PART-II

Effect of Garcinol in Diabetic Nephropathy in STZ induced NIDDM in rats
Introduction
1. Introduction:

Garcinol is an organic compound, known for its various biological activities, is found in the fruit of the plant *Garcinia indica*. Garcinia is a large genus of evergreen dioecious trees, in several cases apomictic in origin (The wealth of India, 1956). Garcinia includes 200 species found in the World tropics, especially in Asia and Africa. Out of the 35 species found in India, 17 are endemic. In India, this plant is extensively found in the tropical rain forests of Western Ghats especially in the Konkan region of Maharashtra, Goa, coastal areas of Kerala, Karnataka and evergreen forests of Assam and Gujarat. It is known by various names: Bindin, Biran, Bhirand, Bhinda, Katambi, kokam, Punarpuli, Ratamba, or Amsool and in English language, Mangosteen, Wild Mangosteen, or Red Mango (Padhye et al., 2009).

The commonly reported Indian species are *G. atroviridi*, *G. cambogia*, *G. cowa*, *G. dulcis*, *G. echinocarpa*, *G. hombroniana*, *G. indica*, *G. lanceaefolia*, *G. livingstonei*, *G. mangostana*, *G. microstigma*, *G. morella*, *G. paniculata*, *G. pedunculata*, (Roberts 1984). Garcinia belongs to the family Clusiaceae (Guttiferae).

![Fig 22: Garcinia indica tree](image-url)
Taxonomic position of Garcinia (Linnaeus, 1753)

- **Kingdom**: Plantae Division
- **Class**: Magnoliopsida
- **Order**: Malpighiales
- **Family**: Guttiferae
- **Genus**: Garcinia

Garcinia possess wide range of applications in the fields of food (Burkill, 1936), pharmaceutical (Padhye et al., 2009) and other industries like soap, candles and cofectionery. Traditionally, the fruit rinds (peel) are widely used for flavoring curries, in south India. The juice of this fruit is used as a cool drink. Kokam butter, extracted from its seed is a demandable product in the market. Lonaval kokam, Pakali kokam, Khanee kokam and Khoba kokam are a few of the trade varities. Due to its significance in the field of pharmacology, *G. indica* is the most extensively researched species among the genus. Xanthones isolated from this genus showed various medicinal properties (Yamaguchi et al., 2000).

**Fig 23** *(a) Garcinia indica Fruit  (b) Dried fruit rinds*

*Garcinia indica* is a rich source of garcinol (molecular weight of 602), a polyisoprenylated benzophenone. These are a class of compounds containing a chain of isoprenoid chain attached to a benzophenone molecule with polyphenolic (OH) groups attached which makes this molecule unique and a powerful antioxidant too.
Garcinol shows strong structural resemblances with curcumin, a well-known antioxidant isolated from Turmeric (Pan et al., 2001). Garcinol shares similar properties with that of curcumin like that of solubility in organic solvents such as methanol, ethanol, chloroform, hexane etc.

Fig 24: Chemical structure of Garcinol

Garcinol is known to be a yellow pigment, which can be eluted as needle like crystals (Krishnamurthy et al., 1981). The dried rinds contain 2-3% of Garcinol by weight (Krishnamurthy et al., 1981, 1982). The chemical structure of garcinol is proposed by Sahu et al., (1989).

Garcinol is responsible for many bio-pharmacological activities such as antioxidant activity (Tamil Selvi et al., 2003), antibiotic (Bakana et al., 1987; Iinuma et al., 1996), anti-ulcer activities (Yamaguchi et al., 2000), ability to suppress colonic aberrant crypt foci (ACF) (Tanaka et al., 2000), formation and inhibit Histone Acetyltransferases (HATs), ability to induct caspases in human leukaemia HL-60 cells, and anti-inflammatory and anticarcinogenic properties {Yamaguchi et al., 2000, Ciochina et al., 2006, Pan et al., 2001, Sang et al., 2001, 2002}.

Thus, garcinol has emerged as an important therapeutic molecule in recent times (Padhye et al., 2009). Since the molecule is hydrophobic and water insoluble and is known to have therapeutic effects, it is important to understand the nature of its transfer in the human
circulatory system. Since time immemorial, the herbal healers of Goa and Maharashtra state in India have been using the kokum rind decoction in the treatment of diabetes and recent studies have validated its antidiabetic effects. Oral administration of the aqueous extract of the kokum rind (100 mg/kg and 200 mg/kg) for a period of 4 weeks to streptozotocin-induced type 2 diabetic rats has been shown to be effective in decreasing both fasting and postprandial blood glucose. Kokum also restored the levels of erythrocyte GSH, an intracellular antioxidant proved to be effective in preventing the risk of developing secondary complications and these observations suggest the usefulness of kokum in treating both hyperglycemia and other complications (kirana & Srinivasan., 2010). ‘Kokum’ contains 2-3%w/w of garcinol (Yamaguchi et al., 2000). Garcinol is a potent antioxidant and anticancer agent among its other biological effects. Its structure makes it a very efficient scavenger of oxygen free radicals and an excellent inhibitor of nitric oxide (Padhye et al., 2009). Also, in vitro studies have shown that garcinol suppressed the protein glycation in the bovine serum albumin/fructose system (Baliga et al., 2011).

Therefore, in light of the previous studies conducted, present study was initiated with the aim to substantiate the nephroprotective role of garcinol in the streptozotocin induced diabetic rats, thereby attenuating the progression of the diabetic nephropathy.
AIM
Of
The Study
2. **Aims & Objectives of the study**: 

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

3.1 Materials

Garcinol (gift sample: Sami Labs, Hyderabad -200mg/kg dispersed in water with 1% CMC); QuantiChromTM α-Glucosidase Assay Kit (by Bioassay systems. Catalog # DAGD-100); Quantichrom creatinine Assay Kit (DICT-500); Rat Albumin ELISA by (Immunology Consultants Lab, Inc.); 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 Measurement of α-glucosidase inhibitory activity in vitro

The α-glucosidase inhibitory activity was determined according to Earnst et al. (2005), by measuring the release of 4-nitrophenol from 4-nitrophenyl α-d-glucopyranoside (4-NPGP). The assay procedure was according to the protocol of a micro-well kit. The assay media contained 200µL (1mM) α-NPG substrate, 20 µL samples and 200 µL of calibrator. Absorbance of the reactants was measured at 405 nm using a microplate reader (Model 550, BIO-RAD Lab, Japan). The rate of reaction is directly proportional to the enzyme activity.

3.3 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 were considered as diabetic. Pups that received 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).

3.4 Eight week daily dosing study

After 6 weeks, the animals were assigned to receive vehicle or Garcinol (200 mg/kg dispersed in 1%CMC); Olmesartan (200mg/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 fixed in phosphate-buffered 10% formalin solution to prepare a paraffin section.

3.5 **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.

<table>
<thead>
<tr>
<th>HOMA-IR = (Glucose x Insulin)/405; HOMA-%B = (20 x Insulin)/(Glucose-63)</th>
</tr>
</thead>
</table>

Where IR is insulin resistance and %B is the β-cell function where Glucose is given in mg/dl and Insulin is given in μU/mL (both during fasting).

3.6 **GLUT- 2 expressions in liver and GLUT- 4 expressions in soleus muscle**

Determination of glucose transporter 2 (GLUT2) protein expressions in liver and glucose transporter 4 (GLUT4) protein expressions in skeletal muscle was done as previously described by (Yoshihiko et al., 2007).

3.7 **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.

3.8 **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).

3.8.1 **Glomerulosclerotic index**

In 4μm kidney sections stained with periodic acid-Schiff’s reagent, 150 glomeruli from each animal were examined. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4, 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:
Where GSI is glomerulosclerotic index, Fi is the % of glomeruli in the rat with a given score (i).

3.8.2 Quantitation of matrix deposition

The accumulation of matrix within the tubulointerstitial was assessed with Masson’s trichrome stain as a blue area on the stained section. The proportional blue colored area of tissue was then semi-quantified to determine the extent of matrix deposition.

3.8.3 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).

3.9 DNA Fragmentation Assay

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).

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. A change was considered statistically significant if P<0.05.
Results
4.0 Results

4.1 Invitro alpha-glucosidase inhibition

*In vitro* studies demonstrated that garcinol possesses α-glucosidase inhibitory activity. The drug clearly showed the decrease in the activity of alpha glucosidase enzyme in concentration dependent manner. Almost 50% inhibition of the enzyme was observed with the concentration of 1000µg/ml of garcinol. (Table 11; fig 25 A-B)

Table 11: Effect of different concentrations of Garcinol on inhibition of alpha glucosidase

<table>
<thead>
<tr>
<th></th>
<th>G0</th>
<th>G1</th>
<th>G10</th>
<th>G100</th>
<th>G1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (U/ml)</td>
<td>6.68</td>
<td>5.586</td>
<td>4.77</td>
<td>3.54</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Fig 25A-B: Activity and inhibition of enzyme alpha-glucosidase by Garcinol
4.2 Body weight and metabolic parameters in blood

When compared with diabetic group, no significant change in the body weight was observed in rats treated with *garcinol* extract after 8 weeks (202.5±8.03g) (Table 12; fig 26). Groups treated with *garcinol* extract showed significant reduction of fasting blood glucose \([p<0.001; \text{ almost 18%}]\), followed by that of olmesartan, reference standard for diabetic nephropathy \([14\%]\). However, the decrease was much less than antidiabetic reference glimepiride treatment \((\sim 27\%)\). Moreover, the reduction of post prandial glucose with *garcinol* treatment \((\sim 36\%)\) was much more than reference standard treatment of olmesartan \((\sim 10\%)\) but was lower than observed with glimepiride treatment \((\sim 43\%)\) when compared to diabetic group which remained nearly constant (Table 12; fig 27). Furthermore, plasma insulin \((p<0.01)\) levels were significantly increased, similar to that of olmesartan and less than glimepiride treatment in the treated diabetic rats as compared with diabetic groups after 8 weeks (Table 12; fig 27).

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>Normal</td>
<td>141.7±3.8</td>
<td>231.7±4.6***</td>
</tr>
<tr>
<td>Diabetic</td>
<td>131.7±4.4</td>
<td>198.3±3.1</td>
</tr>
<tr>
<td><em>Garcinol</em> (200mg/kg)</td>
<td>141.7±8.3</td>
<td>210.2±3.74</td>
</tr>
<tr>
<td><em>Olmesartan</em> (6mg/kg/day)</td>
<td>145.8±7.6</td>
<td>216.7±4.77*</td>
</tr>
<tr>
<td><em>Glimepiride</em> (1mg/kg/day)</td>
<td>140.8±5.6</td>
<td>208.3±3.1</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 26: Effect of Garcinol on Body weight

Fig 27: Effect of *Garcinol* on blood glucose (fasting & post prandial)
4.3 Effect of Garcinol on HOMA-Index, and β-cell function in diabetic rats

Garcinol inhibited insulin resistance assessed by HOMA-Index with significance when compared with the diabetic group. Moreover, β-cell function was significantly improved by Garcinol like that observed with glimepiride (figure 28 and table 13).

Table 13- Effect of Garcinol 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>Garcinol</td>
<td>1.55±0.0827*</td>
<td>31.4±3.17**</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.

Fig 28: Effect of Garcinol on HOMA-Index, and β-cell function
4.4 Expression of glucose transporters in liver and muscle

Reduced GLUT2 and GLUT 4 expression in liver and muscle respectively were improved with the *garcinol* treatment (Fig 29), which were better than observed qualitatively with reference standard olmesartan. Glimepiride, being insulin secretory in nature did not show any improvement in the expressions.

Fig 29: Effects of administration of aqueous extract of Garcinol 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

4.5 Renal function and Glomerular filtration rate

Improvement of Beta cell function also helps in improving insulin secretion from the beta cells of Pancreas, thereby qualifying its antidiabetic nature. To assess renal function, albumin (p<0.05) and total proteins (p<0.001) levels were found to increase significantly in plasma/serum of *garcinol* treated diabetic rats (Table 14, fig 30). The finding of total proteins was equivalent to that of olmesartan and better than glimepiride. However, for serum albumin, the findings were better than either of reference standards. To further substantiate GFR, treatment with *garcinol* extract significantly reduced serum levels of creatinine (p<0.01), cystatin c (p<0.01) and beta 2 microglobulin (p<0.001) (Table 15, fig 31). The results obtained were better than either of the reference standards used. The findings with the administration of extract were more significant than either standard. The area under the ROC curve for cystatin c was observed as 1.00 and that of creatinine was 0.8, indicating better diagnostic efficiency values for cystatin c than creatinine.
Table 14: Effect of Garcinol 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>Garcinol (200 mg/kg)</td>
<td>12.13±1.17**</td>
<td>2.05±0.14***</td>
<td>1.242±0.1324*</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 30: Effect of Garcinol on Plasma Insulin, Total Proteins and Albumin
### Table 15- Effect of Garcinol on Glomerular filtration rate

<table>
<thead>
<tr>
<th></th>
<th>Serum Cystatin C (ng/ml)</th>
<th>BMG (µg/ml)</th>
<th>Serum Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.2298±0.026***</td>
<td>0.4419±0.048**</td>
<td>1.211±0.21***</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.644±0.033</td>
<td>1.362±0.12</td>
<td>3.589±0.18</td>
</tr>
<tr>
<td>Garcinol (200 mg/kg)</td>
<td>0.2933±0.033***</td>
<td>0.3488±0.12***</td>
<td>1.804±0.36**</td>
</tr>
<tr>
<td>Olmesartan (6mg/kg)</td>
<td>0.4617±0.048*</td>
<td>0.6529±0.21*</td>
<td>2.063±0.41*</td>
</tr>
<tr>
<td>Glimepiride (1mg/kg)</td>
<td>0.4098±0.028**</td>
<td>1.42±0.21</td>
<td>1.873±0.56**</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.

### Results

![Graphs showing serum creatinine, cystatin C, and beta-2 microglobulin levels](image)

**Fig 31:** Effect of Garcinol on Glomerular filtration rate (Serum Creatinine; serum Cystatin c; beta 2 microglobulin)
4.6 Inflammatory markers and Growth Factors

However, the extent of inflammation was evident from the decrease in TNF-alpha levels in plasma of garcinol treated diabetic rats when compared with that of diabetic rat \((P<0.05; \text{ Table 16; fig 32})\). However, the decrease was more appreciable than observed with either olmesartan or glimepiride treatment. The values of TNF-alpha were further supported by decrease in nitric oxide levels when compared with diabetic group (Table 16; fig 32). Notably, the decrease in levels of nitric oxide with garcinol treatment though not significant was more than with either of the reference treatments. Moreso, the plasma concentrations of TGF-β1 and VEGF in garcinol treated rats were decreased significantly \((P<0.001)\) when compared with the diabetic group after 8-weeks (Table 16; fig 32). In addition, kidney specific molecular markers viz. adiponectin and erythropoietin were found to increase in plasma with garcinol treatment more than either of reference standards.( Table 16; fig 33)

<table>
<thead>
<tr>
<th></th>
<th>TNF-alpha (pg/ml)</th>
<th>NO (nmol/µl)</th>
<th>TGF-beta (pg/ml)</th>
<th>VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.0176±0.00545**</td>
<td>0.0176±0.00545**</td>
<td>3.361±0.98***</td>
<td>48.5±3.05***</td>
</tr>
<tr>
<td>Diabetic</td>
<td>0.427±0.2198</td>
<td>0.427±0.2198</td>
<td>11.3±1.348</td>
<td>74.08±5.34</td>
</tr>
<tr>
<td>Garcinol (200 mg/kg)</td>
<td>0.04059±0.0092**</td>
<td>0.32±0.026</td>
<td>3.908±0.82***</td>
<td>57.21±3.89**</td>
</tr>
<tr>
<td>Olmesartan (6mg/kg)</td>
<td>0.05665±0.013**</td>
<td>0.34±0.097</td>
<td>5.963±0.896**</td>
<td>66.3±1.14</td>
</tr>
<tr>
<td>Glimepiride (1mg/kg)</td>
<td>0.08563±0.0167*</td>
<td>0.33±0.064</td>
<td>8.316±2.06</td>
<td>61.29±2.54</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 32: Effect of Garcinol on Inflammatory and Growth factors
### Table 17 - Effect of Garcinol on molecular mediators

<table>
<thead>
<tr>
<th></th>
<th>Adiponectin (µg/ml)</th>
<th>Erythropoeitin (mIU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.393±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>Garcinol (200 mg/kg)</td>
<td>3.297±0.56**</td>
<td>0.5141±0.13**</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.

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**Results**

Fig 33: Effect of Garcinol on molecular mediators
4.7 Histopathology

In contrast histopathology showed improved thickening of glomerular basement membrane in most glomeruli, reduced capillary occlusion, and mesangial expansion in diabetic rats treated with *garcinol* (Fig 34, 35; Table 18). Consequently, the decrease in GSI observed in treatment with garcinol was comparable to olmesartan followed by glimepiride. Also, like glimepiride, in garcinol treated rats, collagen is visible in tubulo-interstitium of kidney cortex or medulla unlike in diabetic control rats. Hence, TIMI was decreased with *garcinol* treatment similar to glimepiride. However, the improvement in tubular injury was marked with olmesartan treatment (Fig 34, 35; Table 18). In support to this, nephrin expression which was markedly decreased in diabetic kidney was observed to be improved by the garcinol treatment qualitatively better than treatment with olmesartan or glimepiride (Fig 34; Table 18).

Further, decrease in the DNA fragmentation was clearly observed qualitatively in the kidney of diabetic rats treated with *garcinol* and olmesartan. However, no change in the decrease in apoptosis was observed with the glimepiride treatment (Fig 34; Table 18).
Fig 34: Effect of 8 weeks dosing of Garcinol on histopathological changes and cellular apoptosis (using TUNNEL positive cells) in the kidney of STZ diabetic rats. Periodic acid-Schiff’s reagent-stained, Masson’s trichrome-stained, 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) Garcinol, (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 35: (A) shows the index of glomerulosclerosis in Garcinol, 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. Percentage fractional area of nephrin occupied by different groups in comparison with Garcinol estimated by grid point counting method.
### Table 18: Effect of Garcinol on histopathological parameters.

<table>
<thead>
<tr>
<th>Name of Parameter</th>
<th>Normal</th>
<th>Diabetic</th>
<th>Garcinol (200mg/kg)</th>
<th>Olmesartan (6mg/kg)</th>
<th>Glimepiride (1mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>0.6</td>
<td>4.2</td>
<td>1.4</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>TIMI</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nephrin (%FA)</td>
<td>70</td>
<td>3.7</td>
<td>50</td>
<td>62.5</td>
<td>20</td>
</tr>
<tr>
<td>Apoptosis (%FA)</td>
<td>2.5</td>
<td>87</td>
<td>20</td>
<td>40</td>
<td>90</td>
</tr>
</tbody>
</table>
Discussion
5.0 Discussion

In the present study, nephroprotective activity of garcinol was investigated. The antidiabetic profile of garcinol in vitro and in vivo was outlined along with the renal variables and the extent of apoptosis in kidney.

Alpha-glucosidase inhibitors are the saccharides that act as competitive inhibitors of enzymes, needed to digest carbohydrates in the brush border of small intestine. The membrane bound intestinal alpha-glucosidases hydrolyze oligosaccharides, disaccharides and trisaccharides, to glucose and other monosaccharides. Inhibition of these enzyme systems reduces the rate of digestion of carbohydrates. Less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In diabetic patients, short-term effect of these drug therapies is to decrease the blood glucose levels. The use of α-glucosidase inhibitor for controlling rise in postprandial glucose level is desirable as it constitutes a non invasive mechanism. STOP

NIDDM study published in 2002 has revealed the advantage of alpha glucosidase inhibitors in prevention of progress of potential diabetic patients into type II diabetes in addition to controlling the risk of cardiovascular damage (Abesundara et al., 2004).

Diabetic patients generally suffer from hyperglycemic shoot up after meals, which take almost 4-5 hours to reduce back to the original glucose level. The shoot up is attributed to the increased disaccharidases activity 1.5-fold (Tormo et al., 2002) in diabetic animals as compared to normal (Kwon et al., 2007). The increased glucose level for prolonged time leads to non-specific glycation of proteins initiating a cascade of secondary complications (Peppa et al., 2003). Hence, control of postprandial glucose levels would be valuable in prevention of secondary complications of diabetes. Polyphenols are known to inhibit the activity of digestive enzymes such as amylase, glucosidase, pepsin, trypsin and lipases (Rohn et al., 2002) similar to acarbose, miglitol and voglibose, leading to a decrease in post-prandial hyperglycemia (Bailey. 2001).

From our data, garcinol was observed to inhibit the alpha glucosidase enzyme in vitro in concentration dependent manner. Moreso, percentage inhibition was found to increase by increasing the concentration of garcinol. Moreso, because of its antiulcer properties in gastric mucosa (Hong et al., 2007), garcinol is expected to produce lesser side effects as that observed with acarbose such as flatulence, intestinal disturbances.

Hence, garcinol possesses alpha glucosidase inhibitory activity, thereby lowering postprandial glucose absorption and hence can be used for the treatment of diabetes.
The in vivo findings were also compared with a reference antidiabetic drug, glimepiride and a standard drug for diabetic nephropathy, olmesartan medoxomil. Neonatal–STZ wistar model is a well characterized model of type 2 diabetes, in which persistent diabetes develops rapidly after 6 weeks of age (Arulmozhi., 2004; Daniel., 1991; Masiello et al., 1998; Weir et al., 1981), wherein rats develop renal complications analogous to that seen in human diabetic nephropathy (Kelly et al., 2007). The dose selection has been done on the basis of a previous study (Yamaguchi et al., 2000).

Treatment with Garcinol extract at the dose of 200mg/kg had a significant effect on both fasting and postprandial hyperglycemia in the diabetic rats. Body weight of the diabetic rats was also found to be less during the course of development as compared to normal animals, which is due to continuous excretion of glucose from the body (Sharma et al., 2005). Besides that, decrease in plasma insulin content in the diabetic rats was found to be significantly improved with the garcinol treatment (**P<0.01). These findings were further supported by the qualitatively improved expressions of GLUT 2 and GLUT4 in liver and muscle by garcinol treatment to the diabetic rats, mediating whole body glucose disposal (Maria et al., 2001). These results qualify the anti-diabetic nature of garcinol. Its action in extra-pancreatic tissues and its participation in the overall glucose homeostasis in liver and muscle seem to be directed to the translational or posttranslational level. This could be attributed to polyisoprenylated benzophenone structure of garcinol, possessing a natural histone acetyltransferase (HAT) inhibiting activity, thereby repressing chromatin transcription and hence altering the gene expression. High glucose activates HAT (p300), which in turn acetylates p65 and suppresses histone deacetylase HDAC2 resulting in NF-κB activation and increased transcription of IL-6 and TNF-α in monocytes. In addition, sugar-modified histones can undergo other transformations to form advanced glycosylation end products (AGEs). AGE accumulation associated with histones and other proteins are known to be implicated in the progression of diabetes (Balasubramanyam et al., 2004). Hence, the stimulating action of garcinol on GLUT2 and GLUT4 expression could be a mechanism by which, at least in part, the garcinol exerts its lowering effect on blood glucose. At the same time, reduction of apoptosis was also observed in kidney, which supports the earlier findings (Balasubramanyam et al., 2004), wherein apoptosis causing specific genes were downregulated by garcinol.
Proteinuria, insulin resistance and formation of reactive oxygen species (ROS) are associated with loss of adiponectin level in diabetic rats. 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 (Hsu et al., 2011). The present study confirms the significant reduction in the levels of TNF-alpha, after the 8 weeks chronic dosing of garcinol when compared with diabetic group; thereby inhibiting apoptosis. Interestingly, the decrease is equally significant to olmesartan, but more than with glimepiride treatment. Garcinol has also been shown to strongly inhibit iNOS expression and NO formation in LPS-stimulated RAW264.7 (Padhye et al., 2009). Similar results showing decrease in the total nitric oxide levels in serum was observed on treatment with garcinol 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 (Sonta et al., 2005).

Renal functions were also evaluated with the treatment of aqueous extract of garcinol 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 &Don., 2010). In our study, treatment of STZ induced diabetic rats with garcinol, prevented the development of albuminuria, which was evident from the increase in serum albumin levels and more than the standard treatment. Hence, the decreased expression of nephrin found in STZ induced diabetic rats that correlates with a loss of glomerular filter integrity (Pavensta et al., 2003), was improved by olmesartan treatment.

Further, to substantiate the renal functions, measurement of 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, 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, significant decrease in the serum levels of creatinine, cystatin c and B2M was observed on treatment with garcinol 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 et al., 2006). The improvement of erythropoietin levels by garcinol treatment projects an extended ambit of garcinol in delaying the progression to renal complications.

Besides, kidney also has a finite capacity to regulate intraglomerular pressure by the local synthesis of angiotensin II. This raises intra-glomerular pressure further and induces the expression of fibrotic growth factors TGF-β1 and VEGF via MAPK p42/p44, thereby up regulating molecules like fibronectin, collagen-1, and plasminogen-activator inhibitor-1 (PAI-1) (Chen et al., 2006; Huang et al., 2006) leading to glomerulosclerosis (Brenner, 1983). In the present study, garcinol reduced glomerulosclerosis significantly, which is in turn supported by significant reduction of TGF-β1 and VEGF. Garcinol treatment attenuated tubulointerstitial fibrosis, another important predictor of renal dysfunction (Kelly et al., 2007). The finding was explained by wherein prevention of desmin expression preserves podocytes and protects against interstitial fibrosis (Izuhara et al., 2005).
Conclusion
6.0 Conclusion
In conclusion, present investigation revealed that Garcinol is efficacious in improving glucose homeostasis and metabolic profile in rat model of diabetic mellitus with the defects in insulin sensitivity and secretion. The beneficial effect of Garcinol on diabetic nephropathy is clearly associated with significant increase in the expression of nephrin, decrease in thickening of glomerular basement membrane and hence improving glomerular filtration rate. Allopathic drugs, being not completely safe in the treatment of diabetic nephropathy, these finding suggest the usefulness of garcinol for further development as a therapeutic agent in diabetic nephropathy.