Attenuation of renal dysfunction by anti-hyperglycemic compound isolated from fruit pulp of *Eugenia jambolana* in streptozotocin-induced diabetic rats

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The renal protective effect of an active principle isolated from the aqueous extract of fruit pulp of *Eugenia jambolana* was investigated in streptozotocin (45 mg/kg body weight)-induced severely diabetic rats (FBG ≥ 300 mg/dl). For isolation of active principle, crude aqueous extract of *E. jambolana* fruit pulp was subjected to purification by ion-exchange column chromatography, which yielded a partially purified compound (FII), which on further purification by rechromatography gave a purified active compound (FIIc). Purity of FIIc was confirmed by high pressure liquid chromatography. Detailed UV, NMR, IR spectra suggested that FIIc is a small aliphatic organic compound having molecular formula C₄H₇O₄N. Oral administration of FIIc to diabetic rats (10, 15 and 20 mg/kg body weight per day for a period of 60 days) produced significant (*P*<0.001) fall in fasting blood glucose (FBG) in a dose-dependent manner. Treatment with FIIc (15 mg/kg body wt.) showed significant (*P*<0.001) improvement in body weight, blood urea, plasma creatinine levels, urinary volume, urinary sugar and microalbuminuria. Renal hypertrophy, assessed as the ratio of the weight of the two kidneys to total body weight was also significantly (*P*<0.05) improved after treatment with FIIc. The above results suggest that FIIc possesses significant nephroprotective activity.

**Keywords**: *Eugenia jambolana*, Diabetes mellitus, Streptozotocin, Nephroprotective activity

Diabetes mellitus is known to have diabetic nephropathy as a major long-term complication which occurs in 30-40% of diabetic patients¹². The early stages of diabetic nephropathy are characterized by an elevation of urinary albumin excretion, an increment of glomerular filtration rate and renal hypertrophy³⁴. These symptoms progress to overt proteinuria and finally result in end stage renal failure in diabetic patients.

In spite of the presence of known anti-diabetic medicines in the pharmaceutical market, there is growing interest in herbal remedies due to the fact that synthetic drugs lead to undesirable side effects⁵. Traditional medicinal plants having anti-diabetic property can be used as drugs or simple dietary adjuvant to existing therapies of diabetes. The anti-hyperglycemic activity of a number of plants/plant products have been evaluated and confirmed in animal models⁶⁻⁸, as well as in human beings⁹⁻¹¹. In India, several indigenous plant products have been used by the practitioners of the Ayurvedic system of medicine to treat diabetes¹²,¹³.

*Eugenia jambolana* (Family: Myrtaceae, Common name: Black plum/black berry in English, Jamun/Jambul in Hindi and Neredu in Telugu) is a large tree found in all forests over the greater part of India from the sub-Himalayan tract to extreme south. Fruits are oval to elliptical 1.5-3.5 cm long, dark purple or nearly black, luscious, fleshy and are edible. The anti-hyperglycemic activity of the seeds of *E. jambolana* is well documented¹⁴⁻²³. Aqueous and ethanolic extracts of seeds administered orally to experimental animals and to human adults at various dose levels have shown to be active against diabetes¹⁶,²⁴. Recently, we have demonstrated the anti-diabetic effect of active principle isolated from the seeds²⁵. The water extract of fruit pulp of *E. jambolana* has also been found to show anti-hyperglycemic activity immediately or as early as 30 min¹⁶, while seeds require 24 h for the same effect. However, hot water extract of dried fruit pulp has been found to be inactive in alloxan-induced hyperglycemia¹⁴.

In our previous study²⁶, we have shown that water extract of fruit pulp of *E. jambolana* is more potent in
reducing the fasting blood glucose (FBG), as compared to the ethanolic extract. In our lab, we have isolated/purified an active principle from aqueous extract of fruit pulp and the process of isolation has already been patented both in US and India. Recently, we have also obtained the Indian product patent for the purified compound. In the present study, we have investigated the effect of active compound on the experimentally-induced diabetic nephropathy by measuring the blood glucose, urinary output (urinary volume and urinary sugar) and renal function test (blood urea, plasma creatinine, microalbuminuria and renal hypertrophy).

**Materials and Methods**

**Plant material and preparation of extract**

Fruits of *E. jambolana* were procured from the Azadpur Mandi (Herbal market) at Delhi. The identity was made with the help of a botanist using taxonomic rules (voucher specimen no: P-96/7) and specimen was kept for further references in Botanical Garden, Kolkata, India. For preparation of crude aqueous extract, fresh fruits were washed well with plenty of water and the seeds were separated from the fruit pulp. The 100 g of fruit pulp was mixed with 200 ml of distilled water, grounded in electric grinder, allowed to stand overnight and pulp was filtered through 5-6 layers of muslin cloth. The whole procedure was carried out at 4°C. The filtrate was first centrifuged for 15 min in a refrigerated centrifuge at 10,000 rpm at 4°C and then lyophilized to store it for longer duration. The yield of lyophilized water extract was about 10 g from 650 g of fruit pulp, obtained from 1 kg fruits.

**Purification and characterization of active compound**

Lyophilized aqueous extract of the pulp was subjected to isolation and purification of anti-hyperglycemic compound. The purification was done by ion-exchange column chromatography using diethylamino ethylcellulose-52 (DEAE-52). Fractions were then eluted with 0.1 M phosphate buffer (pH 6.0). The first fraction (FI) showed hyperglycemic activity. Then after elution of some inactive material, a coloured antihyperglycemic fraction (FII) was obtained, followed by other fractions FIII and FIV. The separation of FII and FIII was clear-cut without overlapping. As FII was found to have potent anti-hyperglycemic activity, it was further subjected to purification by rechromatography that resulted FIIc i.e., purified active principle (patents are already granted in India and USA as mentioned in the introduction).

Homogeneity of FIIc was confirmed by HPLC peak after employing it on chromolith column (Chromolith® performance HPLC column RP-18e 100-4.6 mm). FIIc was eluted with mobile phase (water: methanol: acetonitrile (70:15:15) and monitored by PDA detector at wavelength 220 nm). A single peak was observed in chromatogram, suggesting that FIIc was homogenous (Fig. 1). Detailed UV, IR and NMR spectra suggested that purified active compound (FIIc) is an α-hydroxy succinamic acid which is a small aliphatic organic compound having molecular formula C₅H₇O₄N.

**Experimental animals and induction of diabetes**

Male Wistar albino rats (weighing 160-200 g) were procured from Central Animal House of University College of Medical Sciences (UCMS), Delhi, India. The animals were housed in standard conditions of temperature (22 ± 2°C) and at 12 h light-dark cycle and fed with commercial diet (Hindustan Lever Ltd., Mumbai) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of UCMS, Delhi, India. All experimental procedures were conducted in accordance to the ethical guidelines of International Association for the Study of Pain.

For induction of experimental diabetes, a freshly prepared solution of streptozotocin (45 mg/kg in
0.1 M citrate buffer, pH 4.5) was injected intraperitoneally to overnight fasted rats. Streptozotocin (STZ) injected animals exhibited hyperglycemia within 48 h. Fasting blood glucose (FBG) levels were measured after 48 hours and again repeated twice at interval of 3 days. The rats with stabilized diabetes having FBG values of 300 mg/dl or above were considered severely diabetic and included in the study.

**Experimental procedure**

The experiment was carried out on following groups of five rats in each group. Group 1, healthy control receiving vehicle (distilled water); Group 2, diabetic control receiving vehicle only; Group 3 was subdivided into three groups as follows: subgroups A, B and C, diabetic rats treated with FIIc (10, 15 and 20 mg/kg body weight, respectively); and Group 4, diabetic rats treated with glibenclamide (600 µg/kg), a standard anti-diabetic drug. Lyophilized FIIc and glibenclamide were dissolved in water at above-mentioned doses and were administered orally every morning for 60 days using a standard orogastric cannula. Effect of FIIc was studied for its nephroprotective action in STZ-induced diabetic rats on day 0 (before the treatment), 30 and 60.

**Biochemical analysis and renal hypertrophy**

Blood samples were drawn from overnight fasted rats by retro-orbital venepuncture technique. FBG was measured using the glucose oxidase-peroxidase method. Blood urea and plasma creatinine levels were estimated using the standard methods. Weight of individual rat was measured gravimetrically and for collection of 24 h urine sample, each rat was transferred into metabolic cage equipped with accessory for collecting urine. Microalbuminuria levels were determined using ELISA kit (DRG, USA). Urine sugar was estimated by standard colorimetric method. For renal hypertrophy assessment, the rats were sacrificed and the kidney weight was determined gravimetrically. The degree of renal hypertrophy was expressed as the ratio of the weight of the two kidneys to total body weight.

**Statistical analysis**

Values were expressed as the mean ± SEM for five animals in each group. The data were analyzed by using repeated measure analysis of variance (ANOVA), followed by Dunnett’s test and repeated measure ANOVA, followed by Tukey’s test. The results were considered significant at P<0.05.

**Results**

**Plasma glucose**

Preliminary studies conducted with graded doses of FIIc (10, 15 and 20 mg/kg) showed dose-dependent fall in the levels of FBG in diabetic rats (Data not shown). As FIIc produced maximum fall in blood glucose level at 15 mg/kg, this dose was considered as effective dose and further studies were carried out at this dose.

Effect of FIIc and glibenclamide on plasma glucose levels is shown in Table 1. Diabetic control maintained marked hyperglycemia throughout the experimental period and the values were increased about 4-folds as compared to healthy control (P<0.001). Oral administration of FIIc resulted significant (P<0.001) fall in FBG (63.9% fall by FIIc, compared to 46.7% by glibenclamide) after treatment for 60 days. Results showed that FIIc was more effective than glibenclamide in reducing the FBG.

**Body weight**

Effect of FIIc and glibenclamide on body weight is shown in Table 2. Rats in normal control group

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**Table 1—Effect of FIIc on plasma glucose levels STZ-induced diabetic rats**

[Values are mean ± SEM for 5 animals in each group]

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>FBG (mg/dl)</th>
<th>0 day</th>
<th>30 day</th>
<th>60 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control</td>
<td>84.0 ± 9.7</td>
<td>85.80 ± 4.96</td>
<td>84.40 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Diabetic control</td>
<td>306 ± 60.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>369.40 ± 80.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>382.00 ± 44.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Diabetic + Drug-treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIIc (15 mg/kg)</td>
<td>302 ± 51.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>190 ± 68.9&lt;sup&gt;b, c&lt;/sup&gt; (37% Fall)</td>
<td>109.5 ± 21.9&lt;sup&gt;b, c&lt;/sup&gt; (63.9% Fall)</td>
<td></td>
</tr>
<tr>
<td>Glibenclamide (600 µg/kg)</td>
<td>305 ± 18.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>205 ± 39.7&lt;sup&gt;a&lt;/sup&gt; (32.7% Fall)</td>
<td>162.5 ± 64.9&lt;sup&gt;b, c&lt;/sup&gt; (46.7% Fall)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.001 vs. Healthy control, <sup>b</sup>P<0.001 vs. Diabetic control, <sup>c</sup>P<0.001 vs. initial values
showed consistent increase in body weight from day 0 to day 60 ($P<0.01$). Fifteen days after injection of STZ, body weight was significantly decreased, as compared to non-diabetic group. Diabetic controls showed 9.9% fall after 60 days. However, oral treatment with FIIc and glibenclamide resulted in significant improvement in body weight as compared to diabetic control.

### Urinary volume and urinary sugar

Effect of FIIc and glibenclamide on urinary volume is shown in Table 3. Fifteen days after STZ injection, urine volume was significantly higher in diabetic group compared to healthy control. Polyuria in untreated diabetic animals continued till the end of experiment i.e., 40.7 ± 10.3 ml/24 h vs 9.74 ± 2.7 ml/24 h ($P<0.01$) compared to healthy control and 40.7 ± 10.3 ml/24 h vs 22.3 ± 4.8 ml/24 h ($P<0.01$) compared to initial values. Oral administration of FIIc showed significant ($P<0.01$) reduction in urinary output (55.2% fall) whereas glibenclamide showed only 9.0% fall compared to its initial value. Results showed that FIIc was more effective in improving the urinary output in comparison to glibenclamide. Along with urinary output FIIc also caused significant improvement in urinary sugar after 60 days treatment (Data not shown).

### Renal function test

Effect of FIIc and glibenclamide on blood urea, plasma creatinine and microalbuminuria is shown in Table 4. Diabetic control group showed 26% increase in blood urea ($P<0.001$), 45.3% increase in plasma creatinine and 37.7% increase in microalbuminuria ($P<0.001$), as compared to initial values. Oral administration of FIIc (15 mg/kg b.w.) for 60 days resulted in 26.3% fall in blood urea (40.25 ± 5.6 vs 54.6 ± 6.3, $P<0.001$), 24.8% fall in plasma creatinine (1.12 ± 0.13 vs 1.49 ± 0.39, $P<0.001$) and 45.8% fall in microalbuminuria (10.04 ± 2.9 vs 18.53 ± 4.1, $P<0.001$), as compared to their respective initial levels. Whereas oral administration of glibenclamide (600 µg/kg) for 60 days resulted in 11.5% fall in blood urea (48.25 ± 3.4 vs 54.50 ± 8.4, $P<0.001$), 20.9% fall in plasma creatinine (1.17 ± 0.09 vs 1.48 ± 0.36) and 40.6% fall in microalbuminuria levels (11.13 ± 3.4 vs 18.74 ± 3.5, $P<0.001$), as compared to respective initial values. Results showed that FIIc was more effective in improving the renal dysfunction in STZ-induced diabetic rats, as compared to glibenclamide.

### Renal hypertrophy

Figure 2 shows the effect of FIIc and glibenclamide treatment on renal hypertrophy in STZ-induced diabetic rats. Ratio of both the kidney weight to body

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**Table 2**—Effect of FIIc on body weight in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Healthy control</td>
<td>208 ±10.3</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>181 ± 6.5$^a$</td>
</tr>
<tr>
<td><strong>Diabetic + Drug-treated</strong></td>
<td></td>
</tr>
<tr>
<td>FIIc (15 mg/kg)</td>
<td>192.5 ± 15.9$^a$</td>
</tr>
<tr>
<td>Glibenclamide (600 µg/kg)</td>
<td>186.2 ± 16.8$^a$</td>
</tr>
</tbody>
</table>

$^aP<0.01$ vs. Healthy control, $^bP<0.01$ vs. Diabetic control

**Table 3**—Effect of FIIc on urine volume in STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Urine volume (in ml)/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Healthy control</td>
<td>8.30 ±1.7</td>
</tr>
<tr>
<td>Diabetic control</td>
<td>22.3 ± 4.8$^a$</td>
</tr>
<tr>
<td><strong>Diabetic + Drug-treated</strong></td>
<td></td>
</tr>
<tr>
<td>FIIc (15 mg/kg)</td>
<td>24.5 ± 26.1$^a$</td>
</tr>
<tr>
<td>Glibenclamide (600 µg/kg)</td>
<td>22.0 ± 6.7$^a$</td>
</tr>
</tbody>
</table>

$^aP<0.01$ vs. Healthy control, $^bP<0.01$ vs. Diabetic control, $^cP<0.01$ compared to initial values
weight in the various groups of rats was calculated to assess the renal hypertrophy. In diabetic control rats, there was significant increase in ratio, compared to healthy control rats at day 60. However, renal hypertrophy was significantly \( P<0.05 \) improved by administration of FIIc and glibenclamide.

**Discussion**

In our previous study\(^\text{26}\), anti-hyperglycemic effect of the aqueous extract of *E. jambolana* fruit pulp in normal and alloxan-induced (80 mg/kg) diabetic rabbits has already been established. In present study nephroprotective potential of the active principle isolated from the aqueous extract of fruit pulp was evaluated in STZ-induced diabetic rats. The STZ-induced diabetic animals represent a good experimental diabetic state with residual or remnant insulin production by the pancreatic \( \beta \)-cells. Further, STZ-induced diabetes in rodents results in development of nephropathy, similar to early stage clinical diabetic nephropathy\(^\text{36}\). Therefore, this animal model was selected for the study.

The FIIc, like glibenclamide produced significant reduction in blood glucose level of STZ-induced diabetic rats. Our results (Fig. 2) showed that FIIc was more effective than glibenclamide. The capacity of FIIc to significantly bringing down the elevated levels of blood glucose and its complications in diabetic rats shows its antihyperglycemic activity, which is an essential trigger for the development of normal homeostasis during experimental diabetes and its associated complications. It is well established that in the untreated diabetic animals, there is loss in body weight as assessed by us. Treatment with the FIIc prevented the loss in body weight and caused increase in body weight nearly to the normal values (Table 2).

Polyuria is a characteristic symptom of diabetes. Untreated diabetic rats develop severe

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<table>
<thead>
<tr>
<th>Time interval</th>
<th>Experimental groups</th>
<th>In Blood</th>
<th>In Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urea (mg/dl)</td>
<td>Creatinine (mg/dl)</td>
</tr>
<tr>
<td>0 day</td>
<td>Healthy control</td>
<td>27.40 ± 4.0</td>
<td>0.95 ± 0.60</td>
</tr>
<tr>
<td></td>
<td>Diabetic control</td>
<td>54.0 ± 9.8(^a)</td>
<td>1.47 ± 0.40</td>
</tr>
<tr>
<td></td>
<td><em>Diabetic + Drug-treated</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FIIc (15 mg/kg)</td>
<td>54.6 ± 6.3(^a)</td>
<td>1.49 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>54.5 ± 8.4(^a)</td>
<td>1.48 ± 0.36</td>
</tr>
<tr>
<td></td>
<td><strong>After treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 day</td>
<td>Healthy control</td>
<td>28.60 ± 2.07</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Diabetic control</td>
<td>66.80 ± 14.8(^ace)</td>
<td>2.14 ± 0.68(^ac)</td>
</tr>
<tr>
<td></td>
<td><em>Diabetic + Drug-treated</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FIIc (15 mg/kg)</td>
<td>50.49 ± 11.0(^ace)</td>
<td>1.36 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>53.8 ± 8.0(^ace)</td>
<td>1.38 ± 0.32</td>
</tr>
<tr>
<td>60 day</td>
<td>Healthy control</td>
<td>29.60 ± 3.20</td>
<td>0.90 ± 0.058</td>
</tr>
<tr>
<td></td>
<td>Diabetic control</td>
<td>73.20 ± 17.5(^ace)</td>
<td>2.69 ± 0.83(^ac)</td>
</tr>
<tr>
<td></td>
<td><em>Diabetic + Drug-treated</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FIIc (15 mg/kg)</td>
<td>40.25 ± 5.6(^bc)</td>
<td>1.12 ± 0.13(^bc)</td>
</tr>
<tr>
<td></td>
<td>Glibenclamide</td>
<td>48.25 ± 3.4(^bc)</td>
<td>1.17 ± 0.09(^b)</td>
</tr>
</tbody>
</table>

\(^a\)\(P<0.001\) vs Healthy control, \(^b\)\(P<0.001\) vs Diabetic control, \(^c\)\(P<0.001\) vs initial values, \(^d\)\(P<0.001\) vs values on 30th day
hyperglycemia with polyuria as a result of osmotic diuresis. In the present study, 24 h urine volume was significantly increased in diabetic control vs normal controls and treatment with FIIc caused significant decrease in urinary volume (Table 3) along the entire period of study i.e., 60 days, possibly due to the normalization of plasma glucose level or synergistic effect with insulin as shown in other studies.\textsuperscript{37,38}

Microalbuminuria is a established marker of diabetic nephropathy.\textsuperscript{39,40} It begins insidiously and may precede the diagnosis of type-2 diabetes mellitus, occurring with the insulin resistance syndrome and its components. Microalbuminuria refers to the excretion of albumin in urine at a rate that exceeds normal limits, but is less than the detection level of traditional dipstick method. In the present study, microalbuminuria as well as blood urea and plasma creatinine levels were significantly increased in the diabetic control group. However, treatment with FIIc caused a significant decrease in excretion of microalbuminuria and in blood urea and plasma creatinine.

Diabetes induction by STZ has been known to produce increase in kidney weight relative to body weight\textsuperscript{41}. In the present study, the average net weight of both kidneys of diabetic controls was significantly ($P<0.05$) higher than the non-diabetic controls, which was consistent with the previous finding\textsuperscript{42}. Treatment with FIIc significantly improved ($P<0.05$) renal hypertrophy. Previous studies have correlated the degree of renal enlargement with the degree of glycemic control\textsuperscript{43}. However, in the present study FIIc exerted both anti-hyperglycemic effect and prevented renal enlargement as well. Thus, it was likely that mechanism(s) dependent of anti-hyperglycemic property played a role in preventing renal enlargement.

It has been reported that streptozotocin does not possess any significant nephrotoxic potential\textsuperscript{44}. All changes in the kidney function after STZ administration in rats can thus be attributed to altered metabolism in diabetes.\textsuperscript{45} Administration of FIIc to diabetic rats prevented the increase in urine volume, improvement in renal functions (blood urea, plasma creatinine, and microalbuminuria), renal hypertrophy and significantly improved body weight along with significant reduction in plasma glucose levels. This could be attributed to anti-diabetic action of FIIc, resulting in alleviation of altered metabolic status in animal.

In conclusion, our results demonstrated significant protective effect of FIIc on the consequences of hyperglycemia and early stages of experimentally-induced diabetic nephropathy. However, molecular and mechanistic studies on the therapeutic action of FIIc are needed to use it as a possible insulin replacement or adjuvant in the management of diabetes mellitus and its associated complications.

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