Effect of *Aegle marmelos* (L.) Correa on alloxan induced early stage diabetic nephropathy in rats

Rajbir Bhatti¹*, Shikha Sharma¹, Jatinder Singh², Amarjit Singh² & M P S Ishar¹,³

¹ Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar, India.  
² Department of Pharmacology, Government Medical College, Amritsar, India

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Diabetic nephropathy (DN) has a complex pathogenesis and poor prognosis due to the lack of therapeutic interventions. The present study investigates the effect of *A. marmelos* leaf extract (AME) on early alloxan induced DN. The treatment with AME was found to significantly decrease the fasting blood sugar, total cholesterol, blood urea, creatinine and renal TBARS and increased the levels of renal reduced glutathione and catalase significantly as compared to the diabetic control group. The maximum dose-dependent protection was observed at a dose of 200 mg kg⁻¹. Histological examination revealed marked reversal of the morphological derangements with AME treatment as indicated by a decrease in glomerular expansion, tubular dilatation and inflammatory cells. The present results conclude that AME treatment has a significant ameliorative effect on early changes induced in the kidneys by alloxan and improves the outcome of DN.

**Keywords:** *Aegle marmelos*, Alloxan, Antioxidant, Diabetic nephropathy

Diabetes mellitus has assumed an epidemic form worldwide. The easy availability of drugs to control hyperglycaemia has led to an increase in the life expectancy in the diabetic patients but the complications during the course of the disease account for a high rate of morbidity and malaise. The progress of the disease leads to various complications such as retinopathy, neuropathy, nephropathy and cardiomyopathy. DN is one of the most severe complications of diabetes and the prognosis of DN is mostly fatal. Diabetic nephropathy (DN) is one of the most serious and debilitating complications of diabetes and has emerged as the leading cause of end stage renal failure worldwide. DN is marked by an initial increase in glomerular permeability to proteins, microalbuminuria which eventually progresses to proteinuria, azotemia finally culminating into renal failure. The ultrastructural changes in the glomeruli reported in the early stages include thickening of the glomerular basement membrane, expansion of the mesangium, glomerular hypertrophy and in the later stages there is tubulointerstitial fibrosis, large proteinaceous deposits, hyaline droplets and hyaline casts.

The diabetic syndrome has a complex and multifactorial pathophysiology comprising interplay of varied factors such as oxidative stress, negative nitrogen balance, negative lipid balance, hyperglycaemia and endogenous mediators like cytokines that lead to cellular damage. Although the oral hypoglycaemic agents like the thiazolidinediones and biguanides increase the insulin sensitivity in the tissues, the drug therapy is limited by various side effects of these molecules. Therefore, there has been a renewed interest in antioxidants and natural products that offer a large structural diversity and have a lower incidence of side effects. Vitamin E supplementation ameliorated renal injury in experimental diabetes in rats. A polyherbal formulation containing quercetin, rutin, catechin etc. has been documented to be preventive in early stages of diabetic nephropathy and plant extract of *Asparagus racemosus* has been reported to possess antioxidant and hypoglycaemic activity in rats. Also, the natural products are a good source of interesting leads in new drug development due to their diverse bioactive principles. Further, the natural complexity in the structure of the natural products serves to...
appease a wide array of target sites. Studies have revealed, sargaquinoic acid and sargahydroquinoic acid obtained from Sargassum yezoense as novel PPAR α/γ dual agonists.

*Aegle marmelos* (L.) Correa (Rutaceae) is well documented for its hypoglycaemic potential. The plant is also reported to have beneficial effects in diabetic cardiomyopathy. The plant contains a wide variety of chemical constituents such as aegeline, imperatorin, umbelliferone, limonene etc. which have been proposed to aid in the beneficial effects. However, to the best of our knowledge, there are no reports on the effects of *Aegle marmelos* on early stages of diabetic nephropathy. Therefore, the present study is aimed at investigating the effect of leaf extract of the plant on early stages of diabetic nephropathy.

### Materials and Methods

**Chemicals**—Alloxan monohydrate was purchased from Sigma Aldrich, glipizide provided as a gift sample by Q.P. Pharmaceuticals, Derabassi, India, Fasting blood glucose (FBG), total cholesterol (TC), urea and creatinine diagnostic kits were bought from Span Diagnostics, Surat, Gujrat, India, total protein. 

**Materials and Methods**—Dietary and supplied by local firms. All other reagents and chemicals were of analytical grade and supplied by local firms.

**Plant material and preparation of extract**—The leaves of *A. marmelos* were collected from the Botanical garden of Guru Nanak Dev University, Amritsar, authenticated by Dr. A. S. Soodan and a voucher specimen (SR./Bot.Sci./0350) was deposited in the herbarium of the department of Botanical and Environmental Sciences of the same university. 

**Preliminary phytochemical screening**—Preliminary phytochemical screening was done for various compounds such as alkaloids, coumarins, glycosides etc. using standard tests. 

**Experimental design**—Diabetes was induced by a single intraperitoneal (ip) injection of alloxan monohydrate at a dose of 150 mg kg⁻¹ to overnight fasted rats. The groups were: Gr. I – normal non diabetic; Gr. II: diabetic control; Gr. III: diabetic vehicle treated (0.5% CMC, 1 mL); Gr. IV: diabetic glipizide treated (10 mg kg⁻¹, ip); Gr. V: diabetic AME treated (AME 25 mg kg⁻¹, ip); Gr. VI: diabetic AME treated (AME 50 mg kg⁻¹, ip); Gr. VII: diabetic AME treated (AME 100 mg kg⁻¹, ip) and Gr. VIII diabetic AME treated (AME 200 mg kg⁻¹, ip). All the respective treatments were administered for 14 days.

**Biochemical parameters**—At the end of the study, rats were fasted overnight and blood was collected by retro-orbital puncture under ether anaesthesia and plasma separated by centrifugation. Thereafter, the animals were sacrificed, abdomens anaesthetized and the kidneys were isolated. The right kidney was preserved in formalin for histopathological studies and the left kidney processed for biochemical estimations. The estimation of blood parameters including fasting blood glucose (FBG), total cholesterol (TC), blood urea and creatinine was done. For the estimation of the renal parameters, the left kidney was immediately perfused with ice cold saline and homogenised in chilled (1.15%) KCl. The homogenates were centrifuged at 800 g for 5 min at 4 °C. For the estimation of catalase the supernatant was further centrifuged at 4000 g for 60 min.

The total proteins were estimated by Biuret method using commercially available kits. The levels of thiobarbituric acid reactive substances (TBARS) were used as an index of lipid peroxidation and estimated by the methods previously described in literature. Reduced glutathione (GSH) was estimated by the method described by Ellman and catalase (CAT) activity was estimated as per Aebi.
Histopathological studies—The right kidneys were preserved in formalin for histopathological studies. Sections (2-4 µm thick) were prepared with microtome and stained with haematoxylin for 15 min followed by counter stain with eosin for 2 min. The changes were studied through photomicrographs taken with digital camera (Olympus E-520) attached to pathological microscope (Magnus MLXi) linked to a computer.

Statistical analysis—All the values are expressed as mean ± SE. One way analysis of variance (ANOVA) followed by post hoc analysis using Tukey’s multiple range test was used to calculated the statistical significance with the help of Instat software version 3.05 (GraphPad Software Inc., San Deigo, USA).

Results

Preliminary phytochemical screening—The preliminary phytochemical screening of the ethanol extract of A. marmelos confirmed the presence of alkaloids, cooumarins and glycoides in the extract. The HPTLC of the extract yielded three prominent peaks (R_f 0.14, 0.52 and 0.73) and four minor peaks at R_f 0.28, 0.35, 0.44 and 0.67 as reported earlier.

Effect of AME on FBG, TC, urea and creatinine—The diabetic control group showed significant increase in FBG and TC. The treatment with glipizide, used as the standard hypoglycaemic drug was found to decrease the hyperglycemia. Administration of AME produced a dose dependent decrease in FBG and TC, the effect at the dose of 25 mg kg⁻¹ was not significant but at 50, 100 and 200 mg kg⁻¹ the effect was statistically signicant as compared to the diabetic control group. Maximum dose dependent reduction was observed at a dose of 200 mg kg⁻¹ (Table 1). Alloxan induced a marked increase in blood urea and creatinine levels in the diabetic rats. However, treatment with glipizide and increasing doses of AME produced marked decrease in these parameters. While the response with 25 mg kg⁻¹ of AME was not statistically significant, responses at 50, 100 and 200 mg kg⁻¹ of the drug were significant and maximum dose dependent decrease was found at a dose of 200 mg kg⁻¹ (Table 1).

Effect of AME on renal oxidant stress—Induction of diabetes induced a significant increase in the levels of TBARS and decrease in the levels of GSH and CAT as compared to the non-diabetic animals. Treatment with AME was found to produce a dose dependent decrease in TBARS and increase in GSH, CAT in the renal homogenate of the diabetic rats. The effect at a dose of 25 mg kg⁻¹ was not statistically significant but at higher doses (50, 100 and 200 mg kg⁻¹) significant reduction in the level of TBARS and increase in GSH and CAT as compared to the diabetic control was observed. Maximum dose dependent response was found at the dose of 200 mg kg⁻¹ and the response was better than that of the standard drug glipizide (Table 2).

Effect of AME on the histopathological profile of diabetic rats—The histopathological examination of the kidneys revealed marked glomerular expansion, hyaline casts and focal areas of inflammatory exudate in the diabetic control rats. The treatment with glipizide and AME (200 mg kg⁻¹) was found to reverse these changes partially thereby indicating a recovery in the renal architecture. However, AME was found to decrease the histological derangements better than the standard hypoglycaemic drug glipizide (Fig.1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FBG (mg dL)</th>
<th>TC (mg dL)</th>
<th>BUN (mg dL)</th>
<th>Creatinine (mg dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non- diabetic</td>
<td>85.43 ± 1.089</td>
<td>58.15 ± 0.718</td>
<td>14.73 ± 0.39</td>
<td>0.60 ± 0.008</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>171.45 ± 5.17 *</td>
<td>161.55 ± 9.51 *</td>
<td>26.37 ± 2.91 *</td>
<td>3.41 ± 0.033 *</td>
</tr>
<tr>
<td>Diabetic Vehicle</td>
<td>171.40 ± 6.16 *</td>
<td>158.74 ± 3.99 *</td>
<td>27.07 ± 1.97 *</td>
<td>3.43 ± 0.033 *</td>
</tr>
<tr>
<td>Glipizide, 10 mg/kg</td>
<td>109.53 ± 5.37 **</td>
<td>72.92 ± 7.04 **</td>
<td>18.65 ± 0.42 **</td>
<td>1.30 ± 0.085 **</td>
</tr>
<tr>
<td>AME, 25 mg/kg</td>
<td>138.51 ± 7.27 **</td>
<td>87.09 ± 5.34 **</td>
<td>20.78 ± 0.54 **</td>
<td>2.36 ± 0.005 **</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>121.67 ± 4.90 **</td>
<td>67.15 ± 3.74 **</td>
<td>18.71 ± 0.45 **</td>
<td>2.14 ± 0.016 **</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>87.45 ± 7.92 **</td>
<td>59.25 ± 4.47 **</td>
<td>14.90 ± 0.42 **</td>
<td>0.99 ± 0.015 **</td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>85.49 ± 4.75 **</td>
<td>58.43 ± 7.29 **</td>
<td>15.56 ± 0.76 **</td>
<td>0.80 ± 0.021 **</td>
</tr>
</tbody>
</table>

FBG = Fasting blood glucose; TC = Total cholesterol; BUN = Blood urea nitrogen

P values: <0.05; compared to * non-diabetic; ** diabetic control
Table 2—Effect of the various pharmacological interventions on renal oxidative stress in diabetic rats. [Values are mean ± SE]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS (nmoles/mg protein)</th>
<th>Reduced Glutathione (nmoles/mg protein)</th>
<th>Catalase (moles of H₂O₂ decomposed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic</td>
<td>10.18 ± 0.46</td>
<td>45.27 ± 0.44</td>
<td>1.46 ± 0.09</td>
</tr>
<tr>
<td>Diabetic Control</td>
<td>65.05 ± 1.26 *</td>
<td>15.22 ± 0.26 *</td>
<td>0.45 ± 0.007 *</td>
</tr>
<tr>
<td>Diabetic Vehicle</td>
<td>65.23 ± 1.30 *</td>
<td>15.19 ± 0.23 *</td>
<td>0.47 ± 0.01 *</td>
</tr>
<tr>
<td>Glipizide (10 mg/Kg)</td>
<td>20.52 ± 0.35 **</td>
<td>32.87 ± 0.31 **</td>
<td>0.85 ± 0.08 **</td>
</tr>
<tr>
<td>AME 25 mg/kg</td>
<td>30.02 ± 0.51 **</td>
<td>28.16 ± 0.09 **</td>
<td>0.74 ± 0.02 **</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>24.84 ± 0.18 **</td>
<td>33.14 ± 0.32 **</td>
<td>0.79 ± 0.007 **</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>18.91 ± 0.20 **</td>
<td>40.49 ± 0.36 **</td>
<td>0.90 ± 0.009 **</td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>18.93 ± 0.27 **</td>
<td>40.22 ± 0.24 **</td>
<td>0.91 ± 0.013 **</td>
</tr>
</tbody>
</table>

P values: <0.05; compared to * non-diabetic; ** diabetic control

Discussion

The present study was designed to explore the potential of Aegle marmelos leaf extract (AME) in preventing early stage alloxan induced diabetic nephropathy in rats. Alloxan is well documented to have cytotoxic effect in the pancreas which is mediated by the generation of reactive oxygen species and free radicals. Administration of alloxan led to an increase in blood glucose, cholesterol, urea, creatinine and renal TBARS; while there was a decrease in GSH and CAT in the renal tissue. Also, the alloxan treated diabetic rats showed pathological alterations in the kidneys observed in histopathological studies, comprising glomerular expansion, tubular dilatation and interstitial necrosis on the 14th day of study. Acute manifestations of altered renal function are reported to occur as early as 4 days after the induction of diabetes, and diabetic nephropathy has been reported to occur within 14 days of inducing diabetes. Treatment with various pharmacological interventions is well documented to have beneficial effect on DN. Angiotensin (1-7) has been demonstrated to prevent DN after 2 weeks of treatment; extracts of Phaleria macrocarpa have also been reported to protect against DN in a two week treatment schedule; recently, Croatian propolis has been postulated to protect against alloxan induced DN in a 7 day treatment schedule. Volatile oil from Cinnamomum zeylanicum protected the diabetic rats against DN in a two week study. Similar results have been obtained in the present study.

Administration of AME at doses of 25, 50, 100 and 200 mg kg⁻¹ was found to decrease the FBG, TC, blood urea nitrogen (BUN) and creatinine significantly. The blood glucose lowering activity was comparable to that of the standard hypoglycaemic agent glipizide. Alloxan has been reported to cause hyperglycaemia, increased lipid peroxidation and deplete the levels of reduced glutathione and catalase. Similar results were obtained in the present study. Diabetic control group had a marked increase in renal levels of TBARS and decrease in GSH and CAT as compared to the normal rats. Treatment with AME was found to decrease the TBARS and increase the levels of GSH and CAT in the renal homogenate significantly.

Aegle marmelos is well documented for its antioxidant and hypolipidemic activities. Acute diabetic state is associated with mesangial expansion, deposition of extracellular proteins, thickening of the glomerular basement membrane, hyaline casts and renal tubular expansion. Similar alterations have been observed in the present study. Treatment with AME was found to attenuate the histological derangements in the renal tissues.

The pathogenesis of DN involves a myriad of factors comprising increased renal oxidative stress evidenced by an increase in malondialdehyde levels, advanced glycation end products that lead to irreversible tissue damage. Prolonged hyperglycemia as precipitated in diabetes leads to oxidation of proteins and inflammatory changes in the renal tissue. An imbalance between the generation of free radical species and compensatory mechanisms results in oxidative stress. Aegle marmelos is documented to have phytoconstituents with varied chemical structures including cinnamides such as aegeline, coumarins like umbelliferone, furanocoumarins like imperatorin, psoralen and xanthotoxin and others such as limonene, auraptene etc. These diverse phytoconstituents have multiple target sites which account for its protective effect. Aegeline has antihyperglycemic and antihypercholesterolemic
Fig. 1—Photomicrographs of kidney showing structure with well defined glomeruli, no tubular and no inflammatory cells at 10× (A) and 40× (B); diabetic with glomerular expansion and inflammatory cells at 10× (C) and 40× (D); tolbutamide treated with decrease in glomerular expansion and inflammatory cells at 10× (E) and 40× (F); *A. marmelos* extract treated showing reversal of glomerular expansion and almost negligible inflammatory cells at 10× (G) and 40× (H).

Actions, umbelliferone has been reported to have protective effect in DN by decreasing hyperglycemia, hypercholesterolemia and sorbitol accumulation in the tissues. AM has been postulated to decrease calcium overload in cardiomyocytes. Calcium overload is the common denominator of all complications of diabetes. Also, *A. marmelos* has been reported to activate the peroxisome proliferator activated receptor (PPARγ) gamma. PPARγ plays a pivotal role in regulating glucose metabolism and inflammatory responses in various tissues.
From the above discussion it may tentatively be concluded that A. marmelos has protective effect on DN which is mediated through diverse and multiple target sites. DN has multifactor pathogenesis and therapeutic interventions available for treatment of DN are only few. Aegle marmelos due to its numerous structurally unrelated bioactive principles acts through more than one mechanisms and therefore may provide beneficial and useful leads in mitigation of this complicated syndrome.

Acknowledgement

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References


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