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Ocimum sanctum and *Morus alba* Leaves and *Punica granatum* Seeds in Diet Ameliorate Diabetes-Induced Changes in Kidney

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ABSTRACT

The effect of *Ocimum sanctum* (Tulsi) leaves, *Morus alba L.* (mulberry) leaves and *Punica granatum* (Pomegranate) seeds were evaluated for diabetes-mediated effect on kidney of streptozotocin-induced diabetic rats. Rats (both control and diabetic) were fed with tulsi leaves, mulberry leaves and pomegranate seeds individually at 10%, 10% and 7.5 %, respectively, in AIN-76 diet, for a period of two months. Dietary supplements in the diet were able to ameliorate the increase in various parameters such as blood glucose, albumin excretion, glomerular filtration rate and kidney index. All the above-mentioned dietary supplements had positive influence on extracellular matrix components such as laminin and fibronectin of kidney to various extents. The results indicated that plant materials of dietary origin are able to circumvent diabetes-induced changes in extracellular matrix components there by bringing about beneficial effects in the kidney.

Key words: Diabetes, Kidney, Extracellular matrix, Tulsi leaves, Mulberry leaves, and Pomegranate seeds.

INTRODUCTION

Diabetes mellitus is a disorder characterized by sustained hyperglycemia. It is associated with microvascular and macrovascular complications. Diabetes afflicts kidney which in the long run is the causative factor for end-stage renal disease. Our earlier report showed that onset of kidney damage starts as early as first month after induction of diabetes [1]. Common features of diabetes-induced kidney damage in initial stages include hyperfiltration and renal enlargement caused due to increase in extracellular matrix components such as type IV collagen, laminin and fibronectin[2]. As diet is known to play an important role in the management of diabetes, a better understanding of its effect on diabetes in general and kidney in particular would be helpful in making informed choices. It has been an endeavor in our laboratory to determine and elucidate changes in extracellular matrix components of kidney and study their remodeling by various dietary factors [3].

Effect of *Ocimum sanctum* (tulsi) leaves, *Morus alba L.* (mulberry*)* leaves and *Punica granatum* (pomegranate) seeds were determined on diabetes-mediated changes in kidney with respect to GFR, kidney index, microalbuminuria and quantitative changes extracellular matrix components such as laminin and fibronectin. *Ocimum sanctum* commonly known as tulsi in India has been primarily used in Ayurvedic treatment for various ailments[4]. Studies have shown tulsi to be effective in treatment of diabetes by reducing blood glucose levels and showed significant reduction in total cholesterol levels[5]. Mulberry leaves have been known as traditional medicine to cure and prevent diabetes[6]. It is consumed as tea or incorporated into food preparations[7]. 1-deoxynojirimycin (DNJ), which is abundant in mulberry leaf, is believed to be a typical naturally occurring imino sugar with potent biological activity[8].

Consumption of pomegranate fruit and/or juice has been reported to reduce the risk of CVD and prostate cancer[9,10]. Seeds of pomegranate are a rich source of the bioactive compound 9-cis, 11-trans conjugated linolenic acid. Not much information is available for anti-diabetic effect of pomegranate seeds.

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All the three plant materials were so chosen for their health beneficial effects. While tulsi and mulberry are known to be beneficial during diabetic condition, the anti-diabetic effects of pomegranate seeds, in particular, are not known. The present study deals with examining the effect of pomegranate seeds on diabetic rats and the effect of feeding the three dietary factors individually on diabetes-mediated changes in kidney as well as on extacellular matrix components namely laminin and fibronectin which are known to perform key roles in the kidney.

MATERIALS AND METHODS

Chemicals

Streptozotocin, Antibodies to Laminin, Fibronectin, Secondary Antibody and Albumin blue was obtained from Sigma Chemicals Co., St. Louis, MO, USA. Glucose oxidase/peroxidase (GOD/ POD) and creatinine kits were purchased from Span Diagnostics Limited, Surat, India. All other chemicals used were of analytical grade.

Plant materials

Tulsi and mulberry leaves were collected from local places during the month of January 2011 and materials were identified by a taxonomist. The leaves were separated individually from the plant, washed in running tap water and dried in an oven at 60^{0}C for 24 h after draining off the excess water. The dried leaves were powdered and stored in airtight containers for further use. Pomegranate fruits were purchased from market. The fruits were washed thoroughly and the outer peel was separated manually. The juice was separated from the fruit by the juice separator and seeds were collected separately. Collected seeds were washed with distilled water and excess water was drained and then dried in an oven at 60 ^{0}C for 35 - 40 h. The dried seeds were powdered and used for feeding experiments.

Animals

Male Wistar rats of 2 months of age weighing around 110.0 ± 10.0 g were used for the experiment. The study had prior animal ethical clearance from IAEC. The animals were acclimatized for a period of 10 days using standard AIN-76 diet and made sure that they had free access to food and water.

Induction of diabetes mellitus

Diabetes was induced in rats by injecting Streptozotocin at 47 mg/kg body weight dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5)[11]. Control rats were injected with citrate buffer only. The rats were fed with 5% glucose water for 2 days soon after streptozotocin injection to prevent drug-induced hypoglycemic shock.

Preparation of diet

Diet (AIN-76) was freshly prepared. Dietary materials were incorporated into the diet by replacing equal quantities of starch.

Grouping of animals

After 7-10 days of acclimatization with the control AIN-76 diet, the animals were randomly grouped into control and diabetic rats based on the body weight. Rats in diabetic group were injected with STZ to induce diabetes. After one week of STZ injection, diabetic status was confirmed by measuring fasting blood glucose levels. The animals were again sub-grouped into respective control and diabetic rats based on fasting blood glucose level. The groups were tentatively named as follows; starch fed control / diabetic (SFC/SFD), tulsi fed control / diabetic (TFC/TFD), mulberry fed control / diabetic (MFC/MFD) and pomegranate seed fed control / diabetic (PFC/PFD).

Collection and analysis of various parameters in blood and urine

Blood was drawn from retro-orbital plexus into tubes containing heparin (20 U/ml blood). Blood glucose was determined by Glucose oxidase-peroxidase method[12], after prior fasting for 12 h. Albumin in urine was measured by using Albumin Blue 580 method[13]. Creatinine was estimated in urine and serum by Jaffes method using commercially available kit[14]. Glomerular filtration rate was determined after estimating creatinine levels in urine and serum using the formula[15]

$$GFR = \frac{\text{Urine creatinine (mg/dl) X Urine volume (ml) X 1000 (g)}}{\text{Plasma creatinine mg/dl X Body weight (g) X1440 (min)}} = ml/min$$

Relative quantitation of extracellular matrix components

This was done by Elisa of kidney homogenates prepared by homogenizing 10 % tissue (w/v) in lysis buffer (1% Triton X-100, 50 mM Tris, 150 mM EDTA and Protease inhibitor) in ice and centrifuging at 6000 rpm for 15 min. Supernatant was taken for the analyses. Briefly, kidney tissue homogenates (as 10 - 15 µg protein) was coated over night at 4^{o}C. Non-specific sites of antigen were blocked with 2% gelatin. Extracellular matrix components were detected by adding specific antibody (1:3000 in 10 mM Phosphate buffer saline) directed against laminin and fibronectin and in individual wells overnight at 4^{o}C. Secondary antibody conjugated with ALP was added (1:4000 10 mM Tris buffer saline dilutions) and incubated at 37 ^{0}C for 2 h. Paranitrophenol phosphate (PNPP) was used as substrate, and color developed was read at 405 nm in an ELISA plate reader. The fold changes in extracellular matrix components were determined by comparing to non-diabetic control.

Statistical analysis

Data are expressed as mean ± SD, of n = 6 in control groups (SFC, FFC, TFC and PFC) and n=7, 6, 8 and 6 respectively, in diabetic groups SFD, TFD, MFD and PFD. Statistical analysis of data was performed using one-way analysis of variance with a Tukey's multiple comparison post-test and with significance at *P<0.05, **P<0.01, and ***P<0.001. Comparison between SFC and SFD has been denoted by [a], whereas the comparison between SFD and treated SFD groups has been denoted by [b].

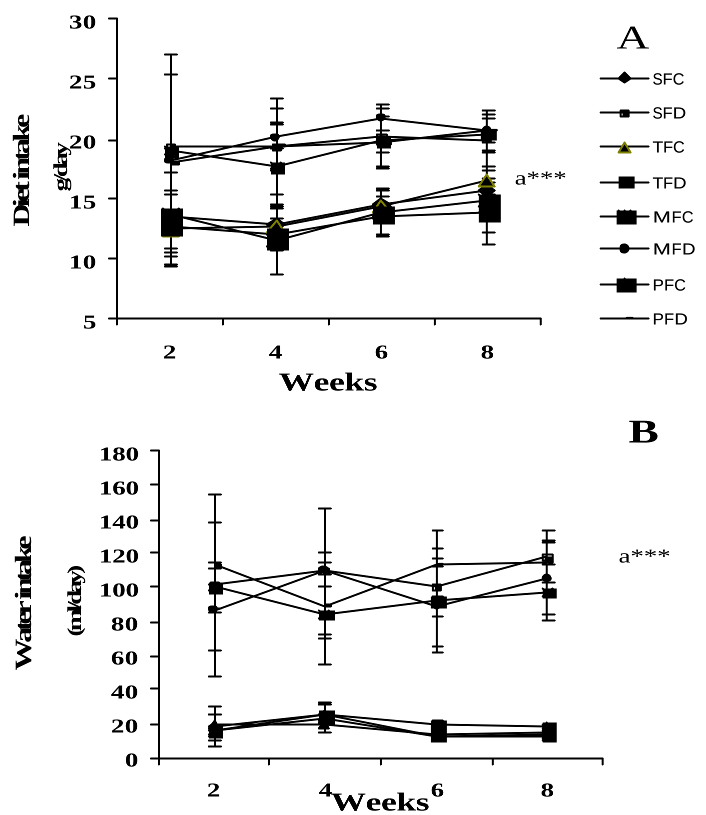
RESULTS AND DISCUSSION

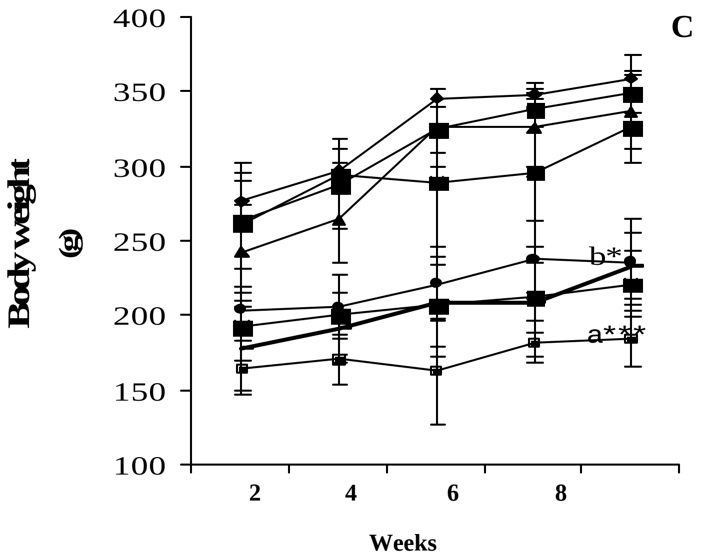
The present study deals with the effect of dietary components - tulsi leaves, mulberry leaves and pomegranate seeds on diabetes-mediated changes on ECM components in experimentally -induced diabetic animals. Diabetes, as a result of sustained hyperglycemia, leads to various secondary complications. Diabetic nephropathy is one such serious complication. Kidney, during diabetes is marked by plethora of changes. It is a complex organ harboring myriad of glycoconjugates as components of ECM which are known to play important role in filtration [16]. However during diabetes, marked changes have been observed in structure-function relationship of these glycoconjugates[17]. The question we have asked is whether dietary materials such as tulsi, mulberry and pomegranate influence these glycoconjugates. Diet, along with drugs and exercise, is one of the cornerstones used in the management of diabetes.

Effect of tulsi, mulberry and pomegranate on diet and water intake and gain in body weight

In this study, animals were monitored for diet intake, water intake and gain in body weight. Diabetic animals in both untreated and treated groups exhibited hyperphagia. Diet intake was significantly higher in all the diabetic groups compared to their respective non-diabetic control groups (Fig. 1A). The presence of dietary factors did not interfere with food intake indicating that there were no issues with palatability. Diabetic animals also exhibited polydipsia which was prominent in all the groups (Fig. 1B). Though diabetic animals consumed more food, there was significant reduction in body weight compared to control animals. Similar trend was exhibited by animals in treated groups (Fig. 1C).

Fig. 1. Effect of tulsi, mulberry and pomegranate on diet intake, water intake and gain in body weight.





Values are mean ± SEM of n rats in control groups (n=6), SFD (n=7), TFD (n=6), MFD (n=8), PFD (n=6). SFC - starch fed control, SFD - starch fed diabetic, TFC- tulsi fed control, TFD- tulsi fed diabetic, MFC- mulberry fed control, MFD-mulberry fed diabetic, PFC- pomegranate fed control and PFD-pomegranate fed diabetic. [a], SFC vs. SFD; [b] SFD vs. treated diabetic groups with significance levels designated as * (P<0.05), ** (P<0.01); *** (P<0.001)

Effect on fasting blood sugar, albumin excretion, GFR and kidney index

In recent years, increased emphasis is being placed on exploration of various dietary factors which are beneficial during pathological conditions [18]. Hence experiments were carried out on STZ-induced diabetic rats. All the three dietary materials tested exhibited beneficial effects on basic parameters such as blood glucose, albumin excretion and GFR. Anti-hyperglycemic properties of tulsi and mulberry leaves in diabetic animals are well documented[19]. Pomegranate is used in treatment of various ailments[20] and anti-hyperglycemic effects of seed during diabetes are not known to the best of our knowledge. Diabetic animals exhibited a significant increase of more than 2-folds in blood glucose levels when compared to control animals. The increase was significantly ameliorated by 23%, 36% and 30% respectively, on feeding tulsi, mulberry leaves and pomegranate seeds. Among the three, mulberry leaves showed more potent anti-hyperglycemic activity in diabetic rats (Fig. 2). Albumin excretion, one of the parameters which are routinely used to assess kidney damage was found to increase significantly in diabetic rats. This was prevented to various extents by the presence of dietary materials (Fig. 3A). The effect on kidney-related parameters was tested to determine their effect on kidney. Significant increase in GFR was observed in diabetic animals compared to the control animals. All the three dietary materials tested showed significant amelioration in increased GFR content (Fig. 3B). Kidney index, measured in terms of g/100 g body weight, showed a significant increase in diabetic animals when compared to non-diabetic control animals which was ameliorated to various extents in animals fed tulsi and mulberry leaves. In diabetic animals fed with diet supplemented with pomegranate seeds, no significant change in kidney index was observed (Fig. 3C), Kidney index due to renal enlargement is one of the common features which occur during early stages of diabetes[21].

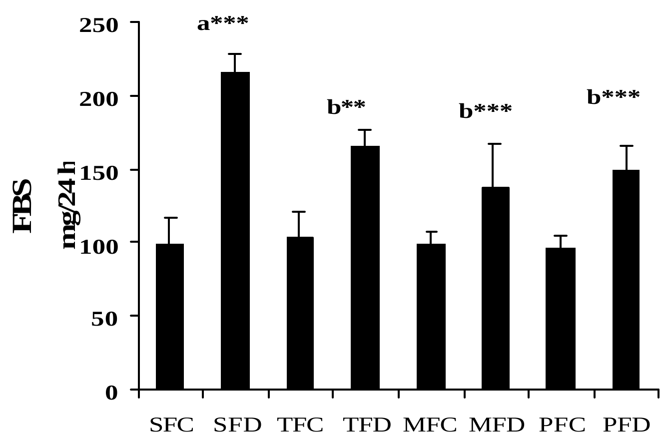


Fig 2

Fig. 2. Effect of tulsi, mulberry and pomegranate on fasting blood sugar. Legend as in Fig 1

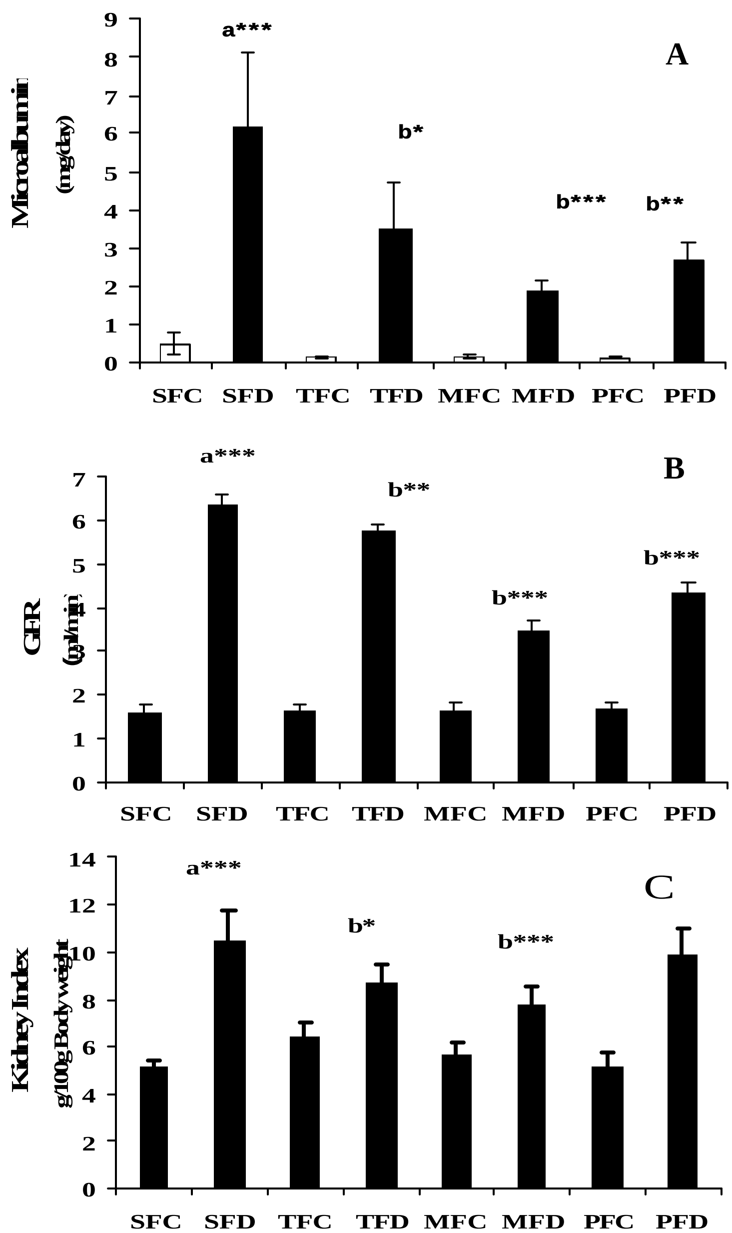
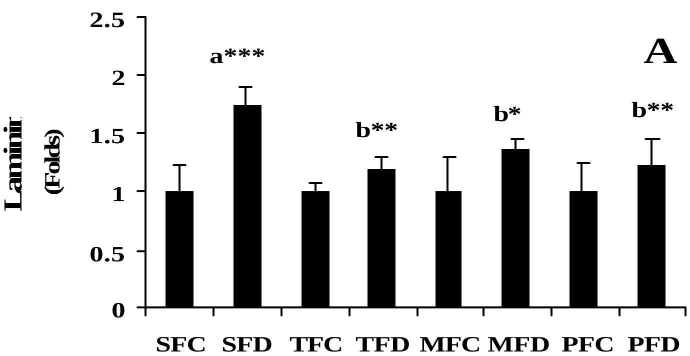


Fig 3

Fig. 3. Effect of tulsi, mulberry and pomegranate on albumin excretion, GFR and kidney index. Legend as in Fig 1

Effect of tulsi, mulberry and pomegrante on extracellular matrix components in kidney

Quantitative changes of major glycoconjugates namely, laminin and fibronectin, of ECM was determined. There was a significant increase in two major ECM components as a result of diabetes. The increase was prevented to various extents by feeding dietary materials (Fig. 4 A,B). There were no significant quantitative changes in non-diabetic controls irrespective of the presence or absence of dietary materials. Accumulation of glycoconjugates such as laminin and fibronectin causes thickening of basement membrane and nephromegaly[22]. Dietary materials are rich in nutraceuticals and in recent years beneficial effects of it are coming to light. Nutrient-gene interactions are being rigorously pursued to understand its molecular mechanisms[24]. Our study shows that some of the dietary materials which were tested are beneficial in terms of preventing renal enlargement by accumulation of ECM components such as laminin and fibronectin to various extents. It is conceivable that some of the nutraceuticals present in them might be playing a role. Tulsi leaves are reported to contain phenolics such as cirsilineol, cirsimaritin, isothymusin ,isothymonin, apigenin , rosmarinic acid, and eugenol[25]. Rosmarinic acid in particular has been demonstrated to be an amylase inhibitor, the activity of which was found to be beneficial during diabetic condition[26]. Mulberry leaves are known to contain 1-deoxynojirimycin (DNJ), a potent glucosidase inhibitor[27]. This inhibitor has been hypothesized to be beneficial for the suppression of abnormally high blood glucose levels and thereby helpful prevention of diabetes mellitus[28]. Likewise, pomegranate seeds also contain phenolic acids such as punicalagin, punicalin, gallic acid, and ellagic acid[29]. The amelioration in diabetic condition is likely brought about by their role as antioxidants.



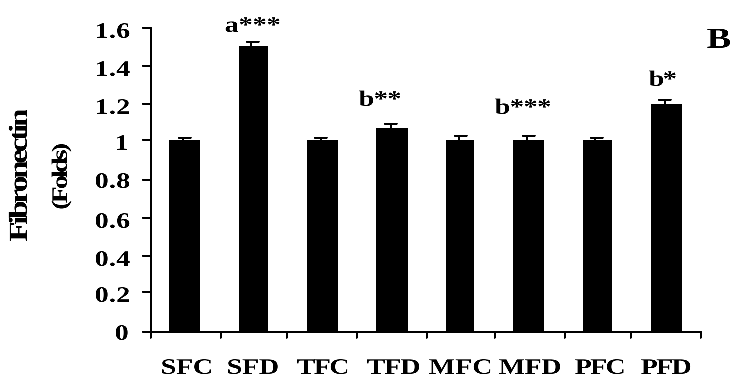


Fig. 4 . Effect of tulsi, mulberry and pomegranate on laminin and fibronectin. Legend as in Fig 1

Apart from phenolic acids, plants sources are also rich in dietary fiber complex. Earlier we had shown that dietary fiber ameliorates decreased synthesis of heparan sulfate in diabetic rats[3]. The changes observed are either as a direct consequence of controlling blood glucose levels or indirectly through generation of short chain fatty acids. Leaves and seeds contain different kinds of polysaccharides along with other nutraceuticals. These polysaccharides produce SCFA by anaerobic fermentation in colon. They inturn modulate various functions by acting at the level of gene expression thereby bringing about health beneficial effects.

Thus tulsi and mulberry leaves and pomegranate seeds exert beneficial effects on kidney by preventing diabetes-mediated synthesis of extracellular matrix components such as laminin and fibronectin.

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