PART-III

Effect of Tribulus terrestris in Diabetic Nephropathy in STZ induced NIDDM in rats
Introduction
1.0 Introduction

*Tribulus terrestris* is a valuable herb known for its application in the folk medicine in various parts of the world. Some Unani physicians described it as “Akhrasul-Ujooj”, which means molar tooth of old lady (Kostova & Dinchev, 2005). This plant is extremely rich in substances having potential biological significance, including: saponins, flavonoids, alkaloids, and other nutrients (Wang et al., 1997)[1]. The fruit and root of *Tribulus terrestris* (Caltrop fruit) contains pharmacologically important metabolites such as phytosteroids, flavonoids, alkaloids and glycosides (Wu et al., 1996) [2]. Preparations based on the saponin fraction of *T. terrestris* are used for treatment of infertility and libido disorders in men and women, as well as for treatment of cardiac diseases. Food supplements containing *T. terrestris* extracts are marketed in USA and Europe with claim of a general stimulating action. The other reported pharmacogolical activities include antioxidant, antihypertensive, antimolluscicidal, aphrodisiac, antidiabetic, anthelmintic, anticancer, antifungal, antiurolithiatic, CNS stimulant activity (Khan et al., 2011) [3].

*Tribulus terrestris* Linn.

<table>
<thead>
<tr>
<th>Tibbi name:</th>
<th>Gokhru</th>
</tr>
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<tbody>
<tr>
<td>English name:</td>
<td>Caltrop</td>
</tr>
<tr>
<td>Botanical name:</td>
<td><em>Tribulus terrestris</em></td>
</tr>
<tr>
<td>Family:</td>
<td>Zygphyllaceae</td>
</tr>
<tr>
<td>Part used:</td>
<td>Seeds</td>
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*Figure 36:* Fruit of *T. terrestris.*
Figure 37: Leaves and flowers of *T. terrestris*.

**Active constituents**

Protodioscin, terrestrosins A-E, desgalactotigonin, F- gintonin, desglucolanatigonin, gitonin, tigogenin, furostanol glycosides, β-Sitosterol, spirosta-3,5-diene, stigmasterol, diosgenin, hecogenin, ruscogenin, Kaempferol, quercetin, tribulusamides A and B (Wu et al., 1999; Mahato et al., 1981).

**Medicinal Uses**

It is well known diuretic plant drug useful in urolithiasis, dysurea, impotence and kidney dysfunction. Goksura is extremely efficacious in most of the urinary tract disorders because it promotes the flow of urine, cools and soothes the membranes of the urinary tract, and aids in the expulsion of urinary stones and gout. It also stops bleeding from the tract and rejuvenates the urogenital system, both in males as well as females. In women, it is beneficial to the uterus, as it boosts the growth of uterus by its anabolic and rejuvenative properties. It is the best panacea for infantile or hypoplastic uterus. It helps to prevent abortion by checking the bleeding and nourishes the genital system. In the males, it is effectively used in impotency. Goksura effectively controls the bleeding, In large doses, it imparts the laxative action, hence is used as an adjunct in the treatment of piles. It is commonly used in treating diabetes, urinary calculi, dysuria, gout and sexual debility (Kritikar and Basu, 1975).
Pharmacological action

*T. terrestris* shows aphrodisiac, diuretic, antiseptic, anti-inflammatory, demulcent, nervine tonic, emenagogue, alterative, astringent analgesic activities. Plant and spiny fruits are used in the form of decoction or infusions in cases of spermatorrhea (Georgiev et al., 1988), phosphaturia, and diseases of the genitourinary system such as dysuria, gonorrhea, gleet, chronic cystitis, calculus affections, urinary disorders, gout, and impotence; also in uterine disorder after parturition, kidney diseases, and gravel. It is used in northern India in cough, and some diseases of the heart.

Recently, a new drug named “Xinnao Shutong” was manufactured from the crude saponin fraction of this plant having significant effects for the treatment of various cardiac diseases (Yang et al., 1991). It has also been shown that the aqueous extract of *T. terrestris* fruits possess significant ACE inhibitory effects in vitro (Somanandhan et al., 1999). Moreover, ACE inhibitors are among the widely used antihypertensive drugs in the treatment of patients with essential and renal complications (Unger et al., 1990). At the same time, hypoglycemic and hypolipidemic activity of *Tribulus terrestris* have also been reported (Tantawyet al., 2007).

However, in several parts of the world, plants containing steroidal saponins have been reported to cause hepatogenous photosensitisation of sheep (Flaøyen et al., 1997; Flaøyen et al., 2000). These plant species include *Agave lecheguilla*, *Tribulus terrestris*, *Brachiaria decumbens*, five Panicum spp., *Nolina texana* and *Narthecium ossifragum* (Abdelkader et al., 1984; Ender. 1955; Holland et al., 1991; Kellerman et al., 1991; Miles et al., 1993). A common feature in photosensitization diseases is the appearance of biliary crystals in liver and bile duct, identified as the insoluble salts of episarsa-sapogenin β-D-glucuronide and/or epismilagenin β-D-glucuronide (Flaøyen et al., 1997; Flaøyen. 2000). Uhlig et al., 2007, also showed that the aqueous extract from *N. ossifragum* contains saponins in concentrations that are low compared with that of plant, but high enough to be toxic to the renal epithelial cells (Unger et al., 1990). Recently, nephrotoxicity by the use of *T. terrestris*’s extract has also been reported (Talasaz et al., 2010).

Despite the evidences on the antihypertensive effect of *T. terrestris*, its beneficial effect and mechanism on kidney is not clear. Therefore, this study has been initiated with the aim to investigate the extent of protection offered by the aqueous extract of *T. terrestris* in developing or delaying the progression of renal complications in streptozotocin induced diabetes model.
AIM
Of
The Study
2.0 Aims and Objectives of the Study:

1. To determine the effect of *Tribulus terrestris* aqueous extract on insulin secretion and resistance (GLUT 2 and GLUT 4 protein expressions)
2. To evaluate the potential nephroprotective activity of *Tribulus terrestris* aqueous extract.
3. To evaluate the effect of *Tribulus terrestris* aqueous extract on Growth factors *i.e.* TGF-β and VEGF
4. To evaluate the effect of *Tribulus terrestris* aqueous extract on biochemical markers for kidney *i.e.* Total Protein, Albumin, TNF-α, NO, adiponectin and erythropoietin
5. To determine the effect of *Tribulus terrestris* aqueous extract on glomerular filtration rate using endogenous markers Serum creatinine, serum cystatin-c and beta 2 microglobulin.
6. To study the effect of *Tribulus terrestris* aqueous extract on structural and functional abnormalities
Materials
&
Methods
3. Material and Methods

3.1 Materials
Aqueous extract of *Tribulus terrestris* (50mg) dispersed in water with 1% CMC (obtained from Sanath Products, Delhi; India); Olmesartan medoxomil (Ranbaxy Lab. Ltd., India.); Glimepiride (Batch no. Po10743397; Provided by: Panacea Biotech ltd. India); Quantichrom creatinine Assay Kit (DICT-500), Rat Albumin ELISA by( ICL), Ultra sensitive Rat Insulin ELISA Kit( by Crystal chem. Inc.), Rat Erythropoietin ELISA Kit (Cusabiotech Co.), Rat beta-2 microglobulin (BMG, Cusabiotech Co.), Rat Cystatin C (Cys-C) ELISA Kit (Cusabiotech co.), Rat transforming growth factor β1 (TGF-β1) ELISA kit (Cusabiotech Co.), Rat TNF-α ELISA Kit (Raybio), Human/Mouse/rat Adiponectin Enzyme Immunoassay Kit (Raybio), Rat VEGF ELISA Kit (Raybio).

3.2 Animals
Healthy albino rats of Wistar strain were kept for breeding. To induce NIDDM, STZ (sigma chemicals, USA) (90 mg/kg) was administered *i.p.* to a group of 2 days old pups. Another group of pups received only saline. The pups were weaned for 21 days, and 6 weeks after the injection of STZ, the animals were checked for fasting glucose level (FPG) ≥ 160 mg/dl, which were considered as diabetic. Pups that receive saline were considered as control animals. All rats were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (*ad libitum*).

3.3 Eight week chronic daily dosing study
After 6 weeks, the animals were assigned to receive vehicle, *T. terrestris* extract (50 mg/kg/day dispersed in 1% CMC), olmesartan (6mg/kg/day) and glimepiride (1mg/kg/day) once daily for 8 weeks. In the morning, after final drug administration, blood samples were collected under fasting conditions and body weight was measured. The kidney was isolated and was fixed in phosphate- buffered 10% formalin solution to prepare a paraffin section.

3.4 Homeostatic model assessment for insulin resistance
The homeostatic model assessment (HOMA) is a method used to quantify insulin resistance and beta-cell function (Matthews et al., 1985). The approximating equation for insulin resistance, in the early model, used a fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant.

\[
\text{HOMA-IR} = \frac{\text{Glucose} \times \text{Insulin}}{405}; \quad \text{HOMA-%B} = \frac{20 \times \text{Insulin}}{\text{Glucose} - 63}
\]
Where IR is insulin resistance and %B is the β-cell function where Glucose is in mg/dl and Insulin in μU/mL (both during fasting).

3.5 GLUT-2 expressions in liver and GLUT-4 expressions in soleus muscle

Western Blot Analysis: 0.3 g of each liver and muscle slices were homogenized with ice-cold 10mM Tris–HCl buffer (pH 7.4) containing 1mM EDTA 2Na, 250mM sucrose, 1mM phenylmethyl sulfonyl fluoride and 1000 U/ml aprotinin, the homogenates were centrifuged at 600 rpm for 10 min at 4°C, and the supernatant was then centrifuged at 11000 rpm for 15 min at 4°C. In this study, the precipitate was designated as the total membrane fraction of the liver. The protein concentration of each sample was determined using BCA Protein Assay Kit.

For determination of glucose transporter 2 (GLUT2) protein expressions in liver and glucose transporter 4 (GLUT4) protein expressions in skeletal muscle; each sample prepared was mixed with 1% sodium dodecyl sulfate and 50mM dithiothreitol, and the mixture was subjected to electrophoresis with 10% polyacrylamide gel. The separated proteins on the gel were electrotransferred to a polyvinylidene difluoride membrane. The membrane was then reacted with anti-GLUT-2 antibody (Abcam, UK) & anti-GLUT-4 antibody (Abcam, UK) for 2 h. Subsequently, it was incubated with horseradish peroxidase conjugated IgG (diluted 1: 2000) [Jackson immunoresearch laboratories, USA] for 2 h at room temperature. The blots were detected qualitatively (Western Blot) (Yoshihiko et al., 2007)[16].

3.6 Measurement of renal function and biochemical parameters

Biochemical estimation for glucose, insulin, albumin and total proteins; glomerular proteins (viz β-2 microglobulin, serum cystatin c and serum creatinine) for the determination of GFR; inflammatory cytokines and growth factors like TNF-α, NO, TGF-β1 and VEGF and other kidney specific molecular markers involving adiponectin and erythropoietin were made in plasma/serum.

3.7 Histopathology

Kidney sections were stained with periodic acid-Schiff’s reagent and Masson’s modified trichrome to assess glomerulosclerosis and collagenous tubulointerstitial matrix, respectively (Kelly et al., 2007).

3.7.1. Glomerulosclerotic index

In 4μm kidney sections stained with periodic acid-Schiff’s reagent, 150 glomeruli from each animal were examined in a masked protocol. The extent of sclerosis in each glomerulus was
subjectively graded on a scale of 0 to 4, as previously described, with the following grades: grade 0 normal, grade 1 sclerotic area <25% (minimal), grade 2 sclerotic area 25–50% (moderate), grade 3 sclerotic area 50–75% (moderate to severe) and grade 4 sclerotic area 75–100% (severe).

A glomerulosclerotic index was then calculated using the formula:

\[
GSI = \sum_{i=0}^{4} F_i (i)
\]

Where GSI is glomerulosclerotic index, \( F_i \) is the % of glomeruli in the rat with a given score (i).

**3.7.2 Quantitation of matrix deposition**

The accumulation of matrix within the tubulointerstitial was assessed with blue area on Masson’s trichrome stained sections. The proportional area of tissue with the color range was then semi-quantified and graded by similar method performed for glomerulosclerotic index as tubulointerstitial matrix index (TIMI).

**3.7.3 Immunohistochemistry for Nephrin:**

Immunohistochemistry was done as previously described according to a modified method using a Polyclonal (C-Terminus) Antibody which is identical to rat nephrin. These experiments were done using 4 micron frozen kidney sections, as previously reported (Davis et al., 2003).

**3.8 DNA Fragmentation**

For detection and localization of apoptosis in kidney, we used the technique of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apo-BrdU-IHC™ In Situ DNA Fragmentation Assay Kit, Biovison, USA). Briefly, sections were deparaffinized, hydrated, and digested with proteinase K (20 μg/ml), and then added biotinylated dUTP to the 3' end of DNA fragments by incubating sections in 0.05 mol/l Tris–HCl buffer (pH 7.6) with 0.03 U/μl TdT and 0.04 nmol/μl biotin-11-dUTP at 37 °C for 1 h. The sections were rinsed in PBS. Endogenous peroxidase was blocked with 0.3% H₂O₂ in distilled H₂O. The sections were rinsed with PBS and covers with 2% blocking solution in 0.1 mol/l sodium maleate to reduce background staining. The sections were then incubated with avidin-peroxidase complexes in PBS (1:50) for 30 min and rinsed with PBS (3×5 min). Peroxidase activity was visualized with 3,3'-diaminobenzidine until the brown product was clearly visible. The sections were then counterstained with methyl green. The positive apoptotic cells were the cells with brown nucleus (Matsuno et al., 1997).

**3.9 Saponin analyses**
3.9.1 Qualitative detection using TLC:

A colorimetric method based on reactive anisaldehyde-sulphuric acid-acetic acid for thin layer chromatography (Fuentes-Alventosa et al., 2009)

**Foam test**: The extract (10 mg) was added to 10 ml of water and the solution was shaken for 30 sec with a vortex and left. The observance of foam in the water solution confirms the presence of saponins

**Liebermann- burchard test**: The extract (2 mg) was dissolved by four drops of acetic anhydride and then a drop of concentrated sulfuric acid was added to the solution. After 15 min, the bluish black color formed in the solution confirms the presence of saponins. (Jong-Keun Son. 1992)

3.9.2 Quantitative detection of Saponins

Protodioscin being major component of T.terrestris (Dinchev et al., 2008) was screened using LC-MS/MS. Frozen plasma and kidney samples were thawed to room temperature prior to extraction. Kidney samples were homogenized using phosphate buffer saline. Protodioscin was determined after protein precipitation by adding 500μl of tri-chloroacetic acid (TCA) to 100 μl of plasma and homogenate. Subsequently, the mixture was vortex mixed for 60sec, followed by centrifugation for 10min at 15000×. A 200 μl aliquot of the supernatant with 0.1% formic acid was injected into the LC-MS-MS system.

HPLC was performed on a Schimadzu system assembly with an Autosampler Plus. Protodioscin was standardized using the diluents Acetonitrile (ACN): Water (10:90) with 0.1% Formic acid (FA).

Protodioscin in the plasma and tissue extracts were separated on a Agilent XDB C18 column, 4.6X50mm, 1.8 μm pre-column filter. Elution proceeded by means of a gradient with a 0.8 ml/min flow rate using mobile phase A: Water with 0.1% FA, B: ACN with 0.1% FA at 40º C of Column temperature and Injection Volume of 10 μL for standards; 50 and 100 μL for samples. The gradient used in column B was 0.01min 20%, 2.00 min 20%, 6.00min 80%, 6.50 80%, 7.00 min 20%. The total run time was 7.5 min.

The HPLC was interfaced to AB Sciex Qtrap 5500 mass spectrometer (Thermo Electron). Ionisation was performed by electrospraying in the negative mode, and the source parameters were as follows: spray voltage, -4.5 kV; GS1 and GS 2 of 40 and 50psi. The
mass analyser was either operated in the full-scan mode (m/z 600-1100), or using data-depended scanning, meaning that the instrument successively performs a full scan and a MS² scan of the most intense ion. Linearity curve generated using multiquad software 2.1 on Qtrap 5500 LCMS system. MRM transitions for protodioscin were observed at 1047.7, 901.6, and 755.5. Normal & Diabetic plasma and kidney samples were analysed for the presence of protodioscin.

3.10 Statistical Analysis

All data are expressed as the mean ±S.E.M The differences in all parameters were analyzed by a one-way analysis of variance (ANOVA) followed by a Dunnett’s Multiple Comparison Test using sigma plot.¹¹ A change was considered statistically significant if P<0.05.
Results
4.0 Results

4.1 Effect of Tribulus terrestris extract on body weight and blood glucose

Before the treatment, there were no significant differences of baseline body weight of the rats (Table 19; fig 38). The change in body weight was not observed in Tribulus terrestris extract treated rats (202.5±8.03g) after 8 weeks when compared with diabetic group.

Before treatment, there was significantly higher fasting and post-prandial plasma glucose (p < 0.001) in diabetic and treated groups when compared with normal (Table 19; fig 39). After 8 weeks, groups treated with Tribulus terrestris extract showed significant reduction of fasting blood glucose [p<0.001; almost 16%] and post prandial glucose [p<0.001; almost 11%] as compared to diabetic group which remained nearly constant (Table 19; fig 39).

<table>
<thead>
<tr>
<th>Table 19- Effect of T.terrestris on body weight and blood glucose</th>
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<tr>
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<tr>
<td></td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>Diabetic</td>
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<tr>
<td>T.terrestris (50mg/kg)</td>
</tr>
<tr>
<td>Olmesartan (6mg/kg/day)</td>
</tr>
<tr>
<td>Glimepiride (1mg/kg/day)</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group. ###p < 0.001 vs. normal group, *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group.
Fig 38: Effect of *T. terrestris* on body weight

Fig 39: Effect of *T. terrestris* on Fasting and Post Prandial blood glucose

Results
4.2 Effect of *T. terrestris* on HOMA-Index, and β-cell function in diabetic rats

Aqueous extract of *T. terrestris* inhibited insulin resistance assessed by HOMA-Index and improved β-cell function but it was not significant when compared with the diabetic group. (figure 40; Table 20).

**Table 20- Effect of *T. terrestris* on HOMA-Index, and β-cell function**

<table>
<thead>
<tr>
<th></th>
<th>HOMA -INDEX</th>
<th>% BETA-CELL FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.47±0.137*</td>
<td>68± 70.5***</td>
</tr>
<tr>
<td>Diabetic</td>
<td>2.08±0.174</td>
<td>14.8± 0.799</td>
</tr>
<tr>
<td><em>T. terrestris</em></td>
<td>1.88±0.16</td>
<td>25.6±1.84</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>1.7±0.095</td>
<td>21.7±1.98</td>
</tr>
<tr>
<td>Glimepiride</td>
<td>1.53±0.144</td>
<td>35.6±5.24**</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from five animals in each group. *p < 0.05; ***p < 0.01; ****p < 0.001 vs. diabetic group.

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**Fig 40: Effect of *T. terrestris* on HOMA-IR and Beta cell function**
4.3 Effect of T. terrestris extract on the expression of GLUT2 in liver and GLUT-4 in soleus muscle
In diabetic rats, GLUT 2 expressions in liver and GLUT4 expressions in soleus muscle was reduced; whereas Tribulus terrestris extract treated rats did not show improvement in both GLUT2 and muscle GLUT-4 expression (Fig 41)

![Image: Effects of administration of aqueous extract of T. terrestris on GLUT-4 expression in the muscles (A) and GLUT-2 expression in the liver (B) of diabetic rats along with reference standards of glimepiride and olmesartan]

**Fig 41**: Effects of administration of aqueous extract of *T. terrestris* on GLUT-4 expression in the muscles (A) and GLUT-2 expression in the liver (B) of diabetic rats along with reference standards of glimepiride and olmesartan

4.4 Biochemical Markers:

4.4.1 Plasma Insulin
Tribulus terrestris extract at the dose 50mg/kg significantly increased plasma insulin (p<0.01) levels in the treated diabetic rats as compared with diabetic groups after 8 weeks (Table 21; fig 42).

4.4.2 Total Plasma Proteins and Albumin
Albumin (p<0.05) and total proteins (p<0.001) levels were found to increase significantly in plasma of Tribulus terrestris extract treated diabetic rats (Table 21; fig 42).
Table 21- Effect of Tribulus terrestris on Plasma Insulin, Total Proteins and Albumin

<table>
<thead>
<tr>
<th></th>
<th>Plasma Insulin (ng/ml)</th>
<th>Total Proteins (mg/ml)</th>
<th>Albumin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17.83±3.2***</td>
<td>2.144±0.076***</td>
<td>1.26±0.26*</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.265±0.36</td>
<td>1.368±0.17</td>
<td>0.1422±0.0151</td>
</tr>
<tr>
<td>Tribulus terrestris (50 mg/kg)</td>
<td>13.14±2.18**</td>
<td>2.108±0.13***</td>
<td>1.09±52.2*</td>
</tr>
<tr>
<td>Olmesartan (6mg/kg)</td>
<td>12.74±1.48***</td>
<td>2.123±0.11***</td>
<td>1.08±37.5*</td>
</tr>
<tr>
<td>Glimepiride (1mg/kg)</td>
<td>14.38±1.29***</td>
<td>1.956±0.09**</td>
<td>1.08±37.5*</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group.

Fig 42: Effect of Tribulus terrestris on Plasma Insulin, Total Proteins and Albumin
4.5 Glomerular Proteins and Glomerular Filtration rate

4.5.1 Serum Creatinine:
Diabetic control rats had elevated serum creatinine (Normal: 1.211 ±0.21mg/dl; diabetic: 3.59mg/dl), which was reduced in treatment with Tribulus terrestris extract, but not significant after 8 weeks chronic dosing (Table 22; fig 43).

4.5.2. Serum Cystatin-C
Serum Cystatin C has been increased in the diabetic rats. Treatment with predetermined dose of Tribulus terrestris extract to the streptozotocin induced diabetic rats decreased the Cystatin c levels in the serum (Table 22; fig 43).

4.5.3 Beta-2 microglobulin
The treatment with Tribulus terrestris extract caused significant decrease in the serum beta-2 microglobulin concentrations in comparison with the diabetic control group (Table 22; fig 43).

| Table 22- Effect of Tribulus terrestris on Glomerular filtration Rate |
|---------------------------|---------------------|---------------------|
|                          | Serum Cystatin C (ng/ml) | BMG (µg/ml) | Serum Creatinine (mg/dl) |
| Normal                   | 0.2298±0.026***      | 0.4419±0.048**     | 1.211±0.21***            |
| Diabetic                  | 0.644±0.033          | 1.362±0.12        | 3.589±0.18               |
| Tribulus terrestris (50 mg/kg) | 0.56±0.056          | 0.9352±0.14       | 2.886±0.31               |
| Olmesartan (6mg/kg)      | 0.4617±0.048*        | 0.6529±0.21*      | 2.063±0.41*              |
| Glimepiride (1mg/kg)     | 0.4098 ± 0.028       | 1.42±0.21         | 1.873±0.56**             |

The values are the means ± S.E.M. from eight animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group.
4.6 Effect of Garcinol on Inflammation and Growth Factors

4.6.1. TNF-alpha
The significant difference in the mean values of TNF-α observed in plasma between normal control and diabetic group (P<0.01). After the 8-week treatment with 50mg/kg dose of Tribulus terrestris extract, the levels of TNF-α significantly decreased when compared with the levels in diabetic rat (P<0.05; Table 23; fig 44).

4.6.2 Nitric Oxide
Nitric Oxide levels were significantly increased in the serum of diabetic rats (p<0.01). On treatment with Tribulus terrestris extract, nitric oxide levels were found to decrease in comparison with the diabetic group, though the decrease is not found to be significant. (Table 23; fig 44)
4.6.3 Transforming Growth Factor-β1 (TGF-β1)

TGF-β1 a key participant in the development of kidney sclerosis significantly differs in diabetic and normal control group (P<0.001). The Plasma concentration of TGF-β1 in T. terrestris extract treated rats was decreased but not significantly (P<0.001) when compared with the diabetic group after 8-weeks. (Table 23; fig 44)

4.6.4 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a critical component in the tissue growth and organ repair processes of angiogenesis and vasculogenesis. VEGF was significantly lower in the normal control groups than in the diabetic group (P<0.001). The Tribulus terrestris extract treatment reduced the plasma concentration of VEGF (P<0.01) (Table 23; fig 44) when compared with the diabetic group.

| Table 23- Effect of Tribulus terrestris on Inflammatory and Growth factors |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                 | TNF-alpha (pg/ml) | NO (nmol/µl)   | TGF-beta (pg/ml) | VEGF (pg/ml)   |
| Normal                          | 0.0176±0.00545** | 0.0176±0.00545** | 3.361±0.98***    | 48.5±3.05***    |
| Diabetic                        | 0.427±0.2198     | 0.427±0.2198    | 11.3±1.348       | 74.08±5.34      |
| Tribulus terrestris (50 mg/kg)  | 0.1055±0.041*    | 0.38±0.048      | 9.019±0.79       | 62.35±4.45      |
| Olmesartan (6mg/kg)             | 0.05665±0.013**  | 0.34±0.097      | 5.963±0.896**    | 66.3±1.14       |
| Glimepiride (1mg/kg)            | 0.08563±0.0167*  | 0.33±0.064      | 8.316±2.06       | 61.29±2.54      |

The values are the means ± S.E.M. from eight animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group.
4.7 Molecular markers

4.7.1 Adiponectin

Adiponectin measured as a surrogate marker for inflammation was significantly lower in diabetic group from normal control group (P<0.01). On treatment with Tribulus terrestris extract, adiponectin levels were increased, which were not significant. (Table 24; fig 45)

4.7.2 Erythropoietin

The erythropoietin concentration in plasma was found to increase significantly after treatment with Tribulus terrestris extract for 8 weeks when compared with diabetic group. (Table 24; fig 45)
### Table 24- Effect of Tribulus terrestris on molecular mediators

<table>
<thead>
<tr>
<th></th>
<th>Adiponectin (µg/ml)</th>
<th>Erythropoietin (mU/ml)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>3.013±0.21**</td>
<td>0.6775±0.026***</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.4±0.089</td>
<td>0.1283±0.064</td>
</tr>
<tr>
<td>Tribulus terrestris (50 mg/kg)</td>
<td>2.402±0.52</td>
<td>0.4796±0.037*</td>
</tr>
<tr>
<td>Olmesartan (6mg/kg)</td>
<td>0.93±0.18</td>
<td>2.277±0.86</td>
</tr>
<tr>
<td>Glimepiride (1mg/kg)</td>
<td>0.2657±0.047</td>
<td>0.3508±0.029</td>
</tr>
</tbody>
</table>

The values are the means ± S.E.M. from eight animals in each group. *p < 0.05; **p < 0.01; ***p < 0.001 vs. diabetic group.

**Fig 45**: Effect of *Tribulus terrestris* on molecular mediators
4.8 Histopathology:
In normal rats, the kidney cortex appeared normal (Fig. 39). In contrast, in diabetic control rats most glomeruli exhibited thickened GBM, capillary occlusion, and mesangial expansion; In addition, many cortical tubules were vacuolated. In diabetic rats treated with Tribulus terrestris extract, glomerular pathology was not observed to be improved (Fig.39).

4.8.1 Glomerulosclerotic index (GSI): Diabetes was associated with an increase in GSI compared to diabetic control rats; GSI was lowered in diabetic rats treated with Tribulus terrestris extract (Fig 46, 47; Table 25).

4.8.2 Quantitation of matrix deposition: In the tubulo-interstitium of kidney cortex or medulla, increased collagen and inflammatory cells were observed in diabetic control rats compared to control rats. Compared to diabetic control rats, interstitial fibrosis was lower in diabetic rats treated with Tribulus terrestris extract (Fig 46, 47; Table 25).

4.8.3 Nephrin Expression
Nephrin expression markedly decreased in diabetic kidney, was not improved by the administration of T.terrestris extract or glimepiride. However, the same was observed to be improved by the olmesartan treatment (Fig 46; Table 25).

4.9 Apoptosis
There was an evident increase in the DNA fragmentation in diabetic kidney compared to the normal kidney (Fig 46; Table 25). 48% of the fractional area was observed to have fragmented DNA. Whereas the kidney treated with Tribulus terrestris extract showed a clear decrease in the extent of DNA fragmentation. (Fig 46; Table 25)
**Fig 46**: Effect of 8 weeks dosing of *Tribulus terrestris* on histopathological along with immunocytochemical changes and cellular apoptosis (using TUNNEL positive cells) in the kidney of STZ diabetic rats. Periodic acid-Schiff’s reagent for glomerulosclerosis, Masson’s trichrome for...
tubular injury, Nephrin expression and methylene green stained (for TUNNEL positive) sections are represented for (A, B, C, D) normal rats, (E, F, G, H) vehicle-treated diabetic rats, (I, J, K, L) *tt- Tribulus terrestris*, (M, N, O, P) Olmesartan (6mg/kg/day) and (Q, R, S, T) Glimepiride treated diabetic rats respectively. Original magnification×400.[GBM: Glomerular Basement Membrane; TBM: Tubular Basement Membrane; I : Interstitium; T: Tubule]. (M) shows the index of glomerulosclerosis [ shown with GBM thickening (+), Mesangial sclerosis (*) and tubular membrane thickening (TBM @)] and (N) tubular injury (represented as tubulointerstitial matrix index) on the basis of qualitative degree of blue staining areas in the interstitium [ 0: No visible blue area, 1: few streaks, 2: Clearly visible, 3: definite patch, 4: pronounced and diffused blue stain] respectively.

**Fig 47**: (A) shows the index of glomerulosclerosis in Tribulus terrestris, glimepiride and olmesartan treated animals when compared with diabetic [shown with GBM thickening (+), Mesangial sclerosis (*) and tubular membrane thickening (TBM @)] and (B) Index of tubular injury (represented as tubulointerstitial matrix index) on the basis of qualitative degree of blue staining areas in the interstitium [ 0: No visible blue area, 1: few streaks, 2: Clearly visible, 3: definite patch, 4: pronounced and diffused blue stain] respectively. The percentage fractional area of nephrin for different groups as quantified using point counting grid method.
Table 25: Effect of Tribulus terrestris on histopathological parameters

<table>
<thead>
<tr>
<th>Name of Parameter</th>
<th>Normal</th>
<th>Diabetic</th>
<th>T. terrestris</th>
<th>Olmesartan (6mg/kg)</th>
<th>Glimepiride</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>0.6</td>
<td>4.2</td>
<td>4.2</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>TIMI</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nephrin (%FA)</td>
<td>70</td>
<td>3.7</td>
<td>2.5</td>
<td>62.5</td>
<td>20</td>
</tr>
<tr>
<td>Apoptosis (%FA)</td>
<td>2.5</td>
<td>87</td>
<td>48</td>
<td>40</td>
<td>90</td>
</tr>
</tbody>
</table>

4.10 Karyopycnosis and Cellular exfoliation

Further, histopathology also showed certain morphological changes in both the cortex and the medulla of the kidneys from rat treated with T. terrestris extract (Fig.48), characteristic of C. perfringens epsilon toxin. In the cortex, glomeruli showed apparent shrinkage resulting in dilatation of Bowman’s space. Proximal tubules were almost intact, while distal tubules and collecting ducts exhibited degenerative changes: a decrease in the height of epithelial cells with a dilated lumen and cellular degeneration with karyopycnosis and cellular exfoliation into the lumen (Fig 48).
Results

Fig 48: PAS stained sections of a rat kidney at 400X with the vehicle alone (A) or Diabetic kidney treated with T.terrestris (B to D). Note the shrinkage of the glomerulus (asterisks), epithelial cells exhibiting karyopycnosis (arrows), and cells exfoliated into the lumen (arrowheads) in the distal tubules and collecting ducts in the toxin group. (Photographs at magnification: (A) at 100 X & (B) at 400X).

4.11 Detection of Saponins:
Saponins were qualitatively detected positive in plasma and the kidney homogenate of the animals, which was confirmed by quantitative analysis LC-MS/MS, specifically for protodioscin. (fig 49-53). Saponin (that is unhydrolysed fraction of Protodioscin) was detected in plasma and kidney samples of both normal and diabetic rats administered with T.terrestris extract. The concentration of Protodioscin was found to be 17ng/100μl and 6 ng/ml of normal and diabetic plasma respectively; 4.9ng/ml and 0.9ng/ml of normal and diabetic kidney respectively.
Q1 MS scan showing parent m/z 1047.6 along with its formate adduct 1093.6 in ESI Negative Ion mode (Done by infusion)

![Q1 MS scan showing parent m/z 1047.6 along with its formate adduct 1093.6 in ESI Negative Ion mode (Done by infusion)](image)

**Fig 49**

Product Ion (MS2) Spectra for Protodioscin m/z 1047.6 Fragmentation

![Product Ion (MS2) Spectra for Protodioscin m/z 1047.6 Fragmentation](image)

**Fig 50**
Results

The Calibration curve with linearity range from 0.5 ng/ml to 500 ng/ml using MultiQuant software 2.1

Linearity range from 0.5 to 500 ng/ml
R²=0.99912

Peak review and calculated concentration (ng/ml) for Diabetic plasma and kidney samples

Standard Protodioscin
Diabetic Plasma (6 ng/ml)
Diabetic Kidney (0.9 ng/ml)

Fig 52
Peak review and calculated concentration (ng/ml) for Normal plasma and kidney samples

**Figure 53**

- **Standard Protodioscin**: 4.06 ng/ml
- **Normal Plasma**: 168 ng/ml
- **Normal Kidney**: 4.9 ng/ml
Discussion
5.0 Discussion

In the present study, we investigated the anti-hyperglycemic actions of Tribulus terrestris extract. Its effect on the renal variables and inflammatory consequences in the serum of STZ induced diabetic rat and apoptosis in kidney were also observed. Neonatal–STZ wistar model is a well characterized model for type 2 diabetes, in which persistent diabetes develops rapidly after 6 weeks of age (Daniel., 1991; Masiello et al., 1998; Weir et al., 1981). Further, when rendered diabetic with streptozotocin (STZ), the rats develop renal which is considered analogous to that seen in human diabetic nephropathy (Kelly et al., 1998). The dose selection in the present study was on the basis of a previous study (Tantawy et al., 2007). The results were further authenticated by the histopathological analysis of the kidney samples of the treated diabetic rats. The findings of the present study formed basis of the hypothesis that unhydrolysed saponins were absorbed by the rats dosed with T. terrestris extract, and elicited the unexpected kidney damage.

Most of the currently available anti-diabetic therapies reduce the fasting blood glucose but have little impact on postprandial hyperglycemia (Ratner., 2001). Treatment with Tribulus terrestris extract at the dose of 50mg/kg had a significant effect on both fasting and postprandial hyperglycemia in the diabetic rats. Body weight of the diabetic rats was found to be less during the course of development as compared to normal animals. Weight loss in diabetes is generally due to continuous excretion of glucose from the body (Sharma et al., 2005). However, the diabetic rats experienced approximately 81 % decrease in plasma insulin content, which was found to be significantly improved with the T. terrestris extract treatment (**P<0.01).

On the contrary, the expressions of glucotransporter isoforms GLUT 2 and GLUT4 in liver and muscle respectively, mediating whole body glucose disposal (Maria et al., 2001), were not improved by the administration of T. terrestris extract. Although the expressions were improved with olmesartan treatment, but not with glimepiride, being an insulin secretagogue. Hence, it can be suggested that although the extract was effective in reducing post prandial and fasting glucose along with the insulin concentration, its action in extra-pancreatic tissues involved in glucose homeostasis viz liver and muscle, does not seem to be regulated. Harmane and norharmane obtained from T. terrestris have been proposed to account for the hypoglycemic property of the extract by stimulating insulin secretion in a glucose-dependent manner (Narender et al., 2011).
The saponin characterized as tigogenin-3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-xylopyranoside showed potent antihyperglycemic activity in streptozotocin induced diabetic rats (Narender et al., 2011). Proteinuria, insulin resistance and formation of reactive oxygen species (ROS) are associated with loss of adiponectin level in diabetic rats (Nakamaki et al., 2011). The diabetic rats showed almost 87% decrease in adiponectin levels as compared to the normal rats. However, the findings of our study showed an improvement of adiponectin levels in the diabetic treated rats. Thus, the significant improvement in insulin secretion might be due to insulin sensitizing effect of adiponectin. Moreover, adiponectin is a surrogate marker for inflammation, which plays an important role in diabetic nephropathy, by the release of proinflammatory cytokines with the special participation of tumor necrosis factor – α (Navarro-González et al., 2009). Present study confirms the significant reduction in the levels of TNF-alpha after the 8 weeks dosing of *T. terrestris* extract when compared with diabetic group. However, the decrease is not more significant than with either glimepiride or olmesartan treatment. *T. terrestris* extract has also been shown to strongly inhibit iNOS expression and NO formation in LPS-stimulated RAW264.7 cells by Hong et al [28]. Similar results showing decrease in the total nitric oxide levels in serum was observed on treatment with *T. terrestris* extract when compared with diabetic. This could be attributed to increase in the renal expression of the p47phox component of NAD(P)H oxidase and eNOS, thereby decreasing the indices of systemic and renal oxidative/nitrosative stress as proposed by Sonta et al., 2005.

Renal functions were also evaluated with the treatment of aqueous extract of *T. terrestris* treatment. A decrease in the plasma proteins trigger pro-inflammatory and pro-fibrotic factors may directly contribute to chronic tubulo-interstitial damage (Allison. 2004). Consequently, an increase in protein levels was achieved. Although, increased urinary albumin excretion (UAE) is the hallmark of diabetic nephropathy (de Zeeuw et al., 2004), we choose serum albumin as an important marker for identification of progression of the renal damage. Lower serum albumin concentration imposes a greater increase in relative risk of mortality among populations (Kaysen et al., 2010). In our study, treatment of STZ induced diabetic rats with *T. terrestris*, prevented the development of albuminuria, which was evident from the increase in serum albumin levels and more than the standard treatment. On the contrary, reduced expression of nephrin in the diabetic
kidney when treated with *T.terrestris* that correlates with a loss of glomerular filter integrity (Pavensta et al., 2003).

Further, to substantiate the renal functions, measuring Glomerular Filtration Rate (GFR) is widely accepted. In clinical practice, an approximation of GFR is often obtained from plasma/urine creatinine concentration alone albeit with limited accuracy (Perrone et al., 1992). But, pure and reliable urinary samples are very challenging to obtain from experimental animals, especially from small rodents (Kurien et al., 2004). Moreover, serum creatinine is particularly insensitive for identifying chronic kidney disease at early and middle stages and also in certain patient groups (e.g. children, females, elderly) (David et al., 2005). It is considered only relatively specific but not very sensitive since its levels significantly increase only when more than 50% of the GFR is reduced. Measurement of freely filtered endogenous low molecular weight proteins viz. cystatin c or beta2-microglobulin (B2M) concentrations have been found to be advantageous over creatinine concentration, for the detection of an impaired GFR (Filler et al., 1997). We preferred to perform the analysis of the filtration markers in serum, as their serum concentration is less dependent on extra renal factors. From the present study, mild decrease which was not significant in the serum levels of creatinine, cystatin c and B2M was observed on treatment with *T. terrestris* extract, when compared with diabetic rats.

In early diabetic nephropathy, damage to the peritubular fibroblasts can occur, leading to erythropoietin deficiency and anemia prior to the loss of filtration. Correction of the anemia not only leads to an improved quality of life of patients but also reduces progression of kidney complications (Janet., 2006). The improvement of erythropoietin levels by *T. terrestris* treatment projects an extended ambit of *T. terrestris* in delaying the progression to renal complications.

It is interesting to note that in the present study, *T. terrestris* extract was not observed to reduce glomerulosclerosis and tubulointerstitial injury when compared with diabetic; however the reduction in serum values of TGF-β1 and VEGF were not compatible with the histopathological results. This was further supported by the decreased expression of nephrin in the diabetic kidney treated with the extract, that correlates with a loss of glomerular filter integrity (Pavensta et al., 2003). Recently, an Iranian male patient using the plant’s extract to prevent kidney stone formation has also been reported to develop nephrotoxicity by Talasaz *et al.*, 2010. The findings of the kidney histopathology in the present investigation on rats supported by the recent case report suggest that there must be some factor responsible for damaging kidney even though the
serum parameters were improved. This could be attributed to the absorption of unhydrolysed saponin fraction of the plant’s extract. The presence of saponins (Protodioscin as major constituent) in plasma and kidney homogenate of both normal and diabetic rats administered with the extract was observed qualitatively and confirmed quantitatively.

Several studies have shown that ingested saponins from saponin-containing plants are almost completely hydrolysed to sapogenins. Hence, kidney was not found to be a relevant route of excretion, for either saponins or their metabolites (Flaøyen., 1998). Notwithstanding, *T. terrestris* extract is a mixture of many different, and partly uncharacterized biomolecules, having varied biological activity. In rats, enzymatic hydrolysis commences after the saponins have been adsorbed to the erythrocyte membranes. However, *T. terrestris* extract possessing partial β-glycosidase inhibitory activity (Guerrero et al., 2004), prevent the hydrolysis of some fraction of saponins in the plant extract, which leads to its partial absorption in rats, thereby reaching the kidney. Actually, saponins are usually not associated with renal damage, but when absorbed, their membrane-permeabilising effect may possibly be detrimental to the renal epithelial cells. Apart from this, histological changes involving shrinkage of glomeruli along with karyopycnosis despite mesangial expansion was observed in certain parts of the kidney sections. These changes are characteristic of epsilon toxin. *C. perfringens*, epsilon-toxin present in the intestinal tract of vertebrates including rats, produces nephrotoxicity. Hyperglycaemia mediated increased intestinal permeability, which was further supplemented by *T. terrestris* extract, facilitate the entry of toxin inside the blood, which is transported to kidney. Its accumulation progressively increases toxicity in the kidney (Tamai et al., 2003). Hence, these observations suggest that hemodynamic and biochemical parameters might not be influenced significantly by the renal effects in the kidney.
Conclusion
6.0 Conclusion
Herbal medicines are being progressively used all over the world. Nevertheless, herbal remedies are not without hazards and several cases of adverse reactions have been described. The findings of the present investigation showed improvement of the renal variables in serum. But, the regression of histopathological observation of the kidney in the treated rat underpinned by an Iranian male patient case that lead to target specific renal damage became an eye opener. Hence, it can be said that 8 week dosing of *T. terrestris* extract showed toxicity in kidney that is independent its antidiabetic action. Furthermore, clinical trials needs to be undertaken to characterize and authenticate the use of *T. terrestris*, so that its potential usefulness in the renal complications can be better determined.