana* treated group shows a small islet structure. A few lymphocytes are seen on the edge of the islet.
**Liver and Heart:** No significant changes were observed in both liver and cardiac sections among any of the three study groups of animals (Figures 5.1.45 - 46).

Figure 5.1.45. Low power photomicrographs from section of liver showing normal hepatic parenchyma (HE x 40). a) normal control group, b) diabetic control group and c) *E. jambolana* treated group
5.1.3.8. Immunohistochemistry

Effect on Bax (Proapoptotic) Protein Immunoreactivity
Immunocytochemistry was performed to establish the alteration in homeostasis of apoptotic gene transcription during the course of this study. Pro-apoptotic protein Bax was localized by using anti-Bax monoclonal antibody in the hemotoxylin and eosin stained sections of retinas of normal control, diabetic control and *E. jambolana* treated groups. Minimum immunoreactivity of protein was seen in the normal retina. However, a marked positive staining for Bax antigen was seen in ganglion cells of nuclear fiber layer (NFL) and outer nuclear layer (ONL) in diabetic control group. However, intensity of positive staining for Bax was lesser in *E. jambolana* treated group as compared to diabetic group (Figure 5.1.47 a-c).

Effect on Bcl-2 (Antiapoptotic) Protein Immunoreactivity
Immunohistochemistry was performed to localize Bcl-2 protein in retinas of the study groups. Anti-Bcl-2 monoclonal antibody was used for the same. As in Bax, Bcl-2 positive retinas also showed brown stain mainly in nuclear fiber layer (NFL) and outer nuclear layer (ONL) of diabetic control group. Thus, it represented maximum apoptosis. Normal
retina did not show brown pigmentation in the ONL. Immunoreactivity in the *E. jambolana* treated group was lesser as compared to that in untreated diabetic group (Figure 5.1.47 d-f).
Figure 5.1.47. Immunohistochemical staining of BAX and BCL-2 in retinae. (a) Normal group showing scanty expression of Bax (arrow) (b) Diabetic control showing intense retinal positivity for Bax (arrow) (c) *E. jambolana* treated group showing occasional brown staining (arrow) for Bax positivity in retina (d) Normal retina showing strong positivity for Bcl-2 (arrow) (e) Diabetic control retina showing occasional positivity for Bcl-2 (arrow) (f) *E. jambolana* treated group showing increased brown staining (arrow) for Bcl-2 positivity in retina

NFL=Nerve Fibre Layer, IPL=Inner Plexiform layer, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer
5.1.4. *Tinospora cordifolia*

5.1.4.1. Effect of *Tinospora cordifolia* on Glycemic Parameters

A. Blood glucose level

Blood glucose level in untreated STZ diabetic rats (423.15±49.28 mg/dl) was significantly higher than in the non-diabetic rats (96.07±2.76; p<0.0001). Oral administration of *Tinospora cordifolia* (TC) suppressed the elevation of blood glucose level to 352.22±47.78 mg/dl, which was significantly lower as compared to diabetic control animals (p<0.001; Figure 5.1.48).

![Graph showing blood glucose levels](image)

Figure 5.1.48. Effect of *T. cordifolia* on fasting blood glucose in type-1 diabetic rats. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Glycated hemoglobin (%HbA1c)

At the end of 24 weeks period, the glycated hemoglobin level in normal group was 4.45 ± 0.31%, while the same was 7.04 ± 0.48% in diabetic control group. The %HbA1c level of
the diabetic control group was significantly increased (p<0.0001), showing poor glucose control. However, in *T. cordifolia* (TC) treatment group, the %HbA1c level was 6.42±0.85, which was significantly less (p<0.01) as compared to diabetic control group (Figure 5.1.49).

![Glycated hemoglobin](image)

**Figure 5.1.49. Effect of *T. cordifolia* on %HbA1c in type-1 diabetic rats. Each value represents mean ± S.D (n=6). ***p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).**

**5.1.4.2. Effect of *Tinospora cordifolia* on Body weight and food and water intake**

Table 5.7 below illustrates the variations in body weight, food and water intake of normal control, diabetic control and diabetic treatment groups. STZ significantly reduced the body weight compared with normal control animals (p<0.0001). The extract of *Tinospora cordifolia* demonstrated a significant beneficial effect in ameliorating the weight loss.

Animals which received STZ also showed a significant increase in water and food intake as compared to normal control. The result was significantly reversed (p<0.0001) by the extract of *T. cordifolia* by the end of 24 weeks of treatment (Table 5.7).
Table 5.7 Effects of the oral administration of TC extract on the body weight and food and water consumption of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>T. cordifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Change in BW (%)</strong></td>
<td>55.21±4.98*</td>
<td>25.34±4.87</td>
<td>37.86±2.56*</td>
</tr>
<tr>
<td><strong>Water intake (ml)</strong></td>
<td>33.08±4.56*</td>
<td>147.21±23.74</td>
<td>81.36±6.42*</td>
</tr>
<tr>
<td><strong>Food intake (gm)</strong></td>
<td>13.45±1.16*</td>
<td>35.81±1.24</td>
<td>27.45±1.06*</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

5.1.4.3. Clinical Ocular Examination

A. Lenticular Changes

Normal group rat lenses had no signs and symptoms of cataract development. However, diabetic rats exhibited a strikingly increased incidence of cataract. In diabetic control group cataractogenesis appeared and invaded the central part of lens (Figure 5.1.50 a-c). On the other hand T. cordifolia protected the lens and exhibited marked amelioration of lenticular lesions. The animals were scored for cataract by counting the number of clock hours affected (Table 5.8). Cataractous changes were observed in diabetic control rat over a period of 24 weeks, which were absent in normal and significantly less (p<0.0001) in the treatment group.

B. Fundus visible changes

After 24 weeks of diabetes, a significant dilation and tortuosity of retinal vessels was seen in diabetic rats. However, fundus of treatment and normal groups presented clear vascular architecture (Figure 5.1.50 d-f).

Mean vessel diameter in normal (18.75 ± 2.36 pixels) was significantly higher than that of diabetic control group (35.96 ± 1.05 pixels; p<0.0001). Vessel diameter in TC treated group was measured to be 28.39 ± 0.72 pixels, which was significantly lower than diabetic rats (p<0.0001) (Table 5.8).
Figure 5.1.50. Photographs showing lenticular and retinal changes at the end of 24 weeks of diabetes (a) Normal rat lens (b) Diabetic lens showing cataract (c) TC treated lens (d) Normal fundus (e) Diabetic rat fundus showing tortuosity (f) TC treated rat fundus

Table 5.8 Effects of the oral administration of TC extract on cataract score and vessel diameter of STZ-induced type 1 diabetic rats (*p<0.0001)

<table>
<thead>
<tr>
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<th>Normal Control</th>
<th>Diabetic Control</th>
<th>T. cordifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clock hours</td>
<td>0*</td>
<td>22</td>
<td>9*</td>
</tr>
<tr>
<td>affected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vessel diameter</td>
<td>18.75±2.36*</td>
<td>35.96±1.05</td>
<td>28.39±0.72*</td>
</tr>
<tr>
<td>(Pixels)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control

C. Fluorescein angiography

Fluorescein angiogram of non-diabetic rat retina represented marked circulation of fluorescein dye into each vein of retina without any leakage or blockage in retinal vessel. This indicated proper blood flow in the eyes. However, fundus of diabetic control rat was not clearly visible due to cataractous changes appearing in anterior segment. Thus, path of flow of dye could not be easily seen. Smooth flow of dye is also hampered due to
ischemia in untreated diabetic animal. On the other hand, the fundus of *T. cordifolia* treated rat exhibited the fluorescein dye in the retinal architecture without any hindrance in the flow, thus indicating absence of ischemia or hypoxia (Figure 5.1.51).

![Fluorescein angiogram of the study groups at the end of 24 weeks](image)

Figure 5.1.51. Fluorescein angiogram of the study groups at the end of 24 weeks (a) Normal rat (b) Diabetic rat (c) TC treated rat

5.1.4.4. Effect of *Tinospora cordifolia* on Angiogenic Parameters

A. Vascular Endothelial Growth Factor (VEGF)

The mean VEGF value in diabetic rats’ retinas showed more than 4-fold increase than that of non-diabetic rats (*p*<0.0001). TC significantly reduced VEGF by 29% in treatment group as compared to diabetic control group (*p*<0.001) (Figure 5.1.52).
B. Protein Kinase - C (PKC-β)

A sharp increase in PKC-β value occurred in diabetic group as compared to normal group over 24 weeks of diabetes (p<0.0001). However, over a period of 24 weeks the PKC level reduced by half in the retina of treated rats (p<0.0001) as compared to that in untreated diabetic group (Figure 5.1.53).
**Figure 5.1.** 53. Effect of *T. cordifolia* on PKC after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

### 5.1.4.5. Effect of *Tinospora cordifolia* on Anti-inflammatory Parameters

**A. Interleukin-1β (IL-1β)**

Diabetic group exhibited about 3-times increase in the retinal IL-1β level to that of the normal control group (p<0.0001). However, there was a significant reduction of 50% in IL-1β value (p<0.0001) in the retina of *T. cordifolia* treated rats in comparison to untreated diabetic animals (Figure 5.1.54).
**Figure 5.1.** Effect of *T. cordifolia* on IL-1β after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Tumor Necrosis Factor (TNF) –α**

The diabetic control group rats had significantly increased expression of TNF-α as compared to normal control group. Treatment with *T. cordifolia* significantly showed 26.7% reduction (p<0.01) in level of anti-inflammatory marker when compared with diabetic control group (Figure 5.1.55).
Figure 5.1. 55. Effect of *T. cordifolia* on TNF-α after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). **p<0.001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.4.6. Effect of *Tinospora cordifolia* on Antioxidant Parameters

A. Reduced Glutathione (GSH)

Reduced GSH in the retina of normal animals was 9.42±1.23 nmol/mg protein (Figure 5.1.56). Significant depletion of this antioxidant (6.23±0.38 nmol/mg protein) was seen in diabetic animals. However, the GSH activity was restored by 39.5% in TC treated group (8.69±2.12 nmol/mg protein), which was significantly higher as compared to diabetic group (p<0.01).
Figure 5.1.56. Effect of *T. cordifolia* on glutathione (GSH) activity after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). **p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

**B. Catalase enzyme**

At the end of 24 weeks of study, the concentration of catalase enzymes was 8.51±0.74 IU/mg protein in retina of normal animals. However, this concentration depleted to about one-third in retina of diabetic control animals. *T. cordifolia* was able to provide significant defense to the retinal enzyme concentration (p<0.01) and restored about 2-times of the catalase activity in the treatment group than that of diabetic control group (Figure 5.1.57).
### Catalase Activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase (IU/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
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</tr>
<tr>
<td>Diabetic control</td>
<td>2.00 ± 4.00</td>
</tr>
<tr>
<td>T. cordifolia</td>
<td>5.06 ± 8.00</td>
</tr>
</tbody>
</table>

**Figure 5.1.57.** Effect of *T. cordifolia* on catalase activity after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 and *p<0.01 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

#### 5.1.4.7. Histological Changes

Histological changes in retina, kidney, pancreas, heart and liver were observed to confirm the potential of TC against diabetic complications. The observed changes are depicted in Figure 5.1.58 – 5.1.63.

**Retina:** High power photomicrographs (x 400) of the normal control, diabetic control and PHC treated groups depicted the blood vessel of hematoxylin and eosin stained section of retina (Ret BV). Retina of non-diabetic animal represented the vasculature with no abnormal changes (--). Whereas an appreciable widening of retinal vascular basement membrane was seen in diabetic rat and the change was graded as severe (+++). As compared to diabetic control group, retinal membrane of *T. cordifolia* treated animal showed only mild changes (+) (Figure 5.1.58; Annexure 3-A).
Figure 5.1.58. High power photomicrographs of retina showing retinal blood vessels (Ret BV) (HE x 400). (a) Normal control (b) Diabetic control showing widening of the basement membrane (c) TC treated

Kidney: Renal parenchyma sections of the study groups were stained using Periodic acid-Schiff (PAS) stain. Renal photomicrograph showed normal architecture of glomerulus in tissue section of non-diabetic rat (--). On the other hand, glomerulus of diabetic control group was overloaded with mesangium cells (+++). Section from the *T. cordifolia* treated group showed only mild presence of the inflammatory cells in glomerulus (+) as compared to untreated diabetic glomerulus (Figure 5.1.59; Annexure 3-A).
**Pancreas**: Sections of pancreas were stained using hematoxylin and eosin and were observed and accordingly graded for islet architecture and insulin-positive cells. Non-diabetic rat showed a normal size of the islet with sufficiently good number of beta cells present (--). No lymphocytes or apoptotic bodies were observed in the pancreas (--). On the contrary, large reduction in islet structure was seen in diabetic group with a very few number of beta cells (++++). A number of inflammatory cells in the form of lymphocytes were between the islet cells, leading to insulinitis (++++). Islet size was also found to be greatly reduced in *T. cordifolia* treated group (++++). However, inflammatory cells were
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comparatively lesser in the treatment group (++) than the diabetic control group. (Figure 5.1.60; Annexure 3-A).

Figure 5.1. 60. High Power photomicrographs of rats’ pancreas (HE x 400) a) Normal group islet with large size and inflammatory cells absent (b) Diabetic control showing reduced islet structure with many lymphocytes infiltrating between the islet cells. (c) *T. cordifolia* treated with few inflammatory cells

**Heart & Liver:** High power photomicrograph of HE stained heart and liver sections showed the coronary arteries and portal triad structures respectively (Figure 5.1.61 and 5.1.62). Coronary arteries showed normal arterial wall in heart section of all the study groups (HE x 400). Also no significant changes were observed in liver sections of any of the groups (HE x 400).
Figure 5.1. High power photomicrographs of heart of rats showing cardiac muscle fibers and coronary artery (HE x 400) (a) Normal (b) Diabetic control (c) T. cordifolia treated
5.1.4.8. Immunohistochemical Changes

The apoptosis induced by high glucose implicates hyperglycemia as a causative factor, thus the role of BAX and BCL-2 in diabetes-induced accelerated death of retinal cells was established.

**Effect on Bax (proapoptotic) Protein Immunoreactivity**

BAX immunoreactivity was seen in the inner retina, with specific staining detected in cells of the Nerve Fiber Layer (NFL) and Outer Nuclear Layer (ONL), the endothelial and medial layers of blood vessels. Maximum positive immunoreactivity was seen in retina of diabetic control animals in the NFL. It selectively altered the retinal BAX expression as it causes increased rates of apoptosis. However, staining intensity was reduced in *T. cordifolia* treated group (Figure 5.1.63 a-c).
Effect on Bcl-2 (antiapoptotic) Protein Immunoreactivity

Immunoreactivity due to BCL-2 was present in the retina, with specific staining detected in cells of the nuclear fiber layer. NFL of normal group retina showed least positivity. Maximum apoptosis was observed in the diabetic control group. Protein expression in *T. cordifolia* treatment group indicated prevention against apoptosis (Figure 5.1.63 d-f).
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Figure 5.1. 63. Immunohistochemical staining of BAX and BCL-2 in retinae. (a) Normal group showing scanty expression of Bax (arrow) (b) Diabetic control showing intense retinal positivity for Bax (arrow) (c) *T. cordifolia* treated group showing occasional brown staining (arrow) for Bax positivity in retina (d) Normal retina showing strong positivity for Bcl-2 (arrow) (e) Diabetic control retina showing occasional positivity for Bcl-2 (arrow) (f) *T. cordifolia* treated group showing increased brown staining (arrow) for Bcl-2 positivity in retina

NFL=Nerve Fibre Layer, IPL=Inner Plexiform layer, INL=Inner Nuclear Layer, ONL=Outer Nuclear Layer
5.1.5. Polyherbal Combination (PHC)

5.1.5.1. Effect of Polyherbal Combination on Glycemic Parameters

A. Blood glucose

Figure 5.1.64 shows the level of fasting blood glucose in normal, diabetic control and polyherbal combination (PHC) treatment groups. The diabetic rats showed more than four times increase in blood glucose when compared with normal animals (p<0.0001). On administration of PHC to diabetic rats for 24 weeks, the level of blood glucose in treatment group reduced significantly (p<0.001) by about 27% than that of diabetic control animals.

![Blood Glucose Graph](image)

Figure 5.1.64. Effect of PHC on fasting blood glucose. Each value represents mean ± S.D (n=6). ***p<0.0001 and **p<0.001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Glycated hemoglobin (HbA1c)

The level of glycosylated hemoglobin in different experimental groups is represented in Figure 5.1.65. The diabetic rats, when compared with non-diabetic group, showed a significant increase in the level of HbA1c (p<0.0001). The administration of PHC to
diabetic rats ameliorated the rise in hemoglobin level and reduced it significantly (p<0.001) as compared to diabetic control group.

![Glycated hemoglobin graph](image)

**Figure 5.1.** Effect of PHC on %HbA1c. Each value represents mean±S.D (n=6). ***p<0.0001 and **p<0.001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

### 5.1.5.2. Effect of Polyherbal Combination on Body Weight and Food and Water Intake

There was no significant difference in the initial weights of animals from the three groups (normal control, diabetic control and treatment groups). By the end of the study, STZ rats showed significantly lesser gain in weight than normal rats (p<0.0001). Treatment with PHC exhibited significant increase in body weight gain in comparison to untreated diabetic rats (p<0.0001, table 5.9).

Similarly, diabetic control animals had significantly higher intake of food and water than healthy rats. Treatment with polyherbal extract altered the food and water consumption in treated diabetic animals as compared to untreated one (p<0.0001, table 5.9).
Table 5.9 Effects of the oral administration of PHC extract on the body weight and food and water consumption of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>Polyherbal combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in BW (%)</td>
<td>55.21±4.98</td>
<td>25.34±4.87</td>
<td>43.54±4.14</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>33.08±4.56**</td>
<td>147.21±23.74</td>
<td>56.88±4.07</td>
</tr>
<tr>
<td>Food intake (gm)</td>
<td>13.45±1.16**</td>
<td>35.81±1.24</td>
<td>18.51±1.94</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). **p<0.001 vs Diabetic control

5.1.5.3. Clinical Ocular Examination

Eye from the healthy rat showed normal structure of lens and was scored zero for cataract development. In diabetic control group cataract originated in the capsular region and extended to margin with opacity beginning at the centre. However signs and symptoms of cataract development were absent in the PHC treated group (Table 5.10; Fig. 5.1.66 a-c).

Changes in vascular structure are presented in Figure 5.1.66 (d-f) and table 5.10. Retinal photographs taken by fundus camera showed a normal pattern in eye of non-diabetic animal with thin and clearly visible vessels. On the other hand retinal vessels from diabetic control group appeared to be dull, hazy and dilated, with vessel diameter significantly higher (p<0.0001) as compared to normal group. These changes were however ameliorated in the group treated with polyherbal extract. PHC treated fundus had significantly lesser vessel diameter than that of untreated diabetic animals (p<0.0001) and represented a marked vascular architecture.
Figure 5.1.66. Photographs showing lenticular and retinal changes at the end of 24 weeks of diabetes (a) Normal rat lens (b) Diabetic lens (c) PHC treated lens (d) Normal fundus (e) Diabetic rat fundus showing tortuosity (f) PHC treated rat fundus

Table 5.10 Effects of the oral administration of PHC extract on cataract score and vessel diameter of STZ-induced type 1 diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Control</th>
<th>Diabetic Control</th>
<th>Polyherbal combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of clock hours</td>
<td>0*</td>
<td>22</td>
<td>4*</td>
</tr>
<tr>
<td>affected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vessel diameter</td>
<td>18.75 ± 2.36*</td>
<td>35.96 ± 1.05</td>
<td>21.35 ± 1.46*</td>
</tr>
<tr>
<td>(Pixels)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± S.D (n=6). *p<0.0001 vs Diabetic control
RESULTS - Type 1 DR

**Fluorescein Angiography**

In fluorescein angiography (FA) of normal control animal, the dye appeared in the retinal vessels within few seconds of injection and the angiogram was clearly visible. Fluorescein angiogram of the diabetic control rat was blurred and the circulation time for fluorescein dye was high, which might be due to formation of micro-aneurysms. However there was no blockage in the angiogram of PHC treated rat and the circulation time of fluorescein dye was less, which appeared within few seconds in retina (Figure 5.1.67).

![Fluorescein Angiogram Images]

Figure 5.1. 67. Fluorescein angiogram of the study groups at the end of 24 weeks (a) Normal rat (b) Diabetic rat (c) PHC treated rat

**5.1.5.4. Effect of Polyherbal Combination on Angiogenic Parameters**

**A. Vascular Endothelial Growth Factor (VEGF)**

There was a significant elevation (p<0.0001) in retinal level of VEGF in STZ rats as compared to normal control group. However, supplementation of polyherbal extract to treatment group for 24 weeks resulted in significant reduction (p<0.0001) of VEGF expression by 69% in comparison to untreated diabetic animals (Figure 5.1.68).
Figure 5.1.68. Effect of PHC on VEGF after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. Diabetic control, One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Protein Kinase – C

PKC content of the retinal tissue in normal control, diabetic control and diabetic supplemented with PHC extract groups are shown in figure 5.1.69. PKC expression significantly increased in diabetic control rats with respect to normal animals (p<0.0001). However, treatment with polyherbal extract led to a significant decrease in this angiogenic expression (p<0.0001). PKC level in treatment group was three times lesser than that of diabetic control group.
5.1.5.5. Effect of Polyherbal Combination on Inflammatory Parameters

A. Tumor Necrosis Factor (TNF-α)

In span of 24 weeks of STZ induced diabetes, there was a significant increase (p<0.0001) in retinal tumor necrosis factor (TNF-α) expression in the diabetic group compared to normal control rats. Treatment with polyherbal extract (PHC) led to about 50% decrease in concentration of TNF-α (p<0.0001) as compared to the diabetic controls, and the cytokine level reached in closer proximity to normal control animals (figure 5.1.70).
RESULTS

Type 1 DR

Figure 5.1.70. Effect of PHC on TNF-α after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

B. Interleukin-1β

As compared to normal control group, the expression of Interleukin-1β (IL-1β) was three folds higher in diabetic control group (p<0.0001). Treatment of STZ diabetic rats with PHC extract led to a significant decrease in retinal level of cytokine IL-1β (p<0.0001) in comparison to diabetic control rats (figure 5.1.71).
Figure 5.1.71. Effect of PHC on IL-1β after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.5.6. Effect of Polyherbal Combination on Antioxidant Parameters

A. Glutathione activity

Retinal level of antioxidant glutathione (GSH) was significantly depleted (p<0.0001) in diabetic control group in comparison to normal control group. Supplementation of polyherbal extract for 24 weeks to the treatment group rats resulted in a significant increase of the GSH activity (p<0.0001) and the level of this antioxidant was resettled towards the normal level (figure 5.1.72).
B. Catalase activity

Similar to GSH, retinal level of catalase enzyme also decreased significantly in diabetic control rats with respect to normal (p<0.0001). After 24 weeks of PHC extract supplementation, there was a significant elevation in catalase enzyme activity (p<0.0001) in treatment group of diabetic animals as compared to their diabetic counterpart (figure 5.1.73).
RESULTS - Type 1 DR

**Figure 5.1.** Effect of PHC on catalase activity after 24 weeks of diabetes. Each value represents mean ± S.D (n=6). ***p<0.0001 vs. DC. One Way Analysis of Variance (Student-Newman-Keuls Method).

5.1.5.7. Histological Changes

*Retina:* Figure 5.1.74 represents the high power photomicrograph (HE x 400) of retina from normal control, diabetic control and PHC treated diabetic rat. Retinal blood vessel (Ret BV) from non-diabetic animal showed normal vasculature with thin, long endothelial cells. In untreated diabetic group, damage to endothelial cell wall due to STZ led to significant widening (+++) of vascular basement membrane. The damage was ameliorated in diabetic rats treated with PHC (--), in which no widening of basement membrane was visible (Annexure-3A).
Figure 5.1. 74. High power photomicrographs showing blood vessel (Ret BV) from the retina of rat. (HE x 400). (a) Normal control (b) Diabetic control with widened basement membrane (c) PHC treated

**Kidney**: Light microscopy of kidney was performed for all three experimental groups. Sections were stained with PAS and observed under high magnification (PASx400). Non-diabetic animal showed a normal pattern with well defined glomerular architecture, mesangium and tubules. On the other hand, glomerulus of diabetic control group was severely affected by large number of mesangial cells (+++). However, the section from treatment group represented no significant abnormal changes as observed in diabetic control group (figure 5.1.75; Annexure-3A).
RESULTS - Type 1 DR

Figure 5.1.75. High power photomicrographs of PAS stained sections of rats’ kidney (PAS x 400) (a) Normal control (b) Diabetic control showing mesangium loaded glomerulus (c) PHC treated showing few mesangium cells

Pancreas: Figure 5.1.76 represents HE stained pancreatic sections of normal control, diabetic control and PHC treated rats. Large size of islet was found in normal control group and apoptotic cells were absent. Most of the beta cells in diabetic group were destroyed and lymphocytes were observed (+++ along with reduced size of islet (+++). However, presence of inflammatory cells was reduced in PHC treated group (++) as compared to diabetic control. But the drug could not maintain the normal architecture (+++ of the islets (Annexure-3A).
Figure 5.1.76. High power photomicrographs of rat pancreas (HE x 400).  

a) Normal group islet with large size and inflammatory cells absent  
b) Diabetic control showing reduced islet structure with many lymphocytes infiltrating between the islet cells  
c) PHC treated with very few inflammatory cells.

**Liver & Heart:** No significant damage was observed to liver and heart of STZ - diabetic animals as observed under high power photomicrograph of HE stained sections of both the organs. The portal triad structures of liver and coronary arteries of heart of the diabetic rats revealed very mild thickness, but the change was not appreciable in comparison to PHC treated and normal controls (Figure 5.1.77 and 5.1.78).
Figure 5.1.77. High power photomicrographs of rats' liver. a) Normal (b) Diabetic (c) PHC treated

Figure 5.1.78. High power photomicrographs of heart of rat. (a) Normal (b) Diabetic (c) PHC treated
5.1.5.8. Immunohistochemical Changes

**Effect on Bax (proapoptotic) Protein Immunoreactivity**

To localize pro-apoptotic protein Bax in retinas of normal, diabetic and treatment groups, immunohistochemistry was performed using anti-Bax monoclonal antibody. NFL and ONL areas were mainly affected. Minimum immunoreactivity due to Bax protein’s was found in the HE stained section of retinas of normal group. As evidenced by intense brown pigmentation, protein’s immunoreactivity was appreciably increased in diabetic control group. However, Bax protein expression was found to be sufficiently reduced in the PHC treated group (Figure 5.1.79 a-c).

**Effect on Bcl-2 (antiapoptotic) Protein Immunoreactivity**

Retinal Bcl-2 protein was localized by performing immunohistochemistry with the help of anti-Bcl-2 monoclonal antibody under defined experimental conditions. It was observed that Bcl-2 positive retinae showed brown stain mainly in nuclear fiber layer (NFL) and Inner nuclear layer (ONL). Whereas normal retina showed purpled coloured ONL of hematoxylin stain (Figure 5.1.79 d-f). However, increase of Bcl-2 expression was found in the retina of PHC treated animal.
RESULTS- Type 1 DR

(a) NFL, IPL, INL, ONL

(b) NFL, IPL, INL, ONL

(c) NFL, IPL, INL, ONL
RESULTS - Type 1 DR

Figure 5.1. 79. High power photomicrographs of retina showing the pattern of staining for BAX (a-c) and BCL-2 (d-f) in the different layers of retina (IHC x 40x). Immunoreactivity present in the inner retina, with specific staining detected in cells of the NFL and ONL. DC group shows intense staining as compared to NC and PHC groups. It selectively alters the expression of BAX and BCL-2 in the retina as it causes increased rates of apoptosis.

NFL: Nerve Fibre Layer  
IPL: Inner Plexiform layer  
INL: Inner Nuclear Layer  
ONL: Outer Nuclear Layer